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The putative role of fumarprotocetraric acid in the manganese tolerance of the lichen *Lecanora conizaeoides*

Hauck & Huneck (2007) and Hauck *et al.* (2007) established that lichen secondary chemistry is related to metal absorption in the apoplast of lichens. Depsidones occurring in the medulla of the Mn^{2+} and Cu^{2+}-sensitive epiphytic lichen *Hypogymnia physodes* were found to reduce the absorption of Mn^{2+} and Cu^{2+} at cation exchange sites (Hauck & Huneck 2007). Several lichen substances, belonging to the depsidones, depsides, anthraquinones, or pulvinic acid derivatives, are known to absorb Fe^{3+} (Engstrom *et al.* 1980; Hauck *et al.* 2007). Occurrence of such lichen substances in lichens of Fe-poor sites, but rarity in lichens from Fe-rich sites suggests that they promote the intracellular uptake of Fe^{3+} (Hauck *et al.* 2007). In *Acarosporion sinopicae* lichens specialized on Fe-rich rock and slag, only two efficient Fe^{3+} absorbers are known, viz. the depsidone norstictic acid and the pulvinic acid derivative rhizocarpic acid. Their occurrence in *Acarosporion sinopicae* lichens is attributed to the fact that they reduce the absorption of Fe^{2+} at cation exchange sites despite their high affinity to Fe^{3+} (Hauck *et al.* 2007). The observations by Engstrom *et al.* (1980), Hauck & Huneck (2007) and Hauck *et al.* (2007), which were made in experiments with isolated lichen substances, suggest that these secondary metabolites control metal homeostasis in lichens by reducing the absorption of selected metal ions in the apoplast and by promoting the uptake of Fe^{3+}. The finding that heavy metal pollution induces the synthesis of the depsidone physodalic acid in *Hypogymnia physodes* (Białońska & Dayan 2005) supports this hypothesis, because physodalic acid is particularly efficient at reducing Cu^{2+} and Mn^{2+} absorption (Hauck & Huneck 2007). Extracellular metal absorption is relevant to metal homeostasis, as cations from the apoplast are belatedly transferred into the cytoplasm (Hauck *et al.* 2006).

Although physodalic acid in *H. physodes* apparently functions as a shield that reduces the absorption of Cu^{2+} and Mn^{2+} in the apoplast (Hauck & Huneck 2007), the species is still sensitive to high ambient concentrations of these ions (Hauck *et al.* 2001, 2002*a*; Hauck & Zöller 2003; Hauck & Paul 2005). *Lecanora conizaeoides*, an epiphyte which often co-occurs with *H. physodes*, is tolerant to high concentrations of Cu^{2+} and Mn^{2+} (Hauck *et al.* 2001, 2003). In the case of Mn^{2+}, tolerance mechanisms in *L. conizaeoides* have been studied in detail (Hauck & Paul 2005). In a comparative study of Mn^{2+} uptake in *L. conizaeoides* and *H. physodes*, effective intracellular immobilization of Mn^{2+} by the mycobiont was shown to be the key factor in determining the high Mn tolerance of *L. conizaeoides* (Paul *et al.* 2003). The intracellular deposits were polyphosphate bodies, which are also known from *H. physodes*, but accumulate less Mn^{2+} in this species, and sulphur-containing compounds (Hauck *et al.* 2002*b*; Paul *et al.* 2003).

Another difference that was observed between *L. conizaeoides* and *H. physodes* was significantly lower extracellular absorption of Mn^{2+} in the former (Hauck *et al.* 2002*c*). Since *L. conizaeoides* and *H. physodes* differ in their secondary chemistry, we tested the hypothesis that the main lichen substance in *L. conizaeoides*, i.e. the depsidone fumarprotocetraric acid, would cause lower absorption of Mn^{2+} at cation exchange sites than the lichen substances of *H. physodes*.

Pure fumarprotocetraric acid from the collection of S. Huneck was dissolved in acetone (2.5 mM) and

TABLE 1. *Absorption of Mn^{2+} from 35 µM $MnCl_2$ by filter paper soaked with 2·5 mM fumarprotocetraric acid dissolved in acetone**

	Mn concentration (µM)	Relative change of Mn concentration (%)†
Fumarprotocetraric acid	22·0 ± 0·3 a	14 ± 2 a
Control	19·3 ± 0·3 b	0 ± 2 b
Control, pretreated with $NiCl_2$	28·1 ± 0·3 c	46 ± 2 c

*Arithmetic means ± standard error. Statistics: Duncan's multiple range test (df=12; R^2=0·97, F=217, $P \leq 0{\cdot}001$); within a column, means sharing a common letter do not differ significantly.

