Comparative Analysis of Epicuticular Waxes from Some High Alpine Plant Species

Cornelius Lütz and Paul-Gerhardt Gülz

Botanisches Institut der Universität zu Köln, Gyrhofstraße 15, D-5000 Köln 41

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Epicuticular waxes were extracted and analysed from leaves of 7 different high alpine plant species, with 3 species harvested at different altitudes: *Salix herbacea* (1950 m, 2825 m), *Leucanthemopsis alpinum* (1950 m, 3050 m), *Loiseleuria procumbens* (1950 m, 2660 m), *Dryas octopetala* (2400 m), *Ranunculus glacialis* (2800 m), *Soldanella pusilla* (2640 m), *Oxyria digyna* (2640m). Two main fractions obtained from waxes were investigated: hydrocarbons and wax-esters, -aldehydes plus -acetates. Individual wax compounds accumulated differently in the respective plant species. The gross composition did not indicate a strict altitude-dependent change in wax composition, though some single components like the C-29/C-31 alkanes *(Loiseleuria)* or the C-22/C-26 fatty acid methyl esters in *Salix,* changed significantly with increasing elevation of plant habitat. The evergreen leaves of *Dryas* and of *Loiseleuria* exhibited exceptionally high amounts of triterpenol esters.

The results indicate that the formation of epicuticular wax layers is not based on a special adaptation to altitude or high mountain habitats in general.

Introduction

In general, all aerial parts of higher plants possess a cuticula. This is the boundary to the environment and therefore of great interest especially under ecophysiological aspects. The cuticula proper is mostly covered by layers of epicuticular waxes. This outermost boundary layer has gained more and more interest in the last years. Briefly, it is composed mainly of long chain homologous series of hydrocarbons, wax esters, aldehydes, acetates, free fatty acids and free alcohols. These constituents occur often substituted, *e.g.* with −COOH or with −OH groups, and they require several analytical methods for separation into individual compounds [1–4]. The diversity in composition of epicuticular waxes could explain the great variability of the three dimensional structures, formed by the waxes and best observed under a scanning electron microscope [1, 5, 6].

There is a large body of literature presenting analytical data on epicuticular waxes. Even though it seems difficult to relate wax compositions measured to the systematic order of the investigated plants. Further, the ecological significance of wax composition and structure achieves more consideration today. However, as far as we know, analyses of epicuticular waxes isolated from plants growing at high altitudes (above timberline) has not yet been presented.

Our ecophysiological and cytological studies on high alpine plants [7–9] have led us to ask after the role of the plant surface in communication with the often extreme growth conditions at high altitudes. Plants living in high mountain habitats have developed several mechanisms to survive under the unfavourable conditions of freezing temperatures, snow covering for long periods, occasionally very high irradiance or strong mechanical forces originated by wind [10, 12].

This study presents an investigation of the epicuticular waxes of selected high alpine species. The data obtained in the experiments are discussed in relation to different altitudes of plant habitats and to different environmental influences on these plants.

Materials and Methods

The following plants have been used to extract epicuticular waxes from their leaves: *Dryas octopetala* (Rosaceae), *Leucanthemopsis alpinum* (Asteraceae), *Loiseleuria procumbens* (Ericaceae), *Oxyria digyna* (Polygonaceae), *Ranunculus glacialis* (Ranunculaceae), *Salix herbacea* (Salicaceae), *Soldanella pusilla* (Primulaceae). The plants were collected in July 1984 at different altitudes ranging from 1950 m to 3050 m above sea level in the Central Alps

Reprint requests to Dr. Cornelius Lütz.

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in Tirol, Austria, either in the vicinity of Obergurgl, Ötztal, or on the slopes of the Hoher Nebelkogel, Stubai Alps.

Leaves were carefully picked from the plants and the fresh weights of the samples were determined. Wax compounds were extracted by immersion the leaves into redistilled chloroform twice for 3 min. The solvent fractions were stored in the cold until they could be transferred to the laboratory in Cologne, where further processing of the samples was done. After extraction, the leaves were dried in a ventilated oven for 6 hours at 110 °C for subsequent dry weight determinations. All these steps have been performed in the laboratory of the "Institut für Hochgebirgsforschung" (Innsbruck), branch Obergurgl/Ötztal (2000 m).

