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Hydroxamate siderophore synthesis by *Phialocephala fortinii*, a typical dark septate fungal root endophyte

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Abstract

The siderophore production of various isolates of *Phialocephala fortinii* was assessed quantitatively as well as qualitatively in batch assays under pure culture conditions at different pH values and iron(III) concentrations. We found a distinct effect of both of these parameters on siderophore synthesis and as well as on fungal growth. In comparative analyses of two of the isolates, maximum siderophore production was found at a pH in the range of pH 4.0 to 4.5 while, under the experimental conditions employed, the optimal concentration of ferric iron was determined to be between 20–40 μg iron (III) l^{-1} (0.36–0.72 μM, respectively). HPLC analysis of the culture filtrate of most of the isolates of *P. fortinii* revealed the excretion of ferricrocin as main hydroxamate siderophore, followed by ferrirubin and ferrichrome C. The pattern of release of these three substances proved to be dependent on pH and iron(III) concentration of the culture medium, and to be specific for each isolate under investigation.

Introduction

The hyphomycete *Phialocephala fortinii* Wang & Wilcox (Wang & Wilcox 1985) belongs to the dark septate endophytes, a heterogeneous group of filamentous fungi with optically dense cell walls and septa, which is found frequently to form mycorrhiza-like associations with plant roots in a great variety of ecosystems, especially in extreme arctic and alpine habitats (Haselwandter & Read 1980; Read & Haselwandter 1981; Currah *et al.* 1987; Summerbell 1989; Currah *et al.* 1990; Holdenrieder & Sieber 1992; Ahlich & Sieber 1996). Dark septate endophytes (DSE) appear to colonize plant roots simultaneously with ectomycorrhizal fungi (Holdenrieder & Sieber 1992; Ahlich & Sieber 1996), arbuscular mycorrhizal fungi (Haselwandter 1987; Fernando & Currah 1996) or ericoid mycorrhizal fungi (Currah & Tsuneda 1993; Currah *et al.* 1993; Ahlich & Sieber 1996).

It is well documented that mycorrhizal infection affects the mineral nutrition of host plants (Smith & Read 1997), including micronutrient uptake (George *et al.* 1994). Under iron-limiting conditions most aerobic microorganisms synthesize siderophores (Winkelmann & Drechsel 1997). While ericoid mycorrhizal fungi have been demonstrated to produce hydroxamate siderophores (Haselwandter *et al.* 1992), and there is some evidence that both, ectomycorrhizal and arbuscular mycorrhizal fungi may release hydroxamate siderophores (for review of data see Haselwandter 1995), nothing was known about DSE and their capacity to produce iron chelating agents. Hence the aim of the present study was to test under pure culture conditions whether DSE would have the capacity to excrete siderophores into the nutrient medium. In addition, the impact of culture conditions like pH and iron concentration on siderophore synthesis was investigated. Finally, the chemical structure of the main siderophores released by various strains of *P. fortinii* was elucidated.

Materials and methods

Strains

In the present study the following isolates of *Phialocephala fortinii* (Wang & Wilcox 1985) were used: TS = type strain CBS 443.86 isolated by Wang & Wilcox (1985) from *Pinus sylvestris* root; CC3 isolated from *Carex curvula* (= isolate C2 of Haselwandter & Read 1982); AA = K 93 202 isolated from *Abies alba* (Ahlich & Sieber 1996); PA = K 92 147 isolated from *Picea abies* (Ahlich & Sieber 1996); PS = K 93 402 isolated from *Pinus sylvestris* (Ahlich & Sieber 1996).

Nutrient medium

The low iron medium (LIM; Szaniszlo *et al*. 1981) was modified with regard to the concentration of amino acids: 1 g l^{-1} L-proline; 1 g l^{-1} L-ornithine · HCl; pH and [Fe^{3+}] were adjusted according to the requirements of each experiment. The medium was prepared with distilled deionized water and deferrated with Chelex 100 according to Haselwandter & Winkelmann (1998) prior to the addition of saccharose, magnesium sulfate and trace elements.

