

Dissociation and metal-binding characteristics of yellow lichen substances suggest a relationship with site preferences of lichens

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• **Background and Aims** Many species of lichen-forming fungi contain yellow or orange extracellular pigments belonging to the dibenzofurans (usnic acid), anthraquinones (e.g. parietin) or pulvinic acid group. These pigments are all equally efficient light screens, leading us to question the potential ecological and evolutionary significance of diversity in yellow and orange lichen substances. Here the hypothesis is tested that the different pigments differ in metal-binding characteristics, which suggest that they may contribute to adaptation to sites differing in pH and metal availability.

• **Methods** UV spectroscopy was used to study the dissociation and the pH dependence of the metal-binding behaviour of seven isolated lichen substances in methanol. Metals applied were selected macro- and micro-nutrients (Cu²⁺, Fe²⁺, Fe³⁺, Mg²⁺, Mn²⁺ and Zn²⁺).

• **Key Results** All the pigments studied are strong to moderate acids with pK_{a1} values between 2.8 and 4.5. Metal complexation is common in the lichen substances studied. Complexation takes place under acidic conditions with usnic acid, but under alkaline conditions with parietin and most compounds of the pulvinic acid group. The pulvinic acid derivative rhizocarpic acid forms metal complexes both in the acidic and the alkaline range.

• **Conclusions** Metal complexation by lichen substances could be a prerequisite for lichen substance-mediated control of metal uptake. Assuming such an effect at pH values where the affinity of the metal for the lichen substance is intermediate would explain the strong preference of lichens with usnic or rhizocarpic acids to acidic substrata. Moreover, it would explain the preference of lichens with parietin and some lichens with compounds of the pulvinic acid group either for nutrient-rich substrata at low pH or for calcareous substrata.

Key words: Anthraquinones, dibenzofurans, pulvinic acid derivatives, usnic acid, parietin, dissociation constant (pK_{a1}), metal complexation, lichenized Ascomycetes, lichen ecology, nutrients, UV spectroscopy.

INTRODUCTION

Many lichens produce lichen substances that absorb ultraviolet radiation, visible light usable for photosynthesis (PAR), or both ultraviolet radiation and PAR (Rikkinen, 1995; Huneck and Yoshimura, 1996). Photoprotection by lichen substances, melanins, calcium oxalate crystals or layers of dead fungal cells is essential for lichens occurring in environments exposed to high solar irradiation (Bjerke and Dahl, 2002; Nybakken *et al.*, 2004; Hauck *et al.*, 2007a). Most PAR-absorbing lichen substances are yellow or orange crystals (Fig. 1) that are produced by the fungus, but are located on the surfaces of the fungal or algal cell walls.

Chemically, these pigments belong to three different groups of substances, viz. dibenzofurans, anthraquinones and pulvinic acid derivatives (Huneck and Yoshimura, 1996). The faintly yellow xanthenes form an additional group occurring, for example, in the lichen genera *Buellia* or *Lecidella* that are not treated in the present study. The yellow usnic acid, which is one of the most common lichen substances occurring in many different lichens, is a dibenzofuran (Cocchietto *et al.*, 2002; Ingólfssdóttir, 2002). The anthraquinones include several orange substances that are synthesized by lichens, such as chrysophanol, emodin, fallacinol, parietin or parietinic acid (Huneck and Yoshimura, 1996).

Parietin is the most common anthraquinone in lichens, which occurs in species of the families Teloschistaceae, Brigantiaceae, Letrouitiaceae and Psoraceae (Solhaug and Gauslaa, 1996; Hafellner, 1997; Johansson *et al.*, 2005) and furthermore in the non-lichenized Plectomycetes (Engstrom *et al.*, 1980), which are thought to have lichenized ancestors (Lutzoni *et al.*, 2001). Pulvinic acid and its derivatives, such as calycin, epanorin, pulvinic acid lactone as well as leprapinic, pinastric, rhizocarpic and vulpinic acids form a group of mostly yellow pigments (Huneck and Yoshimura, 1996). While anthraquinones and dibenzofurans are synthesized through the acetyl-polymalonyl pathway, pulvinic acid is formed on the shikimic acid pathway (Huneck, 1999).

Although it is well known that many lichens contain yellow or orange pigments (Fig. 1), it has not yet been studied why different substances occur in different lichen symbioses. To achieve protection against energy-rich solar irradiation, the evolution of just one pigment with absorption maxima in the blue and ultraviolet range of the electromagnetic spectrum would theoretically have been sufficient. The existence of more than one substance with such properties in lichens could be the result of the repeated development of the lichen symbiosis in the course of evolution (Gargas *et al.*, 1995) or suggest that the yellow pigments have other functions in addition to light absorption that may have contributed to the evolution of different substances.

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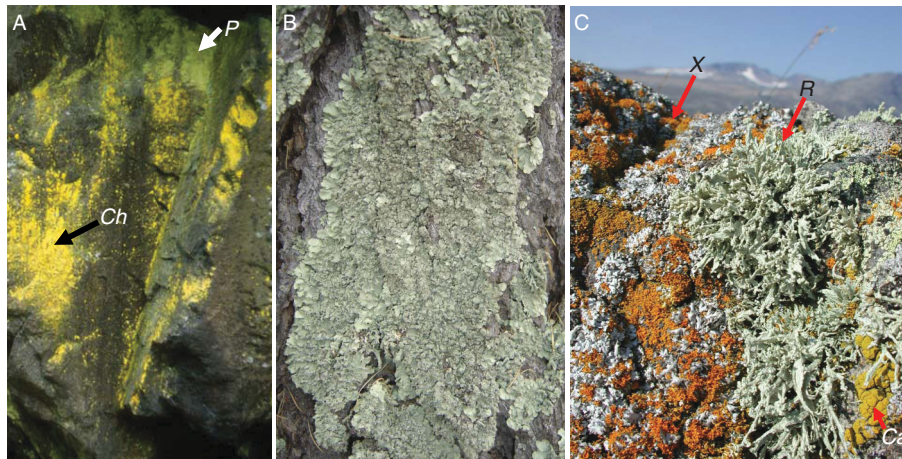


FIG. 1. Examples of lichen symbioses with yellow lichen substances: (A) *Chrysothrix chlorina* (Ch) with the pulvinic acid derivatives calycin, leprapinic acid and vulpinic acid as well as *Psilolechia lucida* (P) with the pulvinic acid derivative rhizocarpic acid growing on siliceous rock; (B) *Flavopunctelia soledica* with the dibenzofuran usnic acid growing on acidic conifer bark; (C) *Candelariella coralliza* (Ca) with pulvinic acid and its derivatives calycin and pulvinic acid dilactone, *Ramalina polymorpha* (R) with usnic acid and *Xanthoria candelaria* (X) with the anthraquinone parietin co-occurring on nutrient-rich siliceous rock. Photographs by M. Hauck (A, B) and E. Tindal (C).