The fractionation, separation and quantification of the different compounds from epicuticular waxes followed conventional procedures. The complex mixture was first separated by column chormatography into three fractions: a) pentane fraction: contained the hydrocarbons, b) 2-chloropropane fraction: contained mainly esters and aldehydes, c) methanol fraction: contained free alcohols and free acids. Identification of individual constituents as esters and aldehydes was achieved by thin-layer chromatography, gas chromatography and chemical derivatisation [2].

Fixation of individual samples for electron microscopy with glutaraldehyde and osmium tetroxide, and embedding in epoxy-resin were done at the research station in Obergurgl, as is described in [7].

Results

Epicuticular waxes were extracted from high alpine plants of 7 different families. Three species *(Salix herbacea, Leucanthemopsis alpinum, Loiseleuria procumbens)* were harvested at different altitudes, and the reamaining 4 species were collected at higher altitudes only (see Table I). In general, leaves could by extracted 2–3 hours after collecting them at their typical growth habitats.

Total composition

The amounts of fresh weights used ranged from 10.8 g to 25.2 g (Table I) and gave sufficient yield of

	Leaves FW [g]	DW [g]	(%FW)	Wax [mg]	%DW	pentane fraction [mg]	%DW	2-CP fraction [mg]	%DW	Methanol fraction [mg]	%DW
Salix herbacea 1950 m	20.2	6.4	31.8	38	0.6	8	0.1	3	0.1	13	0.2
Salix herbacea 2825 m	20.0	6.4	32.0	32	0.5	9	0.1	2	0.03	15	0.2
Leucanthemopsis alpinum 1950 m	13.5	2.3	17.0	37	1.6	5	0.2	4	0.2	26	1.1
Leucanthemopsis alpinum 3050 m	21.0	3.3	15.7	45	1.3	4	0.12	10	0.3	23	0.7
Loiseleuria procumbens 1950 m	22.8	11.4	49.8	760	6.7	40	0.4	200	1.8	380	3.3
Loiseleuria procumbens 2660 m	21.2	9.8	46.0	543	5.6	20	0.2	140	1.4	360	3.7
Dryas octopetala 2400 m	20.0	6.5	32.5	148	2.3	11	0.2	40	0.6	89	1.4
Ranunculus glacialis 2800 m	20.1	3.0	15.1	80	2.6	15	0.5	6	0.2	33	1.1
Soldanella pusilla 2640 m	10.8	4.0	37.0	33	0.8	2	0.1	5	0.1	4	0.1
Oxyria digyna 2640 m	25.2	2.3	8.9	23	1.0	1	0.04	4	0.2	15	0.7

Table I. Fresh weights (FW) and dry weights (DW) from selected alpine plants, which have been used to extract total epicuticular waxes (second column). This extract has been separated into three fractions (next columns), as is described in "methods". 2-CP: 2-chloropropane.

total waxes for further analysis (Yields: 0.5% to 6.7% of dry weight). Epicuticular waxes were fractionated according to their hydrophobicity into three classes (Table I): a) hydrocarbons: they represent the smallest class in respect of % dry weight (DW), ranging from 0.04% DW *(Oxyria)* to 0.5% DW *(Ranunculus)*. Comparing data from plants harvested at different altitudes, a reduction in hydrocarbons of nearly 50% with increasing altitude was found in *Loiseleuria* and in *Leucanthemopsis* leaves, while their content in *Salix* seemed to stay constant. b) wax esters, other esters and aldehydes gave similar percentages from DW as hydrocarbons, except in *Loiseleuria* and in *Dryas,* a severalfold increase could be observed (0.6%−1.8% of DW). An obvious reduction in the amounts of esters and aldehydes with increasing altitude showed *Salix,* to a lesser extent *Loiseleuria,* while an increase from 0.2% to 0.3% DW could be found in *Leucanthemopsis* leaves. Similar low amounts of these compounds were found in *Ranunculus, Soldanella* and *Oxyria.* Only *Ranunculus,* collected at 2800 m, and *Salix,* collected at 2825 m, contained roughly 3 times more hydrocarbons per DW than esters and aldehydes. c) the methanol fraction of epicuticular waxes gave the highest mg-yields, except for *Soldanella,* where the amount is slightly less than in fraction b). Altitude dependent changes occurred similar as has been observed with total hydrocarbons; with increasing altitude of habitats the amounts of free alcohols and fatty acids decreased; the values stayed constant in *Salix* only. *Loiseleuria* and *Dryas* exhibited the highest % DW of wax compounds in this methanolic extract.

