Iron and phosphate uptake explains the calcifuge–calcicole behavior of the terricolous lichens *Cladonia furcata* subsp. *furcata* and *C. rangiformis*

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Abstract Mechanisms causing the calcifuge–calcicole behavior of lichens are largely unexplored. Studying the case examples of two closely related terricolous lichens, the calcifuge *Cladonia furcata* subsp. *furcata* and the calcicole *C. rangiformis*, we found that preference for acidic or calcareous soils in these lichens is related to iron and phosphate uptake as in vascular plants. In laboratory studies, the calcicole species was more efficient in the intracellular uptake of Fe^{3+} and phosphate at pH 8 than the calcifuge species. At pH 3, intracellular uptake of Fe^{2+} in the calcicole species significantly exceeded that in the calcifuge species suggesting that calcicole lichens suffer from toxicity symptoms by excess Fe^{2+} at acidic sites. Though these observations parallel findings from calcifuge and calcicole vascular plants, mechanisms leading to the different iron and phosphate uptake characteristics in the studied calcifuge and calcicole lichens may differ from those in vascular plants and should be the topic of future research. A role of the depside atranorin in facilitating iron uptake by reducing Fe^{3+} in the apoplast is hypothesized.

Keywords Lichenized ascomycetes · Acidic soils · Calcareous soils · Iron · Phosphorus

Introduction

Acidic and calcareous sites strongly differ in their vegetation. This is not only true for vascular plant vegetation, but also applies to lower plants. Only in the case of the vascular plants, however, the mechanisms causing these differences are sufficiently known. Calcifuge vascular plants are excluded from calcareous soils by their lacking ability to fulfill either their Fe or P requirements at high pH (Gries and Runge 1995; Tyler 1996; Zohlen and Tyler 2000). The availability of other transition metals, which are essential micronutrients (including Cu, Mn, Zn), can also be limiting (Gries et al. 1998). Calcicole plants can, in turn, suffer from high concentrations of transition metals or Al at acidic sites (Tyler 1993). In lichens, a marked calcifuge–calcicole behavior is known for terricolous and saxicolous species (Asta and Lachet 1978; Wirth 1972; Paus 1997). Moreover, epiphyte communities differ between acidic, nutrient-poor substrata and bark surfaces with high concentrations of cations at pH values around 7 (Barkman 1958).

Despite the similarities between lichens and vascular plants in the existence of calcifuge–calcicole behavior, the element uptake is principally different between these groups of organisms. While mineral

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uptake in vascular plants is generally limited to the root systems, lichens take up substances from the environment through the entire surface. Moreover, lichens use different element sources (Nash 2008). Aside from the soil or other substrates, minerals are taken up from atmospheric sources, including precipitation and the gaseous phase. A typical character of lichens is their ability to store large amounts of cations in the apoplast that can be taken up intracellularly with delay (Hauck et al. 2006). Cation-binding sites in the cell wall (Paul et al. 2003) and lichen secondary metabolites (Hauck et al. 2009) are apparently involved in this process.

Ecophysiological studies that would explain the calcifuge–calcicole behavior of lichens are virtually absent. Pintaric and Türk (1995) showed that lichens from calcareous sites generally contain significantly more Ca and Mg than lichens growing on acidic substrata. Fletcher (1976) studying the ecology of coastal saxicolous lichens suggested that the high Ca content of sea water, rather than the Na content, would be crucial for the zonation of lichen vegetation at sea shores. In his experiments, Fletcher (1976) found that the calcifuge lichen *Parmelia saxatilis* retained Ca and Mg more efficiently than the calcicole *Xanthoria parietina*, when thalli were rinsed with deionized water. Armstrong (1990, 1993) transplanted lichens from calcareous substrata on siliceous rock and, vice versa, lichens from acidic substrata on artificial lime-rich materials (cement, asbestos). The calcifuge lichens *Parmelia saxatilis* and *Xanthoparmelia conspersa* increased in size 15 months after transplantation to granite or slate, but died on lime. The calcicole lichens *Physcia tenella* and *Xanthoria parietina* could only grow on lime (Armstrong 1993), though the latter survived on slate if a paste of CaCO_3 or MgCO_3 was applied (Armstrong 1990). This effect depended on the anion, and thus evidently on pH, as CaCl_2 had no such positive effect on *X. parietina* (Armstrong 1990).

