Cuticular Waxes and Flavonol Aglycones of Mistletoes

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Cuticular waxes of *Viscum album* subspecies and of *V. cruciatum* have been examined for their micromorphology and chemical composition. Wax crystalloids occur preferably as irregular platelets and rodlets, while deviant structures are found in small areas. Among the triterpenoids forming the wax layer, oleanolic acid is prevailing with some 80 %. The quantitative composition of the long-chain aliphatics, which comprise several classes, is rather variable. Flavonoid aglycones, occurring as very minor components of the cuticular waxes, comprise the flavonols kaempferol and quercetin and a series of their methyl derivatives, in some taxa also the flavanone naringenin. Neither the crystalloid structures nor the chemical composition of the wax allow to discriminate the 2 species, or male and female plants, or plants grown on conifers or on dicotyledoneous hosts.

Key words: Viscum, Cuticular Wax Chemistry, Micromorphology

Introduction

Numerous attempts have been made to divide the European mistletoe, *Viscum album* L. (Viscaceae), into different species, varieties *etc.* These divisions were usually made on the basis of morphological characters like leaf size, fruit morphology and seed number and *e. g.* differences in coloration of shoots and fruit. The often recognized host preferences were also used for characterization of subspecies, suggested extreme host specificities of small populations (even on one single host tree) were applied for formas *etc.* Such attempts resulted in a bewildering number of different taxa (for review of older literature see Tubeuf, 1923). These concepts, however, were untenable in most cases, when either true host preferences or more extended populations were thoroughly examined. Based on morphological and other easily discernible characters as mentioned above, a clear-cut differentiation also proved to be impossible.

Extensive surveys on host specificities accompanied by infection experiments were carried out by Tubeuf (1923) and this work resulted in the differentiation of 3 varieties which parasitize under natural conditions (i) various dicotyledoneous trees, (ii) fir (*Abies* spp.) and (iii) pine (mainly *Pinus sylvestris* and *P. nigra*). This concept, adopted by Gäumann (1951) and further amplified by less meaningful morphological characters, is now widely accepted and led to establish 3 subspecies (*e. g.* Tutin *et al.*, 1968): *Viscum album* ssp. *album* (on dicotyledoneous trees); ssp. *abietis* (on *Abies alba* and *e. g. A. cephalonica*); ssp. *austriacum* (on *Pinus sylvestris* and *P. nigra*, rarely on *Picea abies*).

The red-berried mistletoe (*Viscum cruciatum* Sieber ex Boiss.) from SW-Europe, N-Africa and Asia Minor was always considered as a separate species, being well characterized also by its growth-form, host preferences, and chorology.

Mistletoes obtain from their hosts *via* haustoria not only water and mineral salts. Organic substances are obviously also transferred and should contribute substantially to the carbon nutrition of the parasite (Pfiz *et al.*, 2001; Popp and Richter, 1998). Transfer of alkaloids from the host is known to occur in *Viscum cruciatum* (Martin-Cordero *et al.*, 1997) and even transport of morphogens like plant hormones from host trees to parasitizing mistletoes has been postulated in the case of Australian species which mimic the foliage of their hosts (Atsatt, 1983).

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With respect to these findings, the possibility of chemical influences from the host on various phenotype characters cannot be ruled out and may reflect the host preferences of species and subspecies. The flavonoid patterns of *Viscum album* subspecies from various hosts were investigated by Becker and Exner (1980). They identified quercetin and a series of quercetin methyl ethers, which may be assumed to be accumulated on the plant surface (Wollenweber *et al.*, 2000). Becker and Exner (1980) found important differences only between the European and the Japanese samples, not within the European subspecies. Other authors also reported some flavanones and chalcones as constituents of *Viscum album* (refs. see Wollenweber *et al.*, 2000).