Hydrocarbons

Hydrocarbons were separated into homologous series of alkanes (Table II) and of alkenes. Alkanes showed mainly chain lengths from 23 to 35 carbon atoms, with the predominant species of C-27, C-29, C-31. The C-29 alkane was the main component in all plants, followed by C-27 alkane in *Salix, Dryas,* and *Ranunculus;* and by C-31 alkane in *Leucanthemopsis* and in *Loiseleuria. Oxyria* contained over 83% of the C-29 moiety. A different pattern was found in *Soldanella:* the C-29 alkane counted for only 36.7%, while – in contrast to all other species – the alkene fraction represented 31.6% of the total hydrocarbons.

An altitude related change in composition was only found in *Loiseleuria,* because the C-29 alkane was reduced from 63.7% to 34%, probably in favour of the increasing C-21 alkane (23.7% to 43.9%). *Salix* and *Leucanthemopsis* did not show significant altitude dependent changes in alkane composition.

As mentioned before, the concentration of alkenes found in these alpine species is of interest only in *Soldanella,* in all other plants the sum of alkenes reached maximal 3.3%.

Wax esters, aldehydes and other esters

These compounds are listed in Table III, and only the main species found in the plants will be discussed here.

Salix contained mainly aldehydes (Ald), fatty acid methyl esters (FAME) and wax esters (WE). The relative amount of total WE increased strongly with altitude, while Ald decreased and FAME remained nearly constant. The Ald were composed over 60%

Table III. Composition of epicuticular wax species, as they have been isolated by 2-chloropropane. WE: wax esters, TTE: triterpenol esters, Ald: aldehydes, FAME: fatty acid methyl esters, Ac: acetates, TTAc: triterpenol acetates. Detailed data for WE, TTE, Ald and FAME – compositions have been obtained for all plants, but they are given in the text only for those plants, where these compounds represent major wax species. Values are given in peak area ‰.

	Salix herbacea 1950 m	*Salix herbacea* 2825 m	*Leucanth. alpinum* 1950 m	*Leucanth. alpinum* 3050 m	*Loiseleuria procumbens* 1950 m	*Loiseleuria procumbens* 2660 m	*Dryas octopetala* 2400 m	*Ranunculus glacialis* 2800 m	*Soldanella pusilla* 2640 m	*Oxyria digyna* 2640 m
WE	11.0	19.8	75.1	41.3	1.7	1.7	39.3	66.3	32.8	39.3
TTE	5.5	2.1	–	–	51.5	52.1	56.6	0.6	+	10.3
ALD	38.0	20.6	–	–	2.7	2.3	–	5.0	6.5	9.6
FAME	35.3	39.6	24.7	58.7	–	–	–	15.5	19.0	–
Ac	–	–	–	–	–	–	–	–	–	38.9
TTAc	1.1	6.6	–	–	33.9	40.5	–	0.3	37.8	–
unknown	7.1	2.2	–	–	7.8	2.8	3.3	9.2	1.9	1.1

Table II. Listing of the main alkanes found in the pentane extract of leaves from different alpine plants. Alkenes are given in % of total hydrocarbons and not separated into individual compounds. Only in case of *Soldanella* a detailed fractionation analysis is presented in the text. Values are given in peak area ‰.