In the present paper, the hypothesis is tested that the evolution of different yellow and orange lichen substances is a response to habitats differing in pH and in the availability of metal ions. This hypothesis was tested because the acidity tolerance of lichens was recently shown to be influenced by the content of lichen substances (Hauck, 2008a; Hauck and Jürgens, 2008). Lichens with the dibenzofuran usnic acid were shown to prefer ambient pH values close to the first dissociation constant (pK_{a1}) of this compound (Hauck and Jürgens, 2008). Furthermore, lichen substances were found to be involved in metal homeostasis of lichens (Hauck and Huneck, 2007a, b; Hauck *et al.*, 2007b; Hauck, 2008b). Since both the dissociation and the affinity of lichen substances to metal ions depend on pH (Sharma and Jannke, 1966; Takani *et al.*, 2002), it is reasonable to assume that the evolution of different yellow lichen substances may be an adaptation to different pH conditions. Therefore, pK_{a1} values of yellow lichen substances were determined and the pH dependence of the formation of complexes between these metabolites and metal ions was studied using UV spectroscopy. The results of these experiments were compared with published preferences for ambient pH values of lichens producing yellow or orange lichen substances (Wirth, 1995).

MATERIALS AND METHODS

Determination of pK_{a1} values

Dissociation constants of lichen substances were determined in methanol using an Uvikon 922 UV spectrophotometer (Bio-Tek Kontron, Neufahrn, Germany). Methanol was used as a solvent, because most lichen substances are highly soluble in it and because important physico-chemical properties of methanol influencing the dissociation of dissolved acids resemble those of water. Water, which would be the most realistic solvent in terms of the ecological relevance of the dissociation data, could not be used for the determination of the dissociation constants because of the low solubility of

most lichen substances in water. Methanol was preferred over Brij 35, recently applied by Hidalgo *et al.* (2002, 2005) for the determination of dissociation constants of lichen substances, because many lichen substances are more soluble in methanol than in Brij 35. Furthermore, spectroscopic results obtained in preliminary tests with different solvents were more highly reproducible with methanol than with Brij 35, which might be due to the micellar nature of the Brij 35 solutions. Acetonitrile (Wang *et al.*, 2001) was not used, because its aprotic character suppresses the dissociation of acids and leads, therefore, to very high dissociation constants (Jover *et al.*, 2008), which probably do not realistically reflect their dissociation behaviour in the aqueous environment found in the lichen thallus (Hauck and Jürgens, 2008). Compared with water, methanol is similar with regard to two important characters controlling the dissociation of acids dissolved in these liquids, namely the proticity and the Lewis basicity (donor number 19.0 kcal mol⁻¹ for methanol versus 18.0 kcal mol⁻¹ for water; Stangret, 2002) so that the use of methanol was obvious.

Isolated lichen substances were dissolved in methanol in a concentration of 10 µM. The pH was adjusted with buffer solutions to pH values ranging from 1 to 13 (Fixanal, Sigma-Aldrich, Steinheim, Germany). The methanol solution and the buffer were mixed in a ratio of 1 to 3, so that the final concentration of the lichen substances amounted to 3.3 µM. Lichen substances included commercially available dibenzofuran (+)-usnic acid (Sigma-Aldrich) and anthraquinone parietin (Carl Roth, Karlsruhe, Germany). Five substances of the pulvinic acid group were obtained from the collections of isolated lichen substances of S. Huneck (leprapinic, pinatric, pulvinic and rhizocarpic acids) or G. B. Feige (vulpinic acid) now deposited at the Botanical Museum, Berlin-Dahlem, Germany. Solutions were stirred and left at room temperature for 1 d before measurements were taken.

Absorbance between 220 nm and 500 nm was recorded every 1 nm at pH 1 and pH 13 to detect the absolute and relative absorption maxima of the individual lichen substances.

Absorbance versus pH was then measured at the absorption maxima at 20 °C. Wavelengths selected for these measurements were as follows: (+)-usnic acid, $\lambda=325$ nm; parietin, 438 nm; leprapinic acid, 371 nm; pinastric acid, 347 nm; pulvinic acid, 347 nm; rhizocarpic acid, 325 nm; vulpinic acid, 346 nm. All measurements were replicated six times. Since the buffer solutions could not completely compensate for changes in the pH after the addition of the lichen substance–methanol solutions, the pH was measured subsequent to the spectroscopic analysis with an MP 120 pH meter with an InLab 413 electrode (Mettler-Toledo, Greifensee, Switzerland).

The dissociation constants can be deduced from the plots of the absorbance taken up against the pH. The graphs were fitted with Spline approximations using Xact 7.12 software (SciLab GmbH, Hamburg, Germany). The turning points of these graphs (i.e. the points where the second derivation is zero) equal the dissociation constants (Sharma and Jannke, 1966; Jankulovska *et al.*, 2006). Mean curves calculated from the replicate measurements of the individual lichen substances were used for the determination of the dissociation constants. As the lichen substances dissociate incompletely, only the pK_{a1} values are thought to be physiologically relevant (Hauck and Jürgens, 2008). Therefore, the determination of dissociation constants was limited to the pK_{a1} values.

pH dependence of metal complexation

Complex formation of metal ions with each lichen substance was investigated for selected macro- and micro-nutrients (Cu^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} and Zn^{2+}). These ions were supplied as acetylacetonate salts, because the corresponding acid, acetylacetonone, is a weak acid [pK_a (in water)=9.0] (Pearson and Dillon, 1953) and is therefore less likely to disturb the formation of metal complexes than would be the case if, for example, chloride, nitrate or sulfate salts had been applied. The pH was adjusted with Fixanal buffer solutions varying between pH 2 and pH 12. Isolated lichen substances were dissolved in methanol. Final concentrations both of the lichen substances and the metal ions amounted to 3.3 μM . Absorbance maxima were determined by measuring the absorbance of all pairs of metals and lichen substances between $\lambda=220$ and 900 nm at pH 12, as maximum complex formation was assumed at high pH (Takani *et al.*, 2002). Wavelengths selected for recording absorbance versus pH are compiled in Table 1. Solutions with lichen substances and metal ions were measured against a blank differing only in the lack of the lichen substance. Five replicate measurements