Culture conditions

The fungi were sub-cultured three times in 100 ml iron free LIM in 250 ml Erlenmeyer flasks on a gyratory shaker at 120 rev min^{-1} at 25 °C for 4 days to reduce possible iron storage by the fungi. Four ml of the last sub-culture were used to inoculate the main culture (three replicates) which was incubated under the same conditions as above.

For determination of the optimal pH for siderophore release, the medium was buffered with a glycylglycine piperazine · 2HCl – NaOH buffer (Perrin & Dempsey 1974), adjusted to pH values between 4.0 and 6.0. For assessment of the optimal iron(III) concentration for siderophore production, the growth medium was adjusted to concentrations of 0, 10, 20, 40, 80 and 160 μg $Fe^{3+}l^{-1}$ (0, 0.18, 0.36, 0.72, 1.43 and 2.87 μmol $Fe^{3+}l^{-1}$, respectively) with $FeCl_3 \cdot 6\ H_2O$ standard solutions (50 μg/ml (0.89 mM) and 10 μg/ml (0.18 mM)).

Analyses

During the incubation period, the siderophore production was monitored in samples of the culture filtrate by application of the CAS assay with desferrioxamine B (DFOB, Desferal, Novartis Pharma AG, Basle, Switzerland) as standard compound. This assay is based upon the removal of iron from the blue chrome azurol S iron(III) complex which leads to decoloration of the assay solution (Schwyn & Neilands 1987). Concentrations were calculated by using the molar extinction coefficients of the various siderophores. The incubation was stopped when the siderophore concentration did not increase any further. Dry matter yield was determined by weighing the fungal biomass on pre-dried (2 days at 80 °C) and pre-weighed filters.

Isolation of siderophores

Prior to the qualitative and quantitative determination of the hydroxamate siderophores in the culture filtrate by high performance liquid chromatography (HPLC) the siderophores were extracted following the procedure of Haselwandter *et al.* (1992) and Haselwandter & Winkelmann (1998). The culture filtrate obtained from the three replicate incubations was supplemented with 0.33 g $FeCl_3 \cdot 6\ H_2O$ and the solution was stirred until a brown colour had developed. The siderophores were adsorbed onto XAD-2 (Sigma, München, Germany), washed with three volumes of distilled water and desorbed with one volume of methanol. After evaporation of the solvent under vacuum, the residue was re-dissolved in a known quantity of methanol and filtered through a 0.02 μm ANOTOP® 10 filter (Merck, Darmstadt, Germany).

Identification of siderophores (modified after Konetschny-Rapp et al. 1988)

The siderophore extract was separated on a reversed phase HPLC column (Nucleosil C_{18}, 5 μm, 4.6 mm × 250 mm), fitted with the equivalent pre-column (Sigma-Aldrich) using a gradient of acetonitrile and ammonium acetate (10 mM, pH 3.0). Standard gradient: 4–14% acetonitrile in 4 min., 14–28% in 14 min, 40% for 2 min. Flow rate: 1 ml/min; injected volume: 20 μl. Bio-Rad 1350 soft-start pumps. Detector: ISCO S500 Absorbance Detector, λ = 435 nm. Recording and computing of data with Bio-Rad HRLC 800 software run on an IBM PC.

The siderophores were identified by their retention time in comparison with pure reference samples of known fungal siderophores, and by co-chromatography with these substances using modified solvent gradients (results not shown). In addition,

identification of siderophores was confirmed by on-line HPLC-electrospray ionization mass spectroscopy. Quantification was carried out on basis of peak areas obtained with standard solutions of purified known siderophores.

Results

The various isolates differed in their growth with regard to colour of mycelium, pellet form and incubation time required to reach the maximum siderophore concentration under batch culture conditions. Although all the fungal isolates tested were clearly identified as belonging to *Phialocephala fortinii*, HPLC analysis revealed differences in respect to types and quantities of the excreted siderophores.

Figure 1 shows the pattern of siderophores released by five isolates of *P. fortinii*. Based on the criteria mentioned above, the main peaks detected could be clearly assigned to ferricrocin, ferrirubin and ferrichrome C. Ferricrocin appears to be the prominent siderophore produced in significant quantities by all the isolates investigated in this experiment, while only two of the five isolates released ferrirubin in considerable amounts as well. Strain AA exuded even slightly more ferrirubin than ferricrocin. Ferrichrome C occurred in the culture filtrate of each isolate at low concentrations, except in case of the isolates CC3 and TS, where this peak reached approximately 15% (CC3) and 4% (TS) of the total peak area (cf. Figure 1).