No. of C - atoms Alkanes	*Salix herbacea* 1950 m	*Salix herbacea* 2825 m	*Leucanth. alpinum* 1950 m	*Leucanth. alpinum* 3050 m	*Loiseleuria procumbens* 1950 m	*Loiseleuria procumbens* 2660 m	*Dryas octopetala* 2400 m	*Ranunculus glacialis* 2800 m	*Soldanella pusilla* 2640 m	*Oxyria digyna* 2640 m
23	0.7	0.9	+	+	+	+	+	+	3.2	+
24	0.3	0.4	+	+	0.6	+	+	+	0.5	+
25	2.6	3.3	+	+	0.2	0.2	1.3	2.0	4.1	+
26	0.7	0.5	+	+	0.1	0.2	+	+	0.8	+
27	33.4	30.5	12.0	11.8	2.7	1.2	21.3	10.7	6.9	7.2
28	2.1	2.7	+	+	1.4	0.9	2.2	1.3	1.9	+
29	52.2	55.5	62.6	64.9	63.7	34.0	70.9	71.8	36.7	83.1
30	0.5	0.5	+	+	1.8	3.4	+	0.9	2.2	+
31	5.2	4.7	25.4	23.4	23.7	43.9	4.4	12.8	9.0	9.8
32	0.2	+	+	+	0.8	2.7	+	+	+	+
33	0.6	0.6	+	+	4.5	11.0	+	+	+	+
34	+	+	+	+	0.1	0.3	+	–	–	–
35	0.2	0.1	–	–	0.3	0.7	+	–	–	–
% total alkenes	1.4	< 1	3.3	< 1	1.3	1.5	< 1	< 1	31.6	< 1

by C-28 forms and over 25% by C-30 forms. The FAME contained as main species C-22, C-24, C-26 and C-28 derivatives. Drastically changing with altitude appeared the C-22 form (1950 m: 37.9%, 2825 m: 3.5%) and the C-26 form (1950 m: 19.5%, 2825 m: 46.5%), followed by the C-24 form (1950 m: 10.7%, 2825 m: 17.4%).

Leucanthemopsis showed a simple spectrum in this solvent fraction: almost exclusively WE and FAME were found, other compounds only in traces. Both ester types have strict altitude dependent occurrence: FAME did strongly increase with increasing elevation of plant habitat, while WE decreased. The WE fraction consisted mainly of C-42 to C-48 forms. The latter form increased with altitude of plant growth sites from 8.3% to 14%, while the other forms decreased in concentration. FAME were composed preferentially of the C-26 and the C-28 form, with C-26 increasing and with C-28 decreasing with increasing altitude.

Loiseleuria showed two main components: triterpenol esters (TTE), which represented 52% (altitude independent) and triterpenoid acetates (TTAc), which increased from 33.9% (1950 m) to 40.5% (2660 m) in both plant samples.

An even higher concentration of TTE was found in *Dryas,* while TTAc seemed to be replaced by WE (39.3%, C-42 and C-44 forms mainly).

A different pattern was seen in *Ranunculus:* WE dominated by 66.3% over FAME (15.5%). The pattern of individual WE found in this plant was different from the patterns found in all other plants: the C-38 form represented 40.7% (other plants: from traces to 7%), followed by the C-40 form with 19.4%.

Soldanella displayed three main components: WE: 32.8% (mainly the C-44 and the C-46 forms); FAME: 19% (only C-26, C-28, C-30 forms); TTAc: 37.8%.

Oxyria represented the only plant species with a considerable amount of acetates (38.9%). An equal amount of WE (39.3%) together with TTE (10.3%) and Ald (9.6%) completed this spectrum. Individual WE ranged from C-40 to C-46 mainly, with percentages between 11 and 18.

The methanol fraction prepared from epicuticular waxes yielded most of the material. However, the subsequent analyses have shown a very complex mixture of compounds, which requires careful further investigations. These analyses are in progress now and therefore not reported in this communication.