In the present study, we compared two closely related fruticose, terricolous lichens of the section Ascyphiferae (Stenroos et al. 2002) of the genus *Cladonia* with each other. *C. furcata* subsp. *furcata* grows on acidic soils in patchy grasslands or on siliceous rock outcrops, whereas the morphologically similar *C. rangiformis* prefers calcareous soils (Purvis et al. 1992; Wirth 1995). The two taxa were chosen for the study, because of their close systematic relationship, their morphological similarities, but their

marked differences for acidic versus calcareous soils. By soaking thalli of these species with solutions containing bivalent and trivalent iron or phosphate under acidic (pH 3) or alkaline (pH 8) conditions in the laboratory, we tested the hypothesis that the calcicole *C. rangiformis* is more efficient in the uptake of Fe and P than the calcifuge *C. furcata* subsp. *furcata* both under acidic and alkaline conditions. Verification of this hypothesis would imply that similar mechanisms could cause the calcifuge–calcicole behavior of lichens and vascular plants.

Materials and methods

Study species

C. furcata (Huds.) Schrad. subsp. *furcata* and *C. rangiformis* Hoffm. are two closely related members of the fruticose genus *Cladonia* with erect, dichotomously branched, corticated, hollow thalli (podetia) occasionally scattered with squamules. Morphologically *C. rangiformis* differs by more richly branching and the more areolated cortex from *C. furcata* subsp. *furcata* (Purvis et al. 1992; Wirth 1995). Both lichens have a rich secondary chemistry, including the depsidone fumarprotocetraric acid as the most common lichen substance in *C. furcata* subsp. *furcata* and atranorin as the most common compound in *C. rangiformis* (Huneck et al. 2004). The fatty acids rangiformic and norrangiformic acids are more frequent in *C. rangiformis* than in *C. furcata* subsp. *furcata*. Except for the fatty acid bourgeanic acid, which is only known from the latter species, all lichen substances generally occur in both species, but in different concentrations (Huneck et al. 2004). Samples used in the experiment were collected in Hesse, Germany (*C. furcata*: Hombressen, 10 km NW Eschwege, 51°30' N, 9°28' E; *C. rangiformis*: Frankershausen, 20 km N Kassel, 51°14' N, 9°55' E). It is important to note that *C. furcata* is used in the sense of *C. furcata* subsp. *furcata* throughout the paper, as *C. furcata* subsp. *subrangiformis* prefers calcareous soils (Wirth 1995).

Experimental details

Lichens were stored in air-dry condition at a temperature of -18°C in the dark before the experiment.

Freezing is not harmful to dry lichen thalli and is routinely applied in our work group for storing lichens prior to experiments. After unfreezing, thalli of *C. furcata* and *C. rangiformis* of 2–3 cm length were put on Petri dishes with moist cellulose filters. The thallus base, soil particles and contaminant plant material were carefully removed. Five thalli were put on each Petri dish; each five of these dishes served as replicates. The lichen dry weight of these replicates amounted to 301 ± 14 mg ($N=40$) in the sparsely branched *C. furcata* and 698 ± 18 mg in the more densely branched *C. rangiformis*. The Petri dishes were stored for 2 days at 80% relative humidity, a day temperature (for 13 h daily) of 13°C during a photon flux of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$, and a night temperature of 10°C in the growth chamber for acclimatization. Lichen samples were then exposed for 1 h to 30 mL of salt solution or to a control of deionized water on a shaker. Metal solutions included FeCl_2 , FeCl_3 , and KH_2PO_4 adjusted with HCl and NaOH to pH 3 or 8. The activities of the Fe^{2+} , Fe^{3+} or H_2PO_4^- ions in the solutions amounted to 2 mM. Calculations of activities were carried out with the software PHREEQC 2.0 (U.S. Geological Survey, Reston, Virginia, USA). Following the Henderson–Hasselbalch equation, the ratio of H_2PO_4^- to PO_4^{3-} amounted to 13,490 at pH 3, whereas the PO_4^{3-} ion preponderated at pH 8 with a ratio to H_2PO_4^- of 7.4. In the following, we refer to H_2PO_4^- and PO_4^{3-} as phosphate irrespective of the degree of dissociation.

Viability of lichen thalli was controlled by measuring the chlorophyll fluorescence yield (Φ_2) of the light-adapted samples at photosystem II prior to and after the exposure to the test solutions with a PAM-2100 chlorophyll fluorometer (Walz, Effeltrich, Germany). Two measurements were made per replicate. Φ_2 stayed invariably in a range between 0.64 and 0.69, irrespective of the treatment or the point in time, and the values are, thus, not displayed in the results section.