Two of the isolates, the type strain TS and the isolate CC3 were investigated more closely in order to determine the optima for siderophore synthesis of both, iron(III) concentration and pH of the culture medium. Hence, for comparative purposes the results obtained are being presented in parallel for both of the isolates.

Effect of pH on the siderophore production of P. fortinii

The time course for siderophore production by the type strain TS and the isolate CC3 was monitored with the CAS assay (Figure 2). In case of the type strain TS as well as the isolate CC3 siderophore concentration increased with decreasing pH; both strains reached the maximum siderophore concentration in the nutrient solution at pH 4.0 earlier than at higher pH values. At pH 4.0 strain CC3 as well as TS achieved their maximum within the same time, *i.e.* an incubation period of 24 days.

At the end of the incubation period, HPLC analysis was used for quantification of the siderophores released. The results are presented on a comparative basis in Table 1 together with data on dry weight yield of fungal biomass harvested at the end of the experiment.

In case of both strains siderophore excretion was highest at low pH, where growth was at its maximum as well. In case of the type strain TS, the highest concentration was measured at pH 4.0, while the isolate CC3 reached equally high concentrations of siderophores and dry matter at pH 4.0 and 4.5 (Table 1). Under the experimental conditions employed, both isolates showed a clear minimum in siderophore release at pH 5.5. At any pH tested, the isolate CC3 grew slightly better than TS.

In addition to the quantitative analysis performed at the end of the incubation period, the pH-dependence of the release of specific siderophores was monitored by qualitative HPLC analysis. The relative proportion of each siderophore as percentage of total siderophores excreted is shown in Table 1.

The type strain TS did not produce any measurable quantity of ferrirubin, and ferricrocin represented more than 85% of the total peak area. The pH of the nutrient medium had a clear effect on the siderophore partitioning: the relative proportion of ferrichrome C rose with increasing pH from 7% to 15%, while that of ferricrocin showed a slight decrease with increasing pH. Also the isolate CC3 displayed a clear correlation between the release of specific siderophores and the different values of pH (Table 1). It is interesting to note that strain CC3, too, excreted substantial amounts of ferrichrome C in addition to ferricrocin and ferrirubin. The latter appears to be a major siderophore of strains AA and PA (cf. Figures 1d and 1e).

Effect of iron(III) concentration on the siderophore production of P. fortinii

Figure 3 shows the time course of siderophore production by the type strain TS and the isolate CC3, monitored with the CAS assay. Both isolates reached their highest siderophore content in the nutrient solution at low iron concentrations (TS at 20 μg l^{-1}, CC3 at 40 μg l^{-1}), where the initial presence of iron still allows reasonably good fungal growth, as reflected by the amount of dry matter yield at the end of the incubation period (Table 2). While in case of the type strain TS siderophore production reached its peak after 16 d of incubation at a high concentration of ferric iron