Electron microscopy

A first concomitant ultrastructural study showed details of cell walls, cuticula and epicuticular layers from 4 of the investigated plants (Fig. 1). All leaves

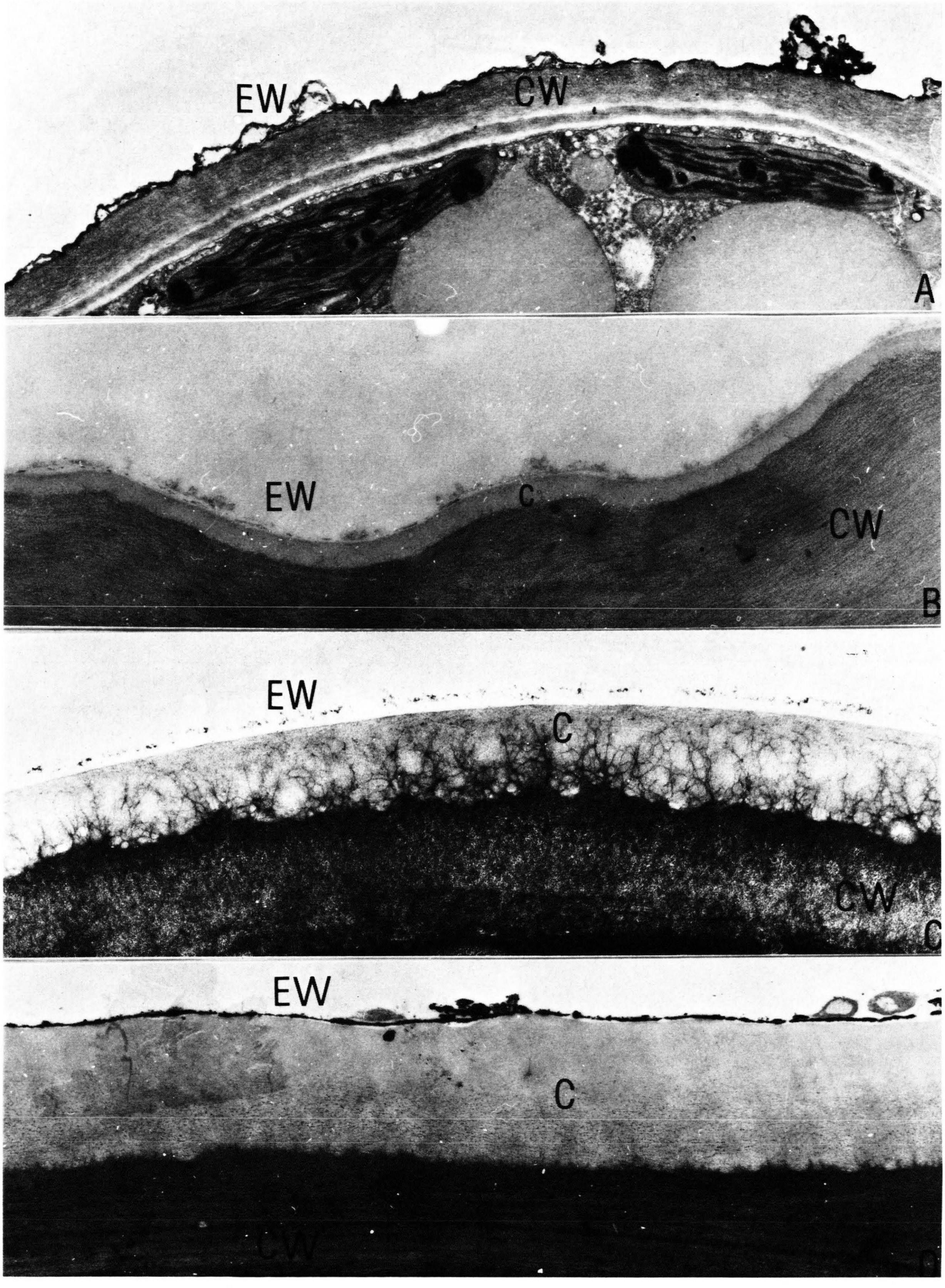

Fig. 1. Structures of the outer epidermal layers from A: *Loiseleuria procumbens* (× 12000), B: *Ranunculus glacialis* (× 68000), C: *Dryas octopetala* (× 43000), D: *Soldanella pusilla* (× 31000). In the *Loiseleuria* – sample a distinct cuticula cannot be resolved. CW = cell wall, C = cuticula, EW = epicuticular wax layer.

exhibited thick epidermal cell walls, which are covered by a cuticula proper of different thickness. Only in case of *Loiseleuria* a clear separation between cell wall, cuticula and epicuticular layer was not possible (Fig. 1A); instead wax formations seemed to be inserted near the cell wall. Fig. 1B shows only a thin cuticula for *Ranunculus* leaves, which is covered by wax structures. *Dryas* leaves have formed a cuticula of 2/3 the thickness of the cell wall. The epicuticular layer is not well preserved in this thin-sectioned sample (Fig. 1C). A cuticula of similar thickness, but covered with a more structured wax layer is seen in sections made from *Soldanella* leaves (Fig. 1D). Altitude dependent differences in ultrastructure of the outer epidermal layers could not significantly be outlined by transmission electron microscopy.