TABLE 1. Wavelengths (in nm) applied in the UV spectroscopic study of complex formation of metal ions with lichen substances

	Cu^{2+}	Fe^{2+}	Fe^{3+}	Mg^{2+}	Mn^{2+}	Zn^{2+}
Usnic acid	282	281	282	286	281	281
Parietin	297	256	295	256	295	294
Leprapinic acid	293	291	293	293	292	294
Pinastric acid	293	293	293	293	293	294
Pulvinic acid	293	294	294	294	294	293
Rhizocarpic acid	293	292	289	293	292	292
Vulpinic acid	294	294	293	294	294	295

were carried out and mean curves were calculated from these replicate measurements. The pH of all solutions was determined after the spectroscopic measurements.

RESULTS

All lichen substances studied are strong to moderate acids with pK_{a1} values varying between 2.8 and 4.5 (Table 2 and Figs 2 and 3). The UV spectroscopic analyses of metal–lichen substance solutions suggest that complex formation took place in all pairwise combinations of metal ions (Cu^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} and Zn^{2+}) and lichen substances (Figs 4–7).

Metal complexation by usnic acid was primarily observed under acidic conditions (Fig. 4). Absorbance of solutions containing Fe^{2+} , Fe^{3+} , Mg^{2+} or Mn^{2+} and usnic acid started to increase between pH 2.0 and pH 2.5 (Fig. 4A and B). Maximum absorbance indicating complete metal binding was achieved at pH 4.5 for Fe^{2+} and Mg^{2+} (Fig. 4A), pH 5.5 for Fe^{3+} and pH 7.5 for Mn^{2+} (Fig. 4B). Absorbance of the solutions with Cu^{2+} and usnic acid increased between pH 3.5 and pH 6.5 (Fig. 4B), whereas the absorbance of solutions with Zn^{2+} and usnic acid increased between pH 4.5 and pH 6.5 (Fig. 4C).

TABLE 2. pK_{a1} values of yellow or orange lichen substances in methanol

Lichen substance	pK_{a1}
Usnic acid	4.0
Parietin	3.9
Leprapinic acid	3.2
Pinastric acid	2.8
Pulvinic acid	2.8
Rhizocarpic acid	4.5
Vulpinic acid	3.4

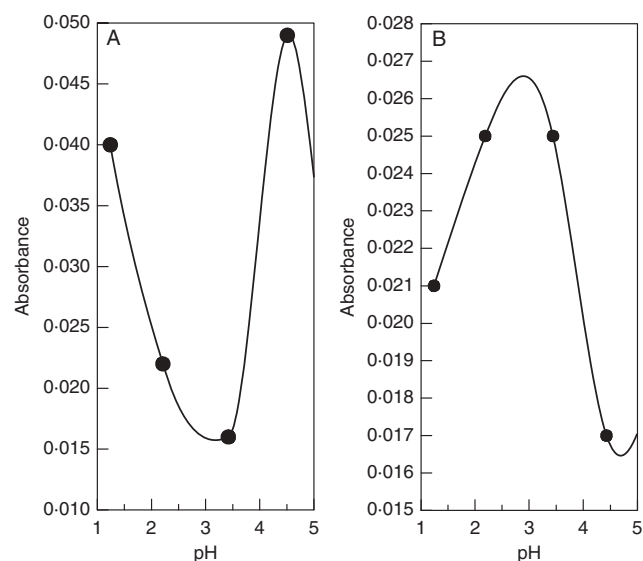


FIG. 2. UV spectroscopic results showing the dissociation of (A) the dibenzofuran usnic acid and the (B) anthraquinone parietin between pH 1 and pH 5. Turning points indicate pK_{a1} values.

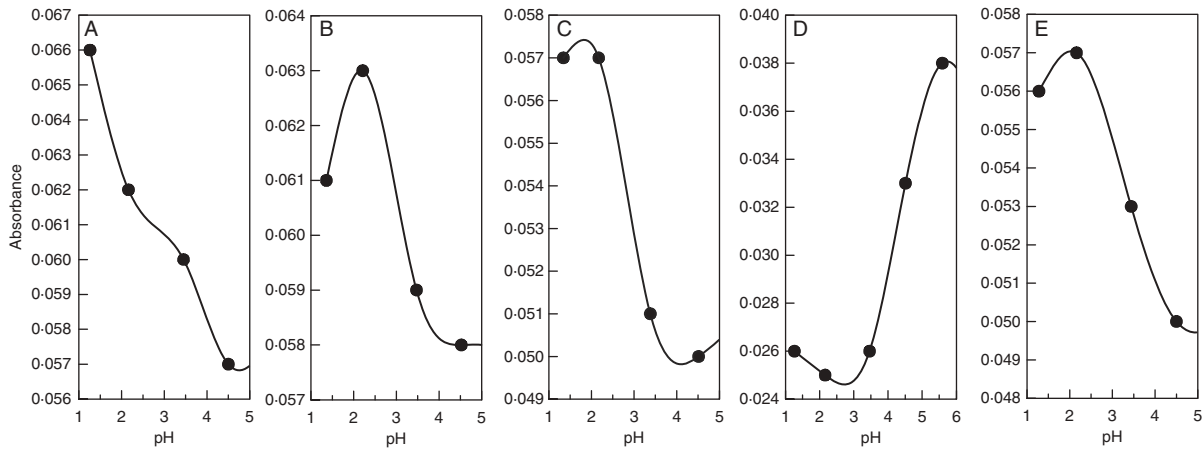


FIG. 3. UV spectroscopic results showing the dissociation of pulvinic acid (A) and its derivatives leprapinic acid (B), pinastric acid (C), rhizocarpic acid (D), and vulpinic acid (E) between pH 1 and pH 5 (A–C, E) or pH 1 and pH 6 (D). Turning points indicate pK_{a1} values.