After the exposure to iron, phosphate or water, extra- and intra-cellular cations were sequentially extracted (Brown and Brown 1991; Hauck et al. 2002). For this purpose, samples were shaken twice with 30 mL of deionized water to remove free apoplastic ions. These water samples were not analyzed. Metal ions bound to hydroxylic or carboxylic exchange sites of the cell wall were exchanged by shaking samples twice each for 20 min with 30 mL

20 mM NiCl_2 . The two NiCl_2 solutions per sample were filtered with ash-free filters (Blue Ribbon Filters, Whatman-Schleicher & Schuell, Dassel, Germany) and pooled for analysis. Afterwards, samples were dried at 105°C , homogenized, and digested with 65% HNO_3 in order to determine the intracellular ions. Total concentrations of Fe, P and additionally K, Ca, and Mg in the NiCl_2 solutions and acid digests were determined with inductively coupled plasma atomic emission spectroscopy (ICP-AES, Spectraflame, Spectro Analytical Instruments, Fitchburg, Massachusetts, USA). A quantitative release of phosphate from the apoplast by the Cl^- ions of the NiCl_2 solution was not expected despite the high concentration of the NiCl_2 solution, because Cl^- is a weak competitor for potential cationic binding sites in the apoplast. Therefore, P concentrations of NiCl_2 and HNO_3 fractions were summated to a total P concentration.

Statistics

All data are given as arithmetic means $\pm$ standard error and were tested for normal distribution with the Shapiro–Wilk test. Samples were tested with Duncan’s multiple range test for the comparison of more than two means. Statistical analyses were calculated with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, USA).

Results

Iron uptake

Thalli of *C. furcata* subsp. *furcata* and *C. rangiformis* not exposed to iron chloride solution did not differ in their Fe content (Table 1). From the cation exchange sites in the apoplast of these thalli, Fe was virtually absent (Table 2). Intracellular uptake of Fe^{2+} was significantly higher in the calcicole *C. rangiformis* than in the calcifuge *C. furcata* at pH 3 (Table 1). At pH 8, no significant differences occurred in the intracellular Fe^{2+} uptake between these lichen species (Table 1). Extracellular Fe^{2+} adsorption was indifferent to pH in *C. furcata* and generally higher than in *C. rangiformis* (Table 2). In *C. rangiformis*, extracellular Fe^{2+} adsorption was negligible at pH 8, but occurred at pH 3 (Table 2). Intracellular Fe^{3+} uptake took place in both species, but was significantly higher in *C. rangiformis*

Table 1 Concentrations of intracellular Fe, K, Ca and Mg (in $\mu\text{mol g}^{-1}$ dry weight) in *C. furcata* and *C. rangiformis* after soaking with deionized water, FeCl_2 , or FeCl_3 ($a=2$ mM) at pH 3 or 8 for 1 h

	pH 3		pH 8	
	<i>C. furcata</i>	<i>C. rangiformis</i>	<i>C. furcata</i>	<i>C. rangiformis</i>
Control				
Fe	5.7±0.7 a ^a	6.4±0.6 a	7.0±0.6 a	4.1±0.3 a
K	56.0±5.9 a	24.8±2.0 bd	46.3±5.0 c	28.2±1.8 bd
Ca	3.5±0.4 a	107±14 bc	3.8±0.3 a	79.5±7.7 b
Mg	7.4±0.6 ab	15.8±3.2 c	6.7±0.4 a	14.0±1.6 c
FeCl_2				
Fe	13.7±1.2 a	43.5±2.7 b	39.7±4.2 bc	31.3±1.4 c
K	34.4±4.3 de	23.5±0.8 b	48.1±2.1 ac	30.3±0.7 bd
Ca	3.1±0.1 a	129±34 c	3.2±0.4 a	97.5±16.4 bc
Mg	5.1±0.7 a	13.1±2.1 bc	6.4±0.2 a	13.6±3.3 c
FeCl_3				
Fe	43.5±1.9 b	59.1±4.4 d	53.4±5.9 d	68.9±4.0 e
K	27.8±1.6 bd	21.6±0.8 b	43.5±5.2 ce	29.8±0.6 bd
Ca	2.4±0.3 a	105±14 bc	3.4±0.4 a	111±27 bc
Mg	5.4±0.3 a	10.8±1.4 abc	6.1±0.6 a	13.1±3.2 bc

^a For a given metal, means (±SE) followed by equal letters do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df=48$)

than *C. furcata* (Table 1). Notable amounts of extracellularly bound Fe^{3+} were found in both species at pH 3, but not at pH 8 (Table 2). The content of extracellular Fe^{3+} at pH 3 was even higher in *C. furcata* than *C. rangiformis* (Table 2).