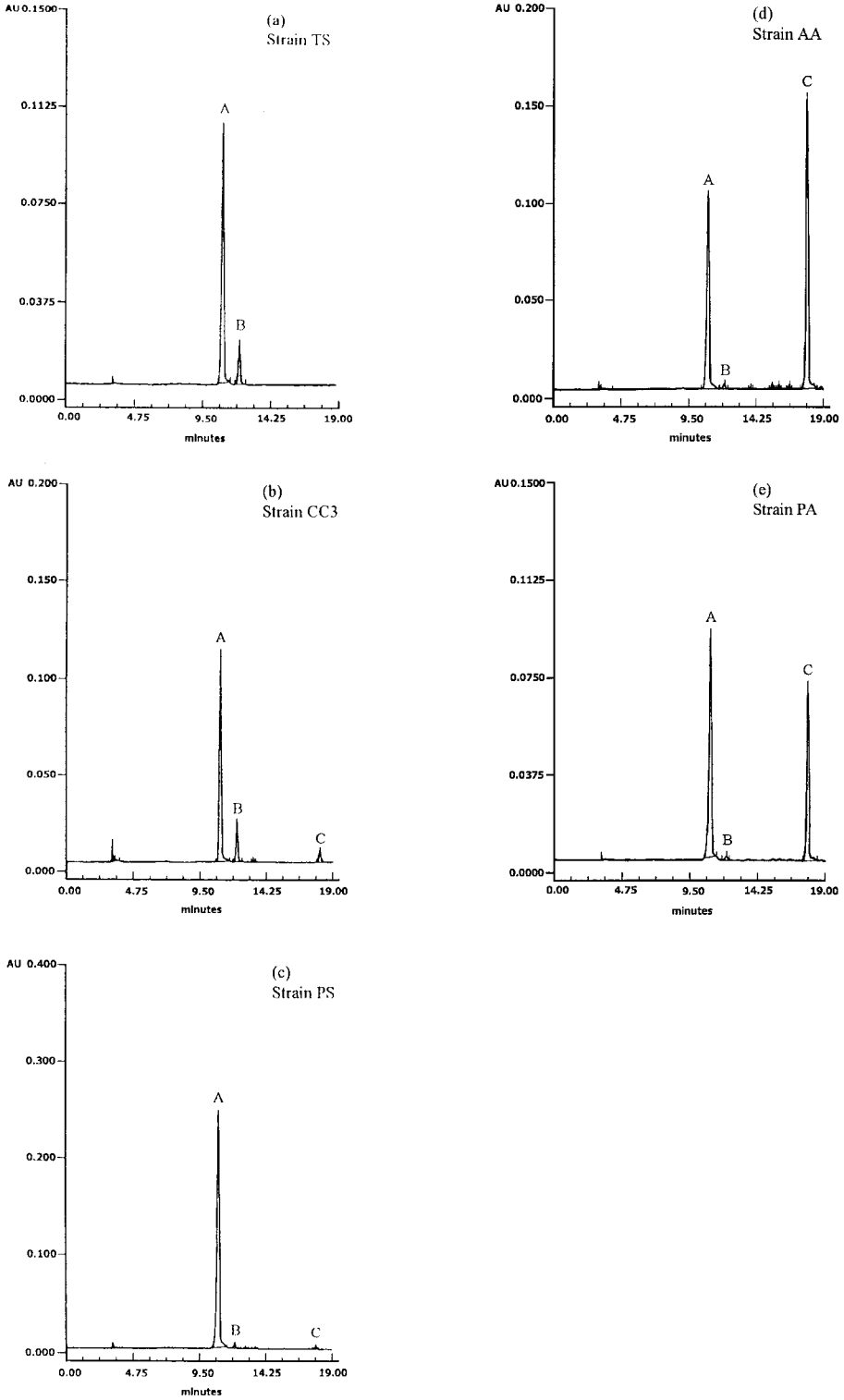

Fig. 1. HPLC chromatograms of culture filtrates of five isolates of *P. fortinii* grown in low iron medium (LIM). Analytic column nucleosil C_{18}, 5 μm, 4.6 $\times$ 250 mm, sample volume 20 μl, photometric detection at 435 nm. Gradient of acetonitrile and ammonium acetate, pH 3: 4–14% acetonitrile in 4 min., 14–28% in 14 min, 40% for 2 min. A = ferricrocin; B = ferrichrome C; C = ferrirubin.