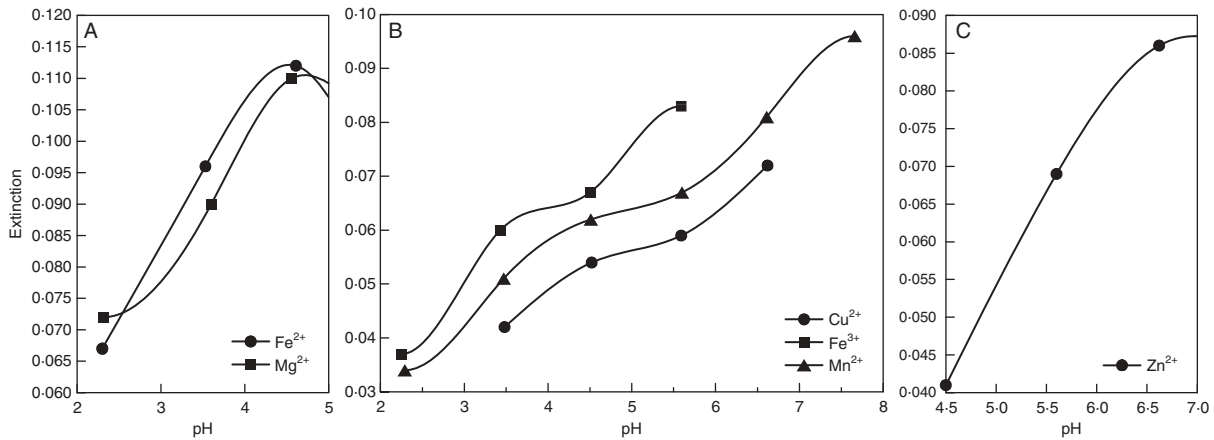


FIG. 4. pH dependence of metal-binding by usnic acid: (A) Fe²⁺, Mg²⁺; (B) Cu²⁺, Fe³⁺, Mn²⁺; (c) Zn²⁺.

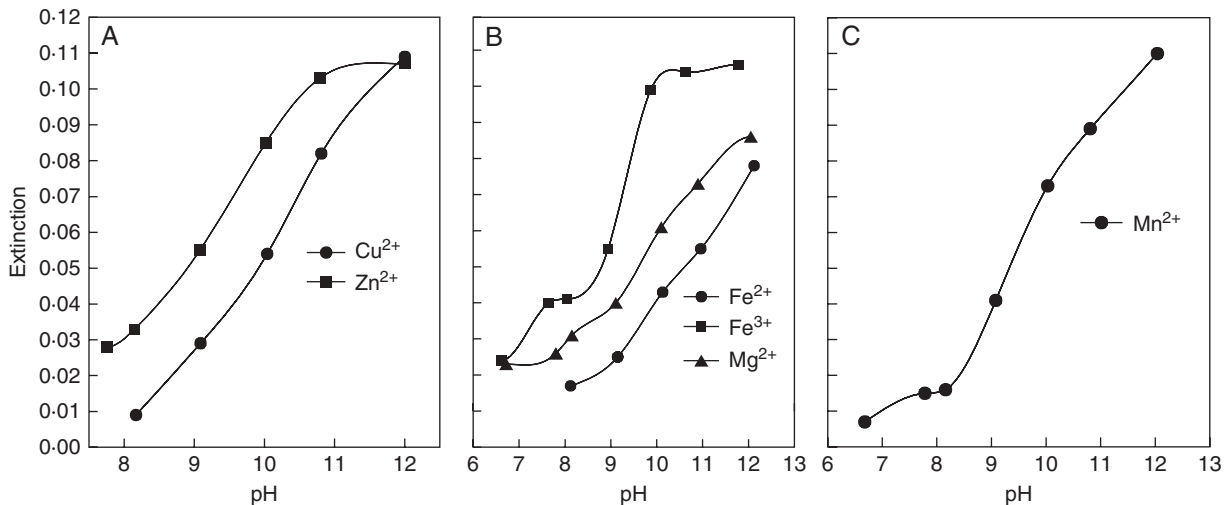


FIG. 5. pH dependence of metal-binding by parietin: (A) Cu²⁺, Zn²⁺; (B) Fe²⁺, Fe³⁺, Mg²⁺; (C) Mn²⁺.

In contrast to the usnic acid, complex formation between parietin and metal ions was found under alkaline conditions (Fig. 5). The absorbance of parietin solutions mixed with Fe²⁺, Mg²⁺ or Mn²⁺ increased at a pH >6.5 (Fig. 5B, C). If parietin was mixed with Cu²⁺, Fe³⁺ or Mn²⁺, absorbance

started to increase around pH 8 (Fig. 5A, B). In all cases, maximum absorbance was reached at pH 12 (Fig. 5).

The pigments of the pulvinic acid group formed complexes with metal ions either in the alkaline range (leprapinic, pinastric, pulvinic and vulpinic acids; Fig. 6) or both at low and high