Cuticular waxes of *Viscum album* ssp. *album* show a high content of oleanolic acid. Aliphatic constituents like alkanes, esters, aldehydes, primary alcohols and free fatty acids are present in much lesser amounts (Wollenweber *et al.*, 2000). Apart from the occurence of oleanolic acid in surface extracts of *Phoradendron juniperinum* (Wollenweber *et al.*, 1999), no other data on the chemistry of cuticular waxes of Viscaceae are available so far. Further results, however, are desirable for chemotaxonomic and ecophysiological studies as well. We therefore extended our studies to all 3 subspecies of *Viscum album* and additionally on *Viscum cruciatum.* To our knowledge this is the first study on wax constituents of *V. cruciatum.*

Materials and Methods

Plant material

Collecting sites and hosts of the mistletoe species and subspecies investigated are as follows: *Viscum album* ssp. *abietis* (Wiesb.) Abromeit on *Abies alba* Mill., Black Forest near Oberkirch, Germany. *Viscum album* ssp. *austriacum* (Wiesb.) Vollmann on *Pinus sylvestris* L., Lampertheim south of Darmstadt, Germany. *Viscum album* ssp. *album* on *Malus domestica* Borkh., Stuttgart-Hohenheim and on *Populus x canadensis* Moench, Meistratzheim (south of Strasbourg), France, for analyses of cuticular wax; on *Crataegus prunifolia* (Lam.) Pers., and on *Populus balsamifera* L, Botanical Garden Darmstadt, for flavonoid analyses. *Viscum cruciatum* Sieber ex Boiss., female plants on artificially infected *Cornus mas* L., and *Hedera helix* L.; male plants on *Ligustrum vulgare* L., and *Pyracantha coccinea* M. J. Roem., Botanical Garden Hohenheim.

Scanning electron microscopy (SEM)

Small pieces of leaves were excised, mounted on specimen holders by means of conductive carbon adhesive tabs and coated with gold/palladium (30 nm) in a Balzers Union SCD 040 sputter coater. For examination of samples a Zeiss DSM 904 SEM was used.

Analysis of cuticular waxes

Triterpenoids and aliphatic compounds

Total cuticular waxes were obtained by dipping at least 6 leaves per sample in $CHCl_3$ for 30 sec at room temp., and subsequently in hot $CHCl_3$ (*ca.* 60 °C) for 120 sec. Leaf areas were determined after wax extraction by means of a Mini MOP area analyzer (Kontron). The combined extracts were fractionated into compound classes by preparative TLC on silica gel G (Riedel-deHaen) with the solvents 1) *n*-hexane and 2) chloroform/*n*-hexane (75:25, v:v). Spray reagent for detection (UV 360 nm) was 0.005 % primuline in acetone/water (80:20, v:v). Individual fractions were analyzed by GC with a Shimadzu GC-17A gas chromatograph equipped with a CP-Sil 8 CB capillary column (25 m × 0.32 mm, Varian-Chrompack), on-column injector and FID. Operating conditions of the chromatograph were: detector temperature 360 °C, linear velocity of helium carrier gas 30 cm/sec. The column temperature was initially set to 160 °C for 2 min and then increased by 8 °C/min to 340 °C (or 360 °C for esters). The mixed fractions of primary alcohols and triterpenols as well as free fatty acids and triterpene acids were analyzed as trimethylsilyl ethers/ trimethylsilyl esters after derivatization with N,O-bis-(trimethylsilyl)-acetamide/ pyridine (1:1, v:v). Appropriate internal standards were employed for each fraction. Data shown in Table I represent mean values of at least 4 independent samples, each analyzed in duplicate.

Flavonoids

Freshly collected twigs were briefly rinsed with chloroform at room temperature to dissolve the epicuticular wax material. The solutions were

Wax composition	Viscum album				Viscum cruciatum	
	ssp. album[a]	*ssp. album*[b]	*ssp. abietis*	*ssp. austriacum*	male	female
Total yield (μg cm^{-2})	201.6	203.4	197.0	202.5	249.0	221.8
Oleanolic acid, c)	80.7	81.1	82.2	82.8	85.0	81.3
Triterpenols, d)	0.4	0.4	0.6	0.4	0.2	0.4
Aliphatics	18.9	18.5	17.2	16.8	14.8	18.3
Aliphatic compound classes (% of total aliphatics)						
Alkanes	26.8	31.4	43.0	26.1	5.4	8.5
Esters	2.0	1.1	1.2	0.9	5.5	2.4
Aldehydes	3.1	2.9	2.5	2.1	0.6	1.8
Primary alcohols	48.8	42.2	28.4	20.4	24.0	26.7
Free fatty acids	19.3	22.4	24.9	50.5	64.5	60.6