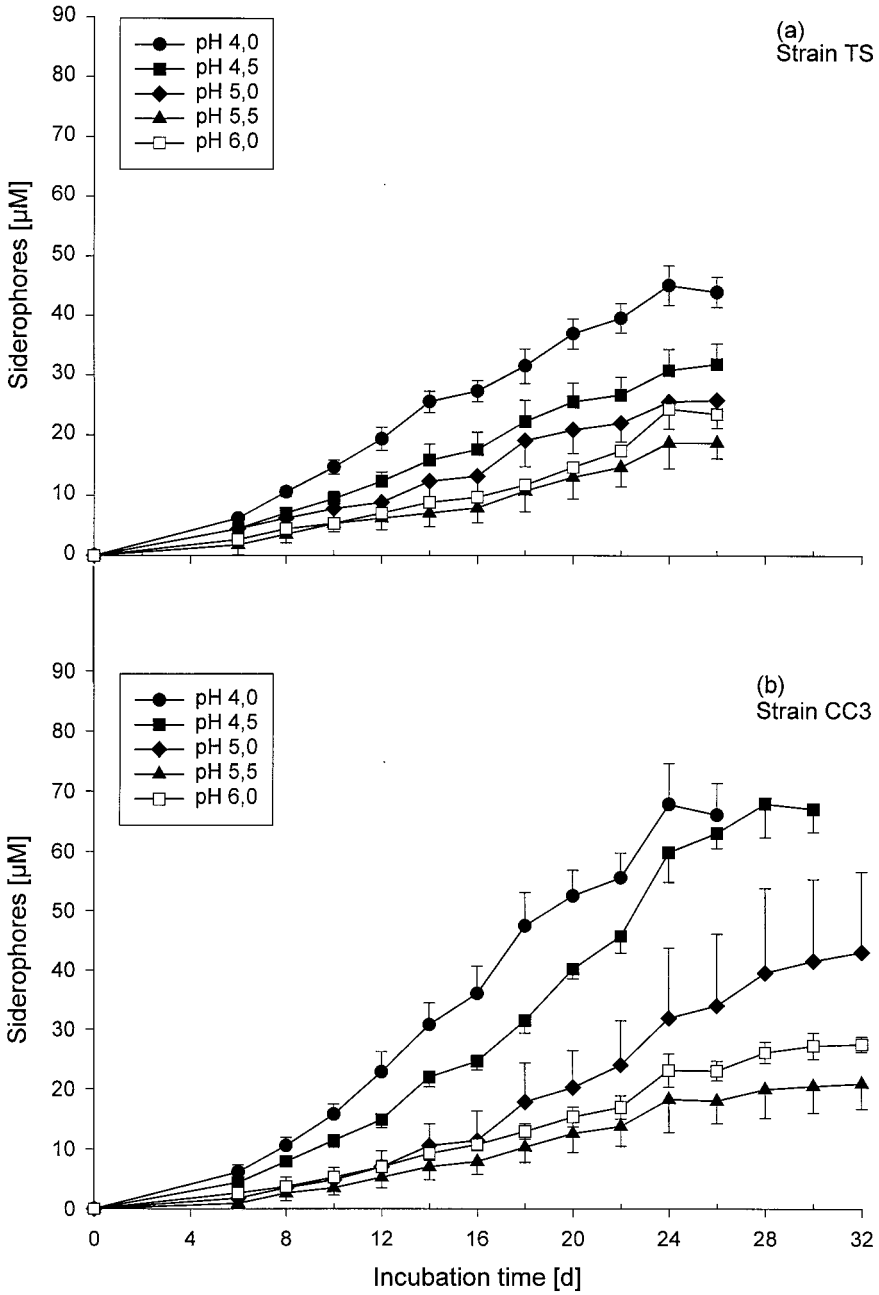

Fig. 2. Time course for siderophore production of the type strain TS (a) and the isolate CC3 (b) of *P. fortinii* in low iron medium (LIM) at different pH values adjusted with glycylglycin piperazine buffer. Siderophore concentration determined with the CAS assay. Vertical bars indicate standard error of the means.