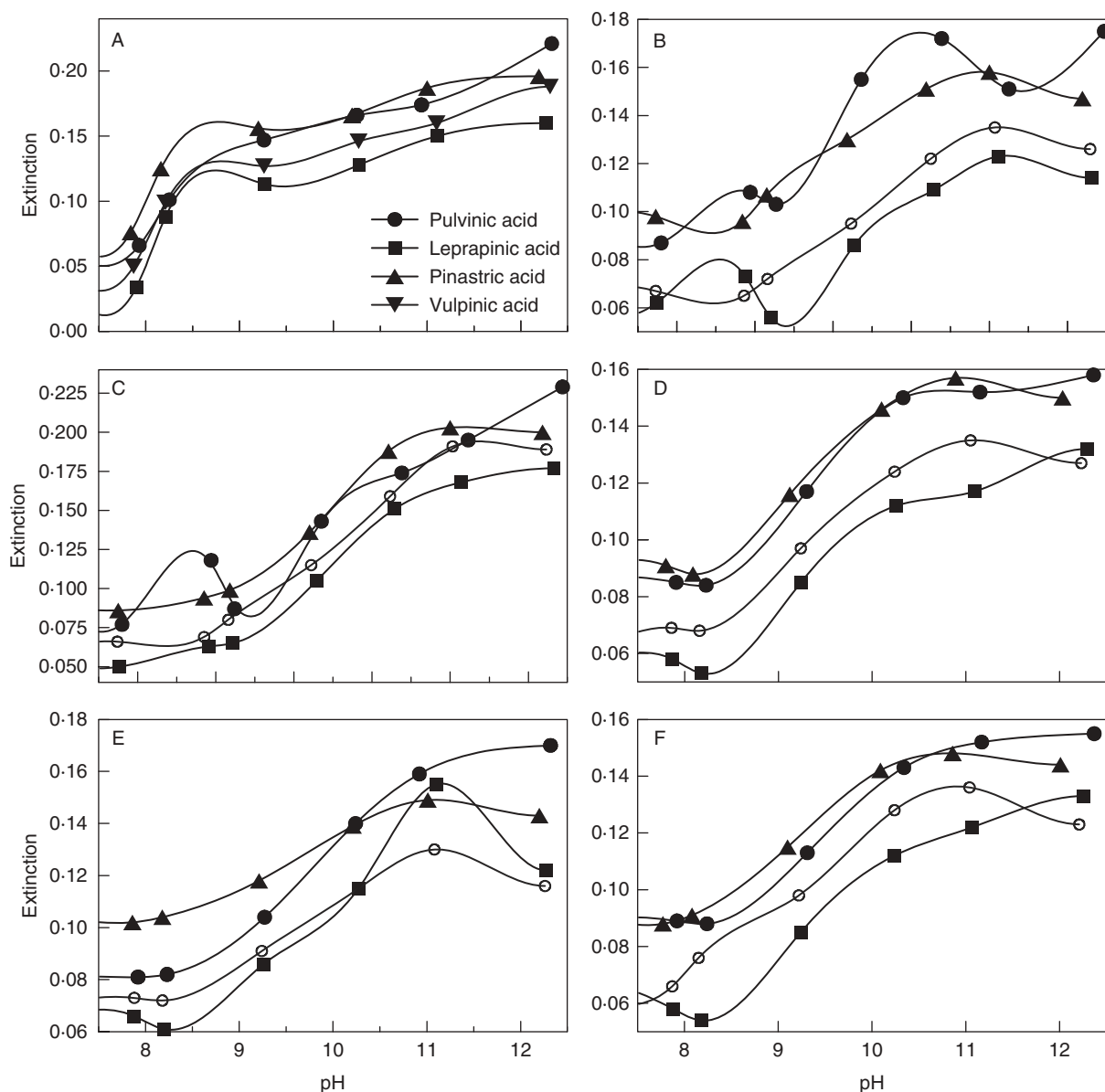


FIG. 6. pH dependence of metal-binding by pulvinic acid and its derivatives leprapinic, pinastric and vulpinic acids: (A) Cu^{2+} , (B) Fe^{2+} , (C) Fe^{3+} , (D) Mg^{2+} , (E) Mn^{2+} , (F) Zn^{2+} .

pH values (rhizocarpic acid; Fig. 7). While all the compounds of the pulvinic acid group investigated showed increased absorbance values at $\text{pH} > 8$ with Mn^{2+} and Zn^{2+} , the metal-binding behaviour differed between the individual lichen substances for Cu^{2+} , Fe^{2+} , Fe^{3+} and Mg^{2+} . Leprapinic, pinastric, pulvinic, and vulpinic acids exhibited increasing absorbance values with Cu^{2+} at $\text{pH} > 7$ and with Mg^{2+} at $\text{pH} > 8$ (Fig. 6). With Fe^{2+} , the absorbance of the leprapinic, pulvinic and vulpinic acids solutions increased at $\text{pH} > 8$, whereas that of the pinastric acid solution increased at $\text{pH} > 7$. With Fe^{3+} , absorbance increased at $\text{pH} > 8$ with pulvinic acid, at $\text{pH} > 6$ with leprapinic and pinastric acids, and at $\text{pH} > 5$ with vulpinic acid (Fig. 6). Rhizocarpic acid exhibited two different pH ranges with increasing absorbance values (Fig. 7). With Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} , absorbance increased

from pH 2 to pH 4 or pH 2 to pH 5 and from pH 8 to pH 12. With Fe^{3+} , increasing absorbance was found between pH 2 and pH 5.5 and between pH 9 and pH 12 (Fig. 7).

DISCUSSION

pK_{a1} values of lichen substances

Dissociation constants have rarely been determined for lichen substances. Published values are available for the dibenzofuran usnic acid (Sharma and Jannke, 1966), the depsidone lobaric acid (Hidalgo *et al.*, 2005), the anthraquinones parietin, emodin and chrysophanol (Wang *et al.*, 2001) and the pulvinic acid derivatives calycin and rhizocarpic acid (Hidalgo *et al.*, 2002). However, the dissociation constants of these