Uptake of Fe^{2+} or Fe^{3+} had no influence on intracellular K, Ca or Mg concentrations (Table 1). Intracellular Ca and Mg concentrations were generally

much higher in *C. rangiformis* than *C. furcata* (Table 2). Ca concentrations were 20 to 40 times higher in the former than the latter species. Intracellular Mg concentrations in *C. rangiformis* exceeded those in *C. furcata* by the factor 2 or higher. Lower Ca and Mg concentrations in the cells of *C. furcata* were compensated by higher K concentrations, though they only differed between the species by the factor 2 in the

Table 2 Concentrations of Fe, K, Ca and Mg (in $\mu\text{mol g}^{-1}$ dry weight) bound to extracellular exchange sites in *C. furcata* and *C. rangiformis* after soaking with deionized water, FeCl_2 , or FeCl_3 ($a=2$ mM) at pH 3 or 8 for 1 h

	pH 3		pH 8	
	<i>C. furcata</i>	<i>C. rangiformis</i>	<i>C. furcata</i>	<i>C. rangiformis</i>
Control				
Fe	0.0±0.0 a ^a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
Ca	11.8±1.0 a	62.8±3.8 b	23.8±2.1 c	66.3±2.2 b
Mg	2.4±0.2 a	39.8±2.2 b	5.7±0.3 a	45.8±3.3 c
FeCl_2				
Fe	8.5±0.7 b	5.8±0.6 c	7.6±0.4 b	0.1±0.0 a
Ca	6.2±0.4 ae	42.2±2.4 d	9.2±0.7 ae	60.8±2.7 b
Mg	1.5±0.1 a	20.0±1.9 d	2.0±0.2 a	35.4±1.7 e
FeCl_3				
Fe	15.0±0.7 d	11.1±1.3 e	0.1±0.0 a	0.1±0.0 a
Ca	4.4±0.2 e	26.0±2.0 c	7.6±0.6 ae	35.4±4.1 f
Mg	1.2±0.1 a	13.3±1.4 f	1.6±0.2 a	14.5±1.4 f

^a For a given metal, means (±SE) followed by equal letters do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df=48$)

controls and even less after Fe uptake (Table 2). In both species, concentrations of Ca and Mg bound to extracellular exchange sites were moderately to appreciably reduced by Fe^{2+} and Fe^{3+} uptake in most treatments, though the differences were only partly significant (Table 2). However, Fe^{2+} uptake in *C. rangiformis* did not affect the extracellular Ca concentrations at pH 8 (Table 2). Extracellular K concentrations are not included in Table 2, because they are highly variable due to the low affinity of the K^+ ion to carboxylic and hydroxylic moieties and thus little informative.

Phosphate uptake

In the controls, the total P content in *C. furcata* exceeded that in *C. rangiformis* approximately by the factor 1.5 irrespective of pH (Fig. 1a). At pH 3, the efficacy of phosphate uptake did not differ between the species, as the total P content doubled during the treatment with KH_2PO_4 in spite of the different initial concentrations (Fig. 1b). At pH 8, the two lichens responded very differently to the exposure to phosphate: uptake was much more efficient in *C. rangiformis*, where the phosphate concentration increased by the factor 2.6, than in *C. furcata*, where the concentration merely increased by the factor 1.3 (Fig. 1b).

Discussion

Efficient intracellular Fe^{3+} and phosphate uptake qualifies *C. rangiformis* for the colonization of calcareous soils. The uptake of Fe^{2+} in *C. rangiformis* was relatively inefficient at pH 8, but this is of subordinate significance, as Fe^{3+} is prevailing under alkaline conditions. In *C. furcata* subsp. *furcata*, uptake of Fe^{3+} at pH 8 was significantly lower than in *C. rangiformis* and uptake of phosphate was nearly absent. This explains that *C. furcata* avoids calcareous soils. Much more efficient uptake of Fe^{2+} under acidic conditions in the calcicole *C. rangiformis* than in the calcifuge *C. furcata* explains the avoidance of acidic soils by *C. rangiformis*. Fe^{2+} is the prevalent form of iron under acidic conditions. The uptake of Fe^{2+} in *C. rangiformis* exceeded that in *C. furcata* by the factor 3.2 at pH 3, whereas the uptake of Fe^{3+} was only 1.3-times higher in *C. rangiformis* than *C. furcata*. Excess

Fig. 1 Total P concentrations of *Cladonia furcata* or *C. rangiformis* exposed for 1 h min to **a** deionized water or **b** KH_2PO_4 ($a=2$ mM) at pH 3 or 8. Means ($\pm$ SE) annotated with equal letters do not differ significantly (one common Duncan's multiple range test for data displayed in **a–b**; $P \leq 0.05$, $df=32$)

amounts both of Fe^{2+} and Fe^{3+} are known to cause chlorophyll degradation in lichens (Garty et al. 1992; Hauck et al. 2003).