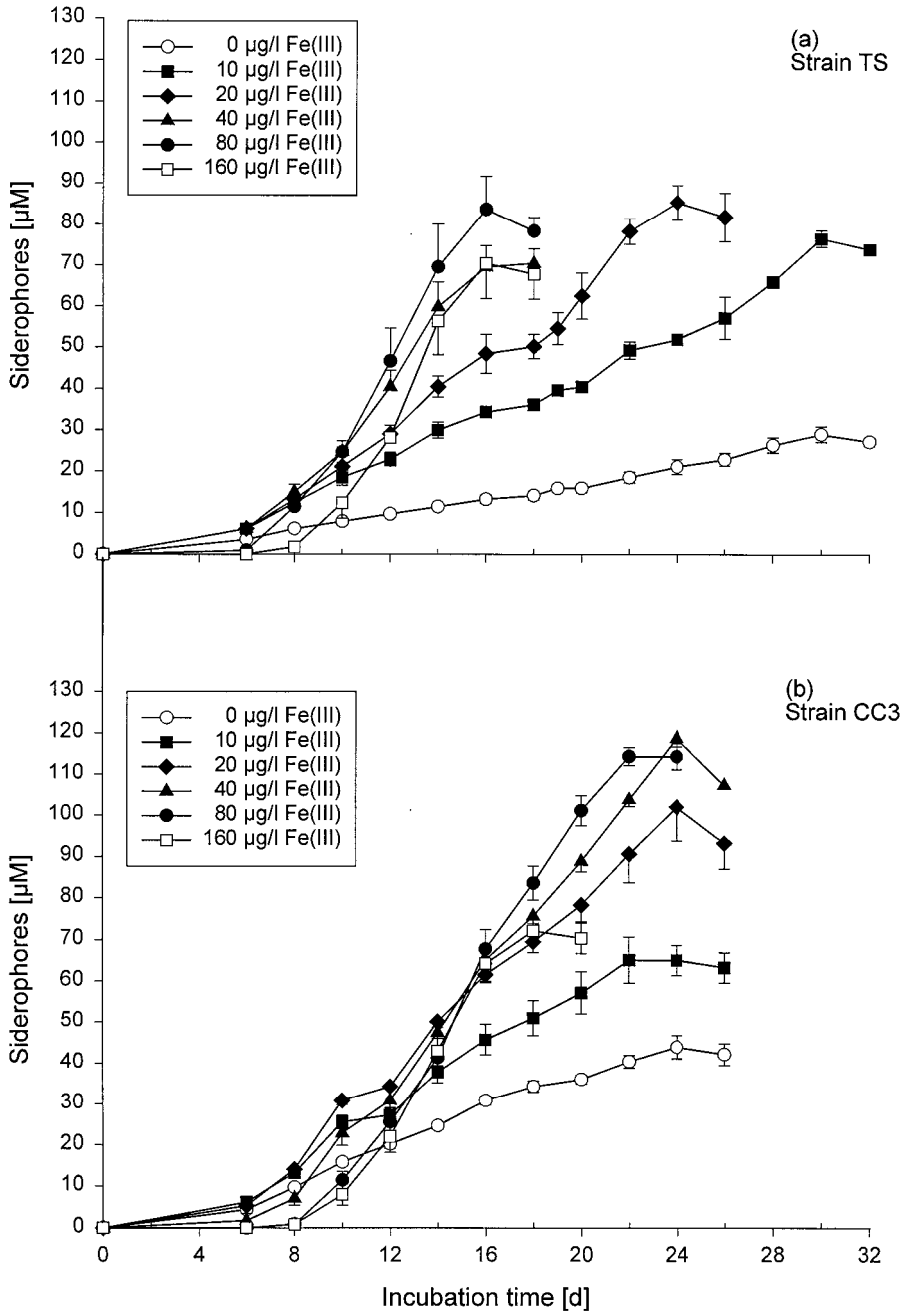

Fig. 3. Time course for siderophore production of the type strain TS (a) and the isolate CC3 (b) of *P. fortinii* in low iron medium (LIM) adjusted to different concentrations of Fe(III). Siderophore concentration determined with the CAS assay. Vertical bars indicate standard error of the means.

Table 1. Effect pH on the siderophore release and dry matter yield of the isolates TS and CC3 of *P. fortinii* after 24 d of incubation in low iron medium (LIM) buffered with glycylglycine piperazine

	pH				
	4,0	4,5	5,0	5,5	6,0
strain TS					
total siderophores [μM]	48,4	36,8	31,7	20,2	28,2
single siderophores [% peak area $A/B/C^*$]	93,3/6,7/0,0	91,3/8,7/0,0	89,7/10,3/0,0	86,8/13,2/0,0	85,5/14,5/0,0
fungal dry matter [g]	$0,44 \pm 0,02$	$0,36 \pm 0,06$	$0,34 \pm 0,01$	$0,30 \pm 0,02$	$0,33 \pm 0,01$
strain CC3					
total siderophores [μM]	83,6	87,1	43,1	22,9	20,2
single siderophores [% peak area $A/B/C^*$]	87,7/8,8/3,5	89,2/7,5/3,3	92,3/5,0/2,7	89,8/6,3/3,9	83,0/10,2/6,8
fungal dry matter [g]	$0,52 \pm 0,02$	$0,52 \pm 0,03$	$0,43 \pm 0,13$	$0,34 \pm 0,04$	$0,44 \pm 0,03$

*Proportion of the three main siderophore peaks (A = ferricrocin, B = ferrichrome C, C = ferrirubin) in relation to the total peak area (A + B + C = 100%).