Discussion

If the gross compositions of epicuticular waxes from the 7 plants studied are compared, great species-specific differences appear: only *Soldanella* shows higher amounts of alkenes; similarly *Oxyria* only has accumulated acetates in the wax fraction. TTE have been found as main constituents only in the dwarf shrubs *Dryas* and *Loiseleuria,* while these plants differ in the amounts of WE and of TTAc. Such variabilities between species are also observed in the chain lenghts of aliphatic compounds, *e.g. Ranunculus* only contains a high relative amount of the C-38 form in WE.

That means, there is no obvious relation of wax composition to different altitudes or to the alpine environment per se. The only trend observed, which certainly needs further studies, is seen in the increase in chain length with increasing altitude found in *Loiseleuria* (hydrocarbons), in *Salix* in the FAME-fraction and in *Leucanthemopsis* in the WE-fraction. The latter, however, also shows in the FAME-fraction a decrease in C-28 and an increase in C-26 with increasing altitude.

This study demonstrates, that it is difficult to discuss the results in regard of an environmental adaptation. Here it is urgently needed that more family specific analyses from contrasting plant habitats are made, together with detailed ultrastructural observations. The diversity in epidermal and in cuticular ultrastructure among species has been shown for four species in this work.

The influence of the environment on the composition of wax constituents has been examined for few species only, and not for high mountain plants. The available data proved difficult to follow clear trends. A waxy outer surface of leaves may indicate a protection against passive diffusion of water from the leaf. This protection is obviously realized in arid environments. However, most alpine plants, including all those investigated in this study, do normally not suffer from limited water supply. *Oxyria, Ranunculus, Salix* and *Soldanella* always grow under humid conditions, but synthesize considerable amounts of wax compounds, which cover a thick cuticula. Körner and Mayr [13] reported an increase in leaf diffusive conductance with increasing altitude, paralleled by increasing stomatal frequency of plants up to 2600 m. The relative high rates of evapotranspiration in plants from higher altitudes explain their higher potential water loss compared to plants growing in the valley. Thus the wax layer should not primarily prevent water loss in high alpine plants.

Hunt and Baker [14] investigated the effects of growth conditions on wax formation in leaves. A considerable increase in wax deposition was found, if they increased the radiant energy rate in the experiment. Insolation and reflectance could avoid overheating of leaves at high irradiance. Whether this protective mechanism is of importance for high alpine plants, remains to be studied. In general, the alpine habitat is not a dry habitat, though in summer the cooling effect of transpiration may occasionally be limited. However, photosynthesis is sensitive to very high energy fluxes, especially from UV-B radiation [15, 16].

An important ecological factor especially in the high mountains is wind. Wind has a strong dwarfing effect on plants [17] and wind affects the plant body by mechanical abrasion with soil particles or snow crystals. *Loiseleuria* and *Dryas* are considered to belong to the most wind-resistant species: wax analysis revealed a very dominant concentration of TTE on the cuticula of these plants, which form amorphous layers or plates; structures, which are most resistant against abraison [18].

The wide diversity in composition of epicuticular waxes among plants was shown for 7 high alpine plant species from different families. Some changes in wax composition seem to be related to changes in altitude of habitats, but clear-cut trends could not be established. Further comparative studies should include species, growing also at low altitudes, like *Ranunculus, Oxyria, Loiseleuria* or *Dryas* in the subarctic region of northern Europe. Scanning electron microscopy of leaf surfaces from plants of selected alpine environments should complete the chemical analysis of waxes, leading to ecophysiological relevant discussions.

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