As to be expected, our results agree with those of Pintaric and Türk (1995) that calcicole lichens contain considerably more Ca and Mg than calcifuge lichens. However, we could not confirm the hypothesis of Fletcher (1976) that calcifuge lichens would release their Ca and Mg less readily in exchange for other ions than calcicole lichens. Low intracellular concentrations of Ca and Mg in the calcifuge *C. furcata* were compensated by the accumulation of K (Table 1).

Our results suggest that, as in higher plants, Fe and P shortages exclude calcifuge lichens from calcareous soils and Fe hyperaccumulation excludes calcicole lichens from acidic sites. Whether the limited availability of essential transition metals other than Fe, including Cu, Mn and Zn (Gries et al. 1998), plays an

additional role at excluding calcifuge lichens from alkaline calcareous soils has still to be investigated. The same is true for the accumulation of potentially toxic amounts of Cu, Mn or Zn by calcicole lichens at acidic sites. It is plausible to assume that relations in saxicolous lichens are similar to those than in terricolous lichens. The extent of parallels to factors controlling the distribution of epiphytic lichens on acidic versus mineral-rich bark should be studied, because the least acidic bark is usually still slightly acidic (around pH 6) to neutral (Wirth 1995).

Despite the common significance of Fe and P in the control of pH preferences both in vascular plants and lichens, mechanisms causing the differences in the Fe and P uptake between calcifuge and calcicole species are likely to be quite different between vascular plants and lichens. Mechanisms of Fe and P uptake in lichens are still largely unexplored. High efficiency of phosphate uptake was demonstrated in two acidophytic lichens under acidic conditions (Farrar 1976; Hyvärinen and Crittenden 1998). Promotion of phosphate uptake in calcicole lichens by the exudation of low-molecular organic acids as in vascular plants (Ström et al. 1994; Tyler and Ström 1995; Zohlen and Tyler 2004) is conceivable, because the release of oxalate from lichens is known in the context of extracellular metal binding (Wilson and Jones 1984). Any involvement in the uptake of phosphate in lichens has not been investigated, though.

Parallels in increasing the uptake of Fe under alkaline conditions between vascular plants and lichens are perhaps limited. Vascular plants respond to Fe deficiency on calcareous soils with increased root formation, H^+ extrusion, increased reductase activity, or the production of phytosiderophores (Marschner et al. 1986; Römheld 1987; Von Wirén et al. 2000). The increase of the absorbing surfaces, analogously to increased root growth, is improbable to be realized in the slow-growing lichens lacking any root system. In contrast to vascular plants, the ability for intracellular uptake of Fe^{3+} by ferric permeases or siderophore permeases is apparently widespread in fungi (Kosman 2003; Eichhorn et al. 2006). The recent discovery that phenolic lichen substances are involved in the metal homeostasis of lichens (Hauck 2008) suggests that lichen substances could also facilitate the acquisition of Fe at high pH. This hypothesis was already published by Engstrom et al. (1980), but has never been scrutinized. A plausible

mechanism not yet discussed would be facilitation of Fe^{3+} uptake by using the reductive power of phenolic lichen substances analogously to caffeic acid in vascular plants. Caffeic acid promotes Fe uptake by supplying electrons for the extracellular reduction of Fe^{3+} to Fe^{2+} (Römheld and Marschner 1983; Deiana et al. 1995). The cortical depside atranorin, being a major lichen substance of the calcicole *Cladonia rangiformis* (Huneck et al. 2004), would be a favorite for a putatively Fe^{3+} -reducing lichen substance, because it functions, like caffeic acid (Lafay et al. 2005), as a strong antioxidant (Valencia-Islas et al. 2007). Remarkably, atranorin, which is rare in the calcifuge *C. furcata* subsp. *furcata*, is also a major secondary metabolite of the calcicole *C. furcata* subsp. *subrangiformis* (Wirth 1995).

Conclusions

Our results from the calcifuge *C. furcata* and the calcicole *C. rangiformis* suggest that Fe and/or phosphate uptake are, like in vascular plants, the key factor determining the preference of lichens for acidic and calcareous sites. Mechanisms causing the different efficacies of Fe and phosphate uptake at different pH in *C. furcata* and *C. rangiformis* or other calcifuge versus calcicole lichens are still unexplored. The present study is the first one substantiating the significance of Fe and P uptake for the calcifuge–calcicole behavior of lichens.

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