Table 2. Effect of iron concentration on the siderophore release and dry matter yield of the isolates TS and CC3 of *P. fortinii* after 24 d of incubation in low iron medium (LIM) at different concentrations of Fe (III)

	Fe (III) concentration [μg l^{-1}]					
	0	10	20	40	80	160
strain TS						
total siderophores [μM]	24,5	74,7	106,5	93,3	95,0	107,4
single siderophores [% peak area $A/B/C^*$]	96,0/4,0/0,0	96,0/4,0/0,0	96,8/3,2/0,0	94,5/5,0/0,5	94,5/5,0/0,5	89,5/9,7/0,8
fungal dry matter [g]	$0,48 \pm 0,05$	$0,83 \pm 0,04$	$1,34 \pm 0,07$	$1,59 \pm 0,12$	$1,82 \pm 0,24$	$1,52 \pm 0,47$
strain CC3						
total siderophores [μM]	40,4	74,6	100,4	134,4	96,5	78,1
single siderophores [% peak area $A/B/C^*$]	87,4/11,1/1,5	86,7/11,8/1,5	84,8/13,8/1,4	85,1/13,9/1,0	85,4/13,8/0,8	81,2/18,2/0,6
fungal dry matter [g]	$0,69 \pm 0,09$	$0,96 \pm 0,08$	$1,44 \pm 0,19$	$1,81 \pm 0,07$	$2,17 \pm 0,04$	$2,18 \pm 0,03$

*Proportion of the three main siderophore peaks (A = ferricrocin, B = ferrichrome C, C = ferrirubin) in relation to the total peak area (A + B + C = 100%).

(80 μg l^{-1}) in the nutrient medium (Figure 3a), the isolate CC3 has been able to excrete sidero-phores for approximately 24 days at each initial iron concentration (Figure 3b) and reached higher final siderophore contents than the type strain at Fe^{3+} concentrations between 20 and 80 μg l^{-1}.

The relative proportion of ferricrocin, ferrichrome C and ferrirubin as produced at different iron concentrations is shown in Table 2. At iron concentrations between 0 and 20 μg l^{-1} the type strain TS did not produce any measurable quantities of ferrirubin in iron free medium, it excreted mainly ferricrocin (more than 95% of the total peak area; see also Figure 1a). However, at iron concentrations of 40 μg l^{-1} or higher this strain proved indeed capable of synthesizing ferrirubin, though only in small quantities, reaching less than 1% of the total peak area (Table 2). The release of ferrirubin paralleled an increase of the concentration of ferrichrome C. The isolate CC3 excreted considerably higher amounts of ferrichrome C (11 to 18% of total siderophores), while ferrirubin, though present in all incubations, never exceeded 2% of the total peak area which it achieved at low iron concentrations (Table 2).

Discussion

Effect of iron

Iron is an essential micronutrient for almost every organism with the exception of certain lactic acid bacteria (Neilands 1972; Archibald 1983). Depending on the nature of the ligand, the redox potential of the Fe(II)/Fe(III) couple may vary over a wide range, thus making it a 'redox-active' metal participating in various enzymatic reactions, such as electron transfer in the respiratory chain, acid-base reactions, DNA syn-

thesis and DNA degradation reactions (Neilands 1972; Matzanke 1994). To gain access to the ferric iron, powerful iron uptake systems have evolved which are activated at low iron concentrations; siderophores are also a component of virulence of microorganisms infecting man, animals, and plants (Neilands 1993).

The optimal iron concentration for a high siderophore yield in batch culture depends upon two apparently incompatible factors: On the one hand, a sufficient initial quantity of iron is required for growth, the amount of biomass being a crucial parameter for the overall siderophore production; but on the other hand, high concentrations of iron are known to inhibit siderophore synthesis. The optimum varies with the organism in question according to the regulation of its biochemical pathways involved in iron uptake and storage.