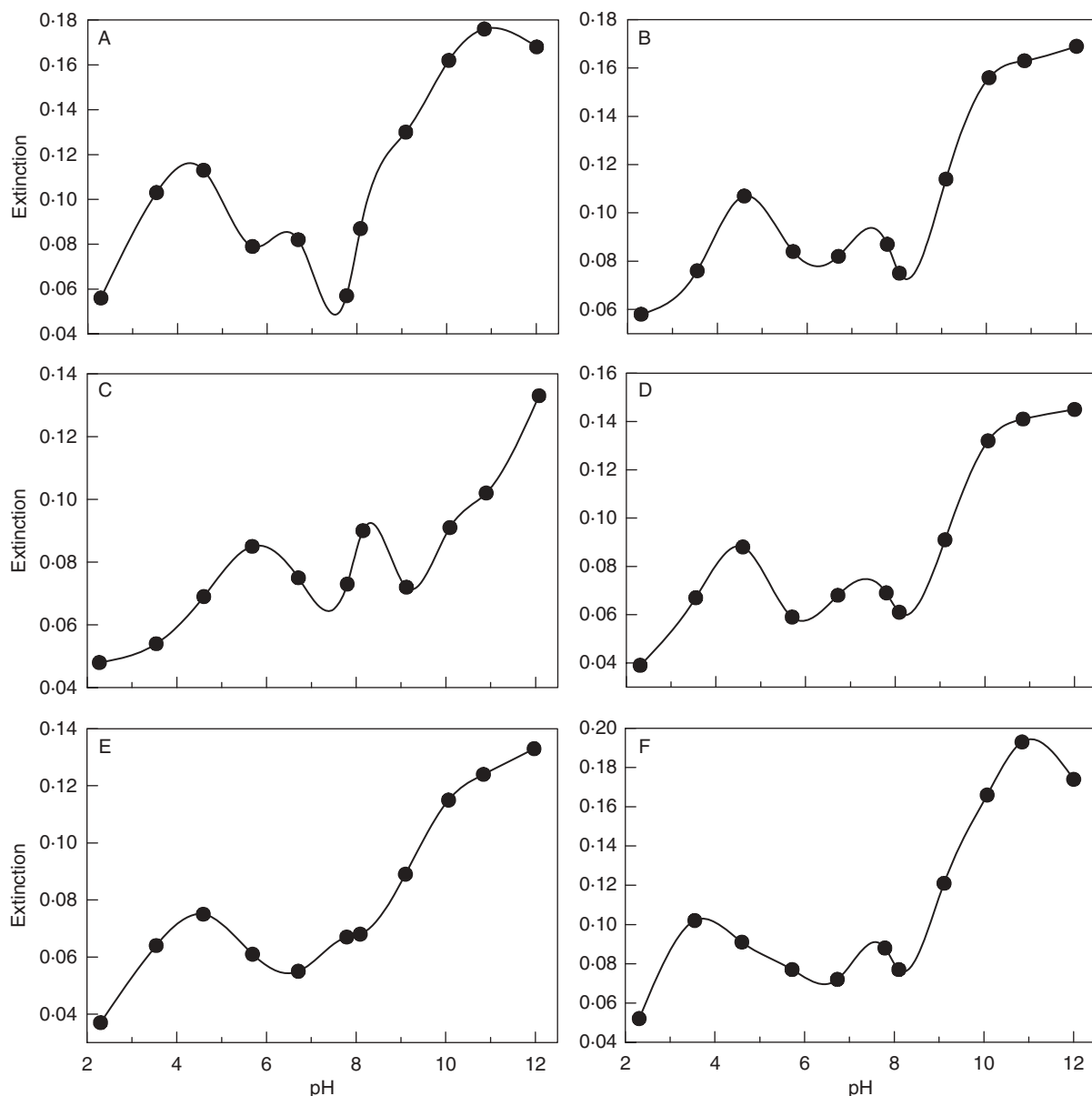


FIG. 7. pH dependence of metal-binding by the pulvinic acid derivative rhizocarpic acid: (A) Cu²⁺, (B) Fe²⁺, (C) Fe³⁺, (D) Mg²⁺, (E) Mn²⁺, (F) Zn²⁺.

compounds were determined in different solvents, limiting comparisons of the results (Bordwell, 1988; Jover *et al.*, 2008). Therefore, pK_{a1} values of usnic and rhizocarpic acids as well as parietin were re-determined in the present study despite the results of Sharma and Jannke (1966), Wang *et al.* (2001) and Hidalgo *et al.* (2002).

The present method for the determination of dissociation constants is most similar to that of Sharma and Jannke (1966) for usnic acid. The difference in the dissociation constants determined in the present study ($pK_{a1}=4.0$) and by Sharma and Jannke (1966) ($pK_{a1}=4.4$) can be attributed to the different solvents, as Sharma and Jannke (1966) used a mixture of chloroform and methanol, whilst pure methanol was the solvent in the present study. The similarity of these results for usnic acid also supports the validity of the present method.

The dissociation constants for rhizocarpic acid differ more between the present study ($pK_{a1}=4.5$) and Hidalgo *et al.* (2002) ($pK_{a1}=5.1$). However, the experimental conditions also differed more strongly than in the comparison made for usnic acid. Hidalgo *et al.* (2002) used Brij 35 as a solvent, which is chemically quite different from methanol. This has also to be kept in mind when the dissociation constants determined in the present study for compounds of the pulvinic acid group are compared with the pK_a of 4.9 published by Hidalgo *et al.* (2002) for calycin, a pulvinic acid derivative not included in the present study. The present result for parietin ($pK_{a1}=3.9$) is very different from that of Wang *et al.* (2001), who published a pK_{a1} of 8.5 in acetonitrile. This difference for parietin is likely to be a solvent effect, as pK_a values in acetonitrile are generally considerably higher than in methanol, because the former is an aprotic solvent, whereas the protic nature of the

latter promotes the dissociation of dissolved molecules (Jover *et al.*, 2008). Furthermore, the higher Lewis basicity of methanol (donor number 19.0 kcal mol⁻¹) than of acetonitrile (14.1 kcal mol⁻¹) supports dissociation (Arnaud-Neu *et al.*, 2003).

Relations between pK_{a1} values of lichen substances and ambient pH values of lichens producing these lichen substances

Hauck and Jürgens (2008) suggested that the acidity tolerance of usnic acid-containing lichens is limited by the ability of protonated usnic acid to shuttle protons into the cytoplasm. The ability of usnic acid to pass phospholipid membranes and thereby to shuttle protons had already been experimentally proven by Abo-Khatwa *et al.* (1996) and Bačkor *et al.* (1997). Abo-Khatwa *et al.* (1996) also found protonophoric properties in the pulvinic acid derivative vulpinic acid, which was also treated in the present study, and in the depside atranorin. The protonophoric properties of lichen substances apparently limit the tolerance of lichens to the acidity of precipitation or leachates from the substratum, as increasing acidity in the environment leads (in the small water-soluble fraction of the lichen substance) to a shift of the equilibrium between the protonated lichen substance and its conjugated base towards the former. If a large proportion of the water-soluble lichen substance is charged with protons in the apoplasts, substances capable of crossing the membrane are assumed to cause an acidification of the cytoplasm and thereby the death of the cells (Hauck and Jürgens, 2008). Support for this assumption was found in the case of usnic acid, as Hauck and Jürgens (2008) established a relationship between the pK_{a1} value of usnic acid and the pH preferences of lichens producing usnic acid. Moreover, lichens with their natural content of usnic acid were found to be less tolerant of acidic solution with SO₂ than lichens where the usnic acid was extracted at the beginning of the experiment (Hauck and Jürgens, 2008). These results explain why usnic acid-containing lichens decline at an environmental pH < 3.5 (Hauck and Jürgens, 2008), where the protonated form of water-soluble usnic acid prevails over the unseated ion.

Given the finding of Abo-Khatwa *et al.* (1996) that protonophoric properties are not limited to usnic acid, but are perhaps a common character of lichen substances, the pK_{a1} value of lichen substances becomes ecologically significant, as it determines the lower limit of the environmental pH range a lichen is able to tolerate. Studies of usnic acid suggest that lichens can tolerate ambient pH values slightly below the pK_{a1} values of their lichen substances. This would mean that all lichens with usnic acid, parietin or the studied compounds of the pulvinic acid group are generally able to grow in acidic environments corresponding to pK_{a1} values between 2.8 and 4.5 (Table 2). Inferred from the pK_{a1} values, the tolerance of lichens producing yellow or orange lichen substances to acidic solutions may increase in the order rhizocarpic acid < usnic acid < parietin < vulpinic acid < leprapinic acid < pinastric and pulvinic acids.