Table I. Cuticular wax composition (% of fractions) and total yield in *Viscum album* subspecies and in *Viscum cruciatum.*

[a] On *Malus domestica*, Stuttgart-Hohenheim;
[b] on *Populus* × *canadensis*, Meistratzheim;
[c] including traces of betulinic acid;
[d] β-amyrin, lupeol, unknown triterpenol and traces of α-amyrin.

taken to dryness, "defatted" (MeOH, – 10 °C, 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).

Results

SEM observations

In the subspecies of *Viscum album* and in *Viscum cruciatum*, no differences of wax microstructure are discernible between the adaxial and abaxial leaf sides and between male and female plants. Epicuticular wax crystalloids occur in a number of different types (Fig. 1). Most frequently, irregular platelets and rodlets can be observed in *Viscum album.* These are not densely arranged, and a considerable part of the basal wax layer is devoid of crystalloids (a). The rodlets are connected with platelet aggregates and oriented parallel to the surface (b). In the first stages of development, the rodlets obviously emerge from the margins of minute platelets (c). Further crystalloid types develop in small areas. There are coiled wax threads together with rodlets (d), crystal-like platelets (e) and parallel oriented wax plates arranged perpendicular to the surface (f). Except for a less frequent occurrence of rodlets and threads in *Viscum cruciatum*, wax microstructures proved to be almost identical. The elaborated crystalloids are found preferably on leaves in the first vegetation period. In the second vegetation period, a continuous degradation of the

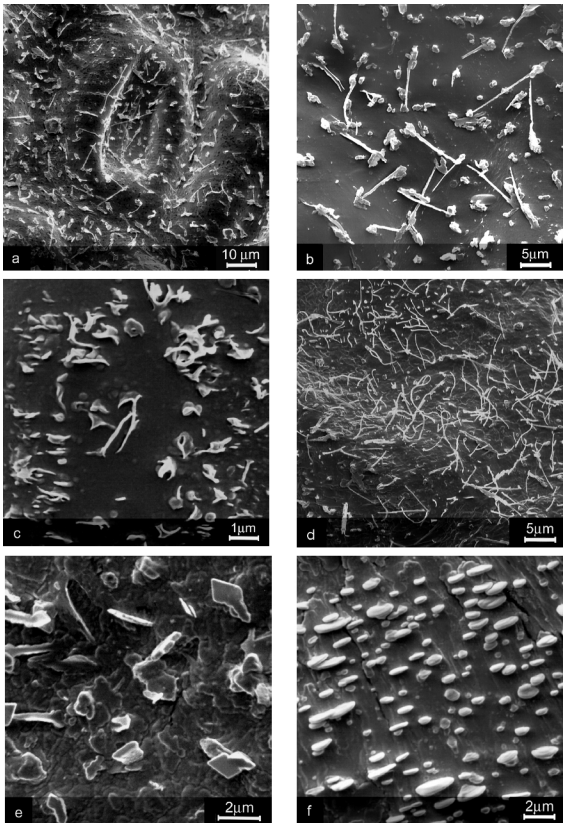

Fig. 1. Types of epicuticular wax crystalloids on leaves of *Viscum album* and *Viscum cruciatum*. a) Epidermal surface with stoma, bearing irregular platelets and rodlets. A considerable part of the basal wax layer is devoid of crystalloids; b) rodlets of variable length connected with platelet aggregates and oriented parallel to the cuticle surface; c) transition stages from minute platelets to rodlets; d) wax threads and rodlets; e) irregular and crystal-like platelets on a wax crust broken up into flakes; f) massive wax plates oriented parallel and perpendicular to the surface.

crystalloids takes place, leading to poorly contoured plates and granules.