†Relative change of Mn concentration after shaking two filter strips impregnated with different fumarprotocetraric acid or not (control) for 1 h in 35 µM $MnCl_2$. Control is set to 100%.

applied to ash-free cellulose filter paper (Blue Ribbon Filters, Schleicher & Schuell, Dassel, Germany) by shaking filter strips (c. 20 cm^2, 160 mg) in the lichen substance solution for 1 h. The filter strips served as standardized surfaces. Cellulose filters consist of randomly interlaced fibres which are littered with exchange sites that bind to metal ions, similar to the cell wall surfaces of lichens (Klemm *et al.* 1998). Untreated filter strips were used as controls. A second set of controls was pretreated with 20 mM $NiCl_2$ for 30 min. Ni^{2+} has a higher affinity to hydroxylic and carboxylic cation exchange sites than Mn^{2+} and is, therefore, thought to prevent most Mn^{2+} from being absorbed at the filter surface (Nieboer & Richardson 1980; Vázquez *et al.* 1999). Hence, comparison of the controls with and without $NiCl_2$ pretreatment provides information on the cation binding capacity of the filters. Two strips of impregnated filters per replicate were exposed for 1 h to 25 ml of 35 µM $MnCl_2$; the concentration of 35 µM referred to the optimum measuring range of the AAS used (Vario 6, Analytik Jena, Germany). After incubation, the filter paper was removed with forceps and the Mn concentration in the solution was analyzed. The experiment was run in five replicates. Statistical analyses were run with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.). Duncan's multiple range test was applied to test for significance of differences between means after testing for normal distribution with the Shapiro-Wilk test.

Approximately 55% of the Mn^{2+} dissolved in the incubation medium was absorbed by the untreated filters (Table 1). Absorption of only 20% of the Mn^{2+} by the filter pieces pretreated with $NiCl_2$ showed that binding at cation exchange sites accounts for most of the Mn^{2+} absorption in the untreated control samples. Fumarprotocetraric acid significantly reduced Mn^{2+} absorption on the filter surfaces (Table 1). Its capacity to reduce Mn^{2+} absorption was about one third of that of Ni^{2+}, which is known for its efficacy at occupying cation exchange sites (Vázquez *et al.* 1999).

The behavior of fumarprotocetraric acid in the experiment suggests that it contributes to the low extracellular Mn^{2+} absorption in *L. conizaeoides*. *Lecanora conizaeoides* is a homoiomerous lichen and the crystals of fumarprotocetraric acid are deposited on the cell walls everywhere in the thallus. The major lichen substance of the cortex in *H. physodes* is atranorin. It exerts no significant influence on the absorption of Mn^{2+} (Hauck & Huneck 2007). This explains the higher Mn^{2+} absorption in *H. physodes* than in *L. conizaeoides*. Another cause of low Mn^{2+} absorption in *L. conizaeoides* is probably the fine structure of the thallus surface, which hampers its soaking with fluid water (Shirtcliffe *et al.* 2006). *Hypogymnia physodes* also produces lichen substances, which are effective at reducing Mn^{2+} absorption at cation exchange site, viz. physodalic and protocetraric acid (Hauck & Huneck 2007). However, these compounds are restricted to the medulla (McCune 2002). Therefore, their contribution to reduction of the overall uptake of Mn^{2+} into the thallus and, with it, the influence on the tolerance to Mn^{2+} is limited. Paul *et al.* (2003) showed that the cell walls of fungal hyphae in thalli of *H. physodes* soaked for 1 h with 5 mM $MnCl_2$ contained 29 ± 5 mmol Mn dm^{-3} in the medulla, but 220 ± 20 mmol Mn dm^{-3} in the cortex. The different affinities of the cortical atranorin and the medullary physodalic and protocetraric acid to Mn^{2+} (Hauck & Huneck 2007) now explain this distribution pattern. Fungal cell walls of *L. conizaeoides* treated with 5 mM $MnCl_2$ contained only 7 ± 1 mmol Mn dm^{-3} (n=12; M. Hauck & A. Paul unpublished data) suggesting a high protective effect of fumarprotocetraric acid in combination with the

hydrophobic surface structure of the thallus.

Fumarprotocetraric acid is also known for its effective absorption of Fe^{3+} (Hauck *et al.* 2007). Assuming that complex formation of lichen substances with Fe^{3+} facilitates the intracellular uptake of this metal (Engstrom *et al.* 1980; Hauck *et al.* 2007), fumarprotocetraric acid would have a double function in the Mn^{2+} tolerance of *L. conizaeoides*. This is because Fe is known to compensate for symptoms of Mn toxicity in lichens and other organisms (Hauck *et al.* 2003; Schmull & Hauck 2003).

Although the present results suggest that a role of fumarprotocetraric acid in the Mn^{2+} tolerance of *L. conizaeoides* is plausible, such function has still to be substantiated with further experiments involving lichen thalli instead of the cellulose filters. Other lichen species with fumarprotocetraric acid often co-occur with *L. conizaeoides* in coniferous forests where high Mn concentrations in bark and stemflow are known to limit epiphytic lichen abundance. Such species include *Cladonia coniocraea*, *C. pyxidata* and *Mycoblastus fucatus* (Hauck *et al.* 2001, 2002*a*). This co-occurrence of unrelated species with fumarprotocetraric acid at sites with high Mn supply provides further support for the hypothesis that fumarprotocetraric acid could increase the Mn tolerance of lichens by simultaneously decreasing Mn^{2+} absorption and increasing Fe^{3+} absorption.

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Markus Hauck and Siegfried Huneck

M. Hauck: Albrecht von Haller Institute of Plant Sciences, Dept. Plant Ecology, University of Göttingen, Untere Karspüle 2, D-37073 Göttingen, Germany.
S. Huneck: Fliederweg 34a, D-06179 Langenbogen/ Saalkreis, Germany.