Metal complexation by lichen substances

The results of the UV spectroscopy suggest that complex formation of lichen substances with metals is common. This

contrasts with a relatively small number of previously published reports on the formation of metal–lichen substance complexes. Crystallographic data that give direct evidence of the formation and molecular structure of such complexes are only available for a few substances (Alagna *et al.*, 1990; Ribár *et al.*, 1993; Takani *et al.*, 2002). One of them is a complex of Cu²⁺ and an usnic acid derivative showing that each Cu²⁺ ion is co-ordinated with two molecules of usnic acid (Takani *et al.*, 2002). Purvis *et al.* (1987, 1990) provided indirect evidence of the formation of complexes of Cu²⁺ with the depsidones norstictic and psoromic acids. Spectroscopic data suggesting the formation of metal–lichen substance complexes have been published repeatedly (e.g. Syers 1969; Iskandar and Syers 1972). One of these studies dealt with a complex of Fe³⁺ and parietin (Engstrom *et al.*, 1980). Arakawa *et al.* (2001) demonstrated the formation of complexes of the anthraquinone chrysophanol with Al³⁺, Ni²⁺ and Zn²⁺.

Prior to this study, the pH dependence of complex formation between metals and lichen substances has only been investigated in a single UV spectroscopic experiment. This was conducted with Cu²⁺ and usnic acid and showed the same sigmoid increase of absorbance at pH > 3.5 as in the present study despite the application of different solvents (methanol versus chloroform) (Takani *et al.*, 2002). The pH dependence is attributable to the release of 1 mol protons per 1 mol Cu²⁺ during the formation of the complex (Takani *et al.*, 2002).

Significance of metal binding by lichen substances for metal homeostasis

Based on their spectroscopic detection of the Fe³⁺–parietin complex, Engstrom *et al.* (1980) were the first to suggest a potential role of lichen substances in the metal homeostasis of lichens; however, they did not conduct any experiments to test this hypothesis. Such a function of lichen substances was later substantiated in a case study with *Hypogymnia physodes* (Hauck, 2008b). The lichen substances produced by *H. physodes* selectively reduced the intracellular uptake of Cu²⁺ and Mn²⁺ (Hauck, 2008b), which, at high concentrations, are known to limit the abundance of this lichen in the field (Hauck, 2003; Hauck and Paul, 2005). The uptake of Fe²⁺ and Zn²⁺, which usually do not occur in high limiting concentrations at the sites of *H. physodes*, was not influenced by the content of lichen substances (Hauck, 2008b). Experiments with isolated lichen substances suggested that depsidone physodalic acid is the most effective metabolite at modifying metal uptake among the seven lichen substances known from *H. physodes* (Hauck and Huneck, 2007a).

The mechanisms by which lichen substances affect metal uptake in lichens are not yet known. Engstrom *et al.* (1980) hypothesized that lipophilic Fe³⁺–parietin complexes could cross phospholipid membranes and could, thus, support the uptake of Fe³⁺. Purvis *et al.* (1987, 1990) found that complexes of the depsidones norstictic and psoromic acids with Cu²⁺ remained in the apoplast and apparently served in the immobilization of Cu²⁺ in lichen thalli from cupriferous substrata.

The pH dependence of metal binding by lichen substances found in the present study and by Takani *et al.* (2002) suggests

that lichen substances may promote metal uptake by temporary intracellular binding at a pH range with modest affinity of the metal to the lichen substance. This hypothesis is supported by the fact that the ambient optimum pH of 4.0–4.5 for usnic acid-containing lichens (Hauck and Jürgens, 2008) is in a range where important nutrients, including Cu^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+} and Mn^{2+} , have a medium–high affinity to usnic acid (Fig. 4). The spectroscopic data suggest that complete binding of metal ions to lichen substances at high pH values could hamper intracellular cation uptake. This is surely an advantage at sites with high metal concentrations (Purvis *et al.*, 1987, 1990), but could lead to deficiencies at sites with low nutrient availability. Rapid binding of nutrients is an important issue for lichens that acquire most of their mineral nutrients during phases with precipitation or when the substratum is wet. Carboxylic and hydroxylic groups in the cell wall or in exopolysaccharide matrices are known to function as cation exchange sites in the apoplast of lichens (Brown and Brown, 1991; Paul *et al.*, 2003), from which delayed uptake into the cytoplasm occurs (Hauck *et al.*, 2006). Lichen substances could be an additional means of enabling temporary extracellular metal binding for subsequent intracellular uptake.

Assuming that pH ranges with complete binding of metal ions to lichen substances are unsuited for lichens producing these lichen substances at sites with normal metal concentrations, the spectroscopic data suggest that usnic acid hampers the uptake of $\text{Fe}^{2+/3+}$ and Mg^{2+} at $\text{pH} > 5$, the uptake of Cu^{2+} and Zn^{2+} at $\text{pH} > 7$ and the uptake of Mn^{2+} at $\text{pH} > 8$ (Fig. 4). In lichens producing parietin (Fig. 5) or with most compounds of the pulvinic acid group (Fig. 6), a reduction of intracellular metal uptake is only to be expected in the alkaline range at $\text{pH} > 11$, which is not ecologically relevant.

The pulvinic acid derivative rhizocarpic acid differs from all other lichen substances studied in having two pH ranges with increasing absorbance values, suggesting the formation of two different types of complexes (Fig. 7). This could imply that rhizocarpic acid facilitates the uptake of these metals at these two different pH ranges. Alternatively, strong binding of metal ions at the acidic binding site could rule out the occurrence of species with rhizocarpic acid at medium or high pH. The preference of rhizocarpic acid-producing lichens for nutrient-poor, acidic sites (Purvis *et al.*, 1992; Wirth, 1995) suggests that the latter alternative is the case.