Triterpenoids and aliphatics

Total cuticular wax is present in the subspecies of *Viscum album* with *ca.* 200 μg cm^{-2} (Table I). No significant differences were found in wax yields from leaves of male and female plants. In *Viscum cruciatum*, wax amounts are markedly higher and the yields from male plants clearly exceed those from females (249.0 and 221.8 μg cm^{-2}, respectively). Oleanolic acid is the most prominent wax constituent, comprising more than 80 % in all samples. This main triterpenoid is accompanied by traces of betulinic acid and small amounts of the triterpenols β-amyrin, lupeol, an unknown triterpenol and traces of α-amyrin. Total aliphatic constituents comprise only 14.8–18.9 % of the waxes.

Predominating fractions of aliphatics in *Viscum album* are alkanes, primary alcohols and free fatty acids with different preponderance in the individual subspecies, *i.e.* primary alcohols in ssp. *album*, alkanes in ssp. *abietis* and free fatty acids in ssp. *austriacum.* Percentages of esters and aldehydes are small throughout. The same fractions of aliphatic constituents are found in cuticular waxes of *Viscum cruciatum.* In this species, free fatty acids represent the predominating compound class in portions of more than 60 %, accompanied by some 20 % of primary alcohols. Alkanes, esters and aldehydes are minor constituents of *V. cruciatum* leaf wax. Differences between male and female plants are small, though a very high portion of oleanolic acid is found in males.

The chain length distributions of individual compound classes proved to be without greater divergencies, both within the subspecies of *Viscum album* and *V. cruciatum.* Profiles of primary alcohols, aldehydes and free fatty acids range from *ca.* C_{22}–C_{30} with C_{26} as main constituent (ca. 40 % of the fractions). In the aldehydes of *Viscum album* ssp. *album* and ssp. *abietis*, the homologues C_{31} and C_{32} are additionally present in noticeable quantities. The comparatively flat profiles of alkyl esters are in the range of C_{36}–C_{56} with C_{42} as major chain length in *Viscum album*, whilst C_{48} predominates in *V. cruciatum.* Small amounts of triterpenol esters were also detected. Chain length distributions of alkanes (C_{21}–C_{31}) are concentrated to C_{29} (up to ca. 80 %), in the alkanes of *Viscum album* bimodal profiles are present with a small second maximum at C_{25}.

Flavonoids

The epicuticular material of the *Viscum album* as well as the *Viscum cruciatum* samples now analyzed contains preferably the flavonol quercetin and its methyl derivatives, occasionally also the flavonol kaempferol and some of its methyl derivatives. Only rarely has the flavanone naringenin been found. The results are given in detail in Table II.

Discussion

The most remarkable feature of epicuticular wax micromorphology in the *Viscum* species and subspecies is the high diversity of crystalloid structures. These include platelets of various shapes, rodlets and wax threads. Platelets and plates are very often found in plant epicuticular waxes, but the variety of forms which occur together (irregular, crystal-like and massive plates in parallel orientation) is outstanding. Platelet crystalloids are often associated with high contents of primary alcohols or triterpenoids in waxes, and triterpenoids may also be involved in the formation of special rodlet types (Barthlott *et al.*, 1998). Thread-like crystalloids mostly consist of flavonoids (Barthlott and Wollenweber, 1981; Wollenweber, 1984), and additionally, there are examples where triterpenoids are main constituents of wax threads (Barthlott and Wollenweber, 1981; Markstädter *et al.*, 2000). Thus, the triterpenoids, aliphatic primary alcohols and flavonoid aglycones present in the wax of *Viscum* species could well be responsible for the crystalloid forms. While irregular platelets and rodlets are the main structures, threads and parallel oriented plates are restricted to small areas. The reason for this local formation of special crystalloids is not known. It could be caused by punctually altered wax compositions or otherwise varying conditions of crystallization.

In addition to the common irregular platelets, further crystalloid types were also noted in previous studies on epicuticular waxes of the Viscaceae (Ditsch and Barthlott, 1997; Weber, 1981). Within the order of Santalales, transversely ridged rodlets were reported for some Viscaceae, Loranthaceae and Santalaceae (Ditsch and Barthlott, 1997). Wax

Table II. Occurrence of flavonoid aglycones in epicuticular waxes of *Viscum album* subspecies and *Viscum cruciatum*. (Kae = kaempferol; Qu = quercetin; Me = methyl ether).