The pattern of siderophore release, varying with the iron concentration, points towards an influence of iron concentration on the regulation of the various pathways of siderophore synthesis. The smaller and structurally simpler molecule of ferricrocin might be predominant because its biosynthesis may require less energy compared with ferrirubin, but the culture conditions themselves might also favour the synthesis of this siderophore type. However, it needs to be pointed out that the capability to excrete different types of siderophores under complex natural situations might convey a great ecological advantage, e.g., in the competition with other microorganisms provided with similar iron uptake systems.

Effect of pH

Highest siderophore concentrations were measured at low pH where solubility of iron is high and fungal growth at its maximum. This has also been observed with *Neurospora crassa* by Winkelmann and Huschka (1987) who suggested that the proton translocating ATPase of the plasmic membrane could be responsible for the acidification of the medium. Pumping protons out of the cell would create an electrochemical gradient which may facilitate nutrient uptake and thus link acidification and growth with each other. Slower growth at higher pH values might then be explained by an additional expense of energy spent on acidifying the growth medium, which can be considered as a rather unspecific measure to improve micronutrient uptake. To tolerate a wide pH range might be advantageous to such a microorganism, thus facilitating a wide distribution in various ecosystems.

Siderophore pattern of different isolates

Almost all of the five isolates under investigation released the same three main compounds found in the siderophore extract: the hydroxamate siderophores ferricrocin, ferrirubin and ferrichrome C. However, there were some remarkable differences between the isolates, concerning morphology as well as siderophore distribution. In relation to the total siderophore content the relative proportion of each siderophore type differed clearly between the isolates (cf. Figure 1). Nevertheless, the five strains can be classified into two main groups: The first group is represented by the isolates AA and PA which released almost equal amounts of ferricrocin and ferrirubin into the culture medium (Figures 1d and 1e), while the second group (TS, CC3 and PS) excreted mainly ferricrocin under the experimental conditions employed (Figures 1a–c).

Despite of their very different origin (*Carex* vs. *Pinus* roots) the isolates CC3 and the type strain TS, which have been studied in more detail, show many similarities, although they seem to be regulated in a slightly different way, in particular with regard to the excretion of ferrirubin at low iron concentration. Molecular biological data confirmed their very close relationship (Jumpponen & Trappe 1998). The strains TS and PS were both isolated from *P. sylvestris* roots; nevertheless, the isolate PS (as well as AA and PA) does not seem closely related to the type strain TS. This was revealed by studies of isozyme patterns by Ahlich Schlegel (1997), and confirmed in the present study by the spectrum of siderophores produced by the various isolates (Figure 1). The isolates AA, PA and PS do not release any significant quantities of ferrichrome C.

Concluding remarks

The role of dark septate endophytes in association with their host plants has been frequently discussed since their first characterisation (see review by Jumpponen & Trappe 1998). While some authors described the association of DSE with plant roots as being parasitic (Melin 1922; Wilcox & Wang 1987), others assumed a mutualistic relationship similar to those of mycorrhizal fungi (Haselwandter & Read 1982; Read 1991; Stoyke & Currah 1991). When one seedling of each of four plant species were grown together, inoculation with *P. fortinii* led to a significant increase in shoot dry weight of one of the plant species tested in contrast with the results of the same symbiosis under

monoculture conditions (Fernando & Currah 1996). A recent study on mycorrhizal functioning of *P. fortinii* associated with *Pinus contorta* revealed increased uptake of phosphorus and nitrogen resulting in an enhanced growth of inoculated plants compared with non-inoculated plants, at least under some circumstances (Jumpponen *et al.* 1998). The potential of DSE to acidify the environment (data not shown) may be regarded as an important mechanism for facilitating micronutrient uptake (Marschner 1995). All that together with the capacity for siderophore biosynthesis which can be anticipated to play a key role in iron nutrition similar to mycorrhizal fungi (Fett *et al.* 1998), and in the interaction with soil microorganisms and plants hosting DSE, strongly support the assumption that the association between DSE, *P. fortinii* in particular, and host plants is of a mutualistic rather than parasitic nature.

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