Potential role of yellow lichen substances at determining site preferences of lichens

The dissociation and metal-binding behaviour of yellow and orange lichen substances can be used for a hypothetical model to explain the ability of lichens with such pigments to colonize substrata of different pH or metal availability. Lichens with usnic acid are excluded from sites with $\text{pH} < 3.5$, as the protonophoric activity of usnic acid is increased, if the pH falls significantly below the $\text{pK}_{\text{a}1}$ value of 4.0 (measured in methanol; Hauck and Jürgens, 2008). At $\text{pH} > 3.5$, lichens could benefit from the production of usnic acid, as the uptake of Mg^{2+} and transition metals could be promoted by temporary binding. The high affinity to metal ions at $\text{pH} > 5$ would explain the

upper pH limit of usnic acid-containing lichens (Hauck and Jürgens, 2008). Only a few species of lichenized ascomycetes with usnic acid occur on alkaline substrata, such as the saxicolous *Lecanora muralis* as well as the terricolous *Cladonia convoluta* or *Vulpicida tilesii* and *V. tubulosus* (Purvis *et al.*, 1992; Mattsson, 1993; Wirth, 1995). However, the occurrence of these species at high ambient pH values does not contradict the assumption that metal-binding by usnic acid determines the upper pH limit of lichens, because (a) the mentioned terricolous lichens are only loosely attached to the soil and can, therefore, be assumed to take up most of their nutrients from the atmosphere, and (b) these lichens contain other lichen substances with intermediate affinity to metal ions under slightly alkaline conditions, including pinastric and vulpinic acids in *V. tilesii* and *V. tubulosus* (Fig. 6) and fumarprotocetraric acid in *C. convoluta* (M. Hauck *et al.*, unpublished UV spectroscopic data). *Lecanora muralis* primarily contains usnic acid in the apothecia, which have no physical contact to the substratum, and produces less usnic acid on highly alkaline mortar than on more acidic sedimentary rock (Huneck *et al.*, 2004). Furthermore, usnic acid is only one of several lichen substances in *L. muralis* (Huneck *et al.*, 1979, 2004), which may influence metal uptake into this species.

The $\text{pK}_{\text{a}1}$ of 3.9 of parietin together with the metal-binding at high pH values (Fig. 5) may explain the wide pH range of substrata that are inhabited by parietin-containing lichens (Wirth, 1995). The largest genera of lichenized fungi producing parietin include the Teloschistaceae *Caloplaca*, *Teloschistes* and *Xanthoria* s.l. In *Caloplaca*, species of both acidic and alkaline substrata are found (Purvis *et al.* 1992; Wirth, 1995; Brodo *et al.*, 2001). Furthermore, *Xanthoria elegans* colonizes acidic rock and limestone (Purvis *et al.*, 1992). The spectroscopic results show that parietin is not able to support the uptake of metals at low pH (Fig. 5). This suggests that parietin-containing lichens of acidic substrata may be restricted to nutrient-rich sites because a support of metal uptake is not necessary in these conditions, whereas lichens with usnic acid prefer nutrient-poor sites. On calcareous soils and rock, the capability of parietin to complex metal ions at high pH can be expected to facilitate the uptake of micronutrients and Mg^{2+} under conditions of poor availability of these metals, a problem that generally excludes calcifuge plants from calcareous sites (Tyler and Ström, 1995).

Similar to parietin, substances of the pulvinic acid group putatively enable lichens to colonize a wide range of substrata. Among the compounds of the pulvinic acid group studied, the physico-chemical properties of leprapinic, pinastric, pulvinic and vulpinic acids differ significantly from those of rhizocarpic acid. The low $\text{pK}_{\text{a}1}$ values of leprapinic, pinastric, pulvinic and vulpinic acids between 2.8 and 3.4 allow lichens containing these pigments to grow on very acidic substrata, such as conifer bark as well as nutrient-poor, acid siliceous rock and sandy soils. Metal adsorption of these acids is limited to the alkaline range. This suggests that lichens with leprapinic, pinastric, pulvinic or vulpinic acids are generally able to colonize sites of a wide pH range. Indeed, lichens with these pigments include marked acidophytes (*Calicium*, *Chaenotheca*, *Chrysothrix*, *Letharia* and *Vulpicida*) as well as species of calcareous rock (*Candelariella aurella* and *C. medians*) (Purvis *et al.*, 1992; Wirth, 1995; Brodo *et al.*, 2001). In the genus

Candelariella, both species from acidic and calcareous substrates are known. As with the parietin-containing lichens, the acidophytes among the species of *Candelariella* (e.g. *Candelariella coralliza*, *C. kuusamoensis*, *C. vitellina*) are confined to nutrient-rich sites (Wirth, 1995). *Vulpicida* produces usnic acid in addition to pulvinic acid derivatives, the former being capable of binding metals under the acidic conditions preferred by most species of the genus (Mattsson, 1993). The species of *Calicium*, *Chaenotheca* and *Chrysothrix* with pulvinic acid lack usnic acid. However, these species apparently have extremely low nutrient requirements, as they are very sensitive to eutrophication (Wirth, 1995).

CONCLUSIONS

The pK_{a1} value of lichen substances has already been shown to determine the lower limit of the ambient pH range preferred by species of lichen-forming fungi in a case study with usnic acid (Hauck and Jürgens, 2008). The present results suggest that the dissociation behaviour of yellow or orange pigments generally allows all lichens producing these metabolites to grow on acidic substrata, at least as long as they are not acidified by atmospheric deposits. The different metal-binding characteristics of the various pigments can be used to explain different site preferences of the species of lichen-forming fungi producing these pigments. The high affinity of metal ions to usnic acid under alkaline conditions could explain why most usnic acid-containing lichens are calcifuge. At moderately acidic sites, lichens may benefit from temporary binding of nutrients to usnic acid. Such benefit is not possible for parietin and most compounds of the pulvinic acid group studied, as metal binding fails to occur at low pH. On the one hand, this could explain the preference of many lichens with these pigments for nutrient-rich sites at low pH, whereas lichens with usnic acid generally prefer nutrient-poor sites. On the other hand, metal-binding at low pH would probably cause nutrient deficiency for lichens at high pH. This may explain the occurrence of lichens with parietin, pulvinic acid and some related substances, but not of lichens with usnic acid or rhizocarpic acid on neutral or alkaline substrata, including limestone.

While a limitation of the acidity tolerance by usnic acid and the control of metal homeostasis by lichen substances have been experimentally proven (Hauck, 2008b; Hauck and Jürgens, 2008), other aspects related to the present conclusions deserve further study to detect the mechanisms behind the correlations demonstrated in here. The hypothesis that temporary binding of metals to extracellular lichen substances facilitates metal uptake, but permanent binding of metals to lichen substances at high pH impairs intracellular metal uptake has still to be substantiated by experimental work involving lichen thalli in addition to experiments based on isolated lichen substances.

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