Species/subspecies	Flavonols													Flavanone
	Kae	Kae-3-Me	Kae-7-Me	Kae-3,7-diMe	Kae-3,4′-diMe	Qu	Qu-3-Me	Qu-7-Me	Qu-3′- Me	Qu-3,7-diMe	Qu-3,3′-diMe	Qu-7,3′-diMe	Qu-3,7,3′-triMe	Narin-genin
Viscum album ssp. album on Crataegus prunifolia		X		X			X			X	X		X	
Viscum album ssp. *album* on *Populus balsamifera*	X					X	X			X	X	X	X	
Viscum album ssp. *abietis* on *Abies alba*		X		X	X		X	X		X	X	X	X	
Viscum album ssp. *austriacum* on *Pinus sylvestris*				X			X			X	X			X
Viscum cruciatum, female on *Cornus mas*				X						X			X	
Viscum cruciatum, female on *Ligustrum vulgare*								X		X		X	X	X
Viscum cruciatum, male on *Hedera helix*	X		X	X		X		X	X	X		X	X	X
Viscum cruciatum, male on *Pyracantha coccinea*										X		X	X	X

crystalloids of this type are usually indicative for the presence of palmitone (Meusel *et al.*, 1999). However, we could find neither rodlets of this type nor palmitone (hentriacontan-16-one) in the wax extracts.

Cuticular waxes of the two *Viscum* species are similar in general composition, being characterized by the marked preponderance of oleanolic acid and minor portions of aliphatic constituents. This is also reflected by the similar micromorphology of epicuticular waxes. Taking into account the aliphatic compound classes, *Viscum cruciatum* is well separated from *Viscum album* by the low alkane content and the abundance of free fatty acids. Additionally, *Viscum cruciatum* shows very high total wax amounts clearly exceeding those of *Viscum album.* The subspecies of *Viscum album* contain either primary alcohols, alkanes or free fatty acids as major aliphatic fractions, but these compound classes occur in more balanced relations. Though primary alcohols are the dominant constituent class of aliphatics in *V. album* ssp. *album*, the 2 populations examined show divergencies in the relative amounts of these and the other constituents. This makes the varying percentages of the aliphatic compound classes less meaningful and it seems a case of doubt, whether the differences found are sufficient to separate the subspecies. There is also no clear discrimination possible with respect to the chain length distributions, as these are comparatively similar. Some small variations would rather allow to differentiate the *Viscum* species, but not the subspecies examined. Additionally, no characteristics of wax compositions are observed which would be indicative of gymnospermous vs. dicotyledoneous hosts.

The situation is much the same when externally accumulated flavonoids are considered. As has been shown earlier, methyl ethers of quercetin are the dominating flavonoid constituents of *Viscum* epicuticular waxes. In an earlier study on *V. album*, grown on *Crataegus prunifolia*, we found four quercetin methyl ethers and two kaempferol derivatives (Wollenweber *et al.*, 2000). Three further quercetin methyl ethers and quercetin proper, which had earlier been reported for *V. album* from various host plants (Becker and Exner, 1980), have now also been detected. Although the latter authors analyzed samples from a number of different host trees, they were unable to detect any kaempferol derivative. They even suggested that 4′-O-substitution of ring B is not revealed. We now found not only kaempferol and its methyl ethers, but also naringenin, a flavanone with 4′-O-substitution. Becker and Exner (1980) also found that Qu-3-Me was lacking on *V. album* from *Abies* and from *Pinus*, while it was present on our material from the same host tree species. No characteristic accumulation trend was observed for mistletoes grown on conifers and on deciduous trees, nor seem constant patterns to exist for male or female plants; not even the two species we analyzed can be told apart by their cuticular flavonoid patterns. By and large, the flavonoid results presented here confirm the earlier finding (Becker and Exner, 1980) that the flavonoid profiles expressed in *Viscum* epicuticular waxes are not as characteristic as to be of taxonomic value.

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