

Detection and identification of ferricrocin produced by ectendomycorrhizal fungi in the genus *Wilcoxina*

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Received 7 July 1995; revised version received 5 December 1995; accepted for publication 11 December 1995

The ectendomycorrhizal fungi *Wilcoxina mikolae* isolates CSY-14 and RMD-947 and *W. rehmsii* isolate CSY-85 were grown in pure culture under iron-limiting conditions. All three isolates tested positive for siderophore formation using both the ferric perchlorate assay and a sensitive HPLC iron-binding assay. A peptide siderophore was isolated from the culture medium by HPLC and shown to contain the amino acids serine, glycine and ornithine in a 1:2:3 ratio. This siderophore was identified as ferricrocin on the basis of electrospray mass spectroscopy and its co-chromatography in two different HPLC systems with ferricrocin isolated from *Aspergillus fumigatus*. Ferricrocin was the only siderophore isolated from these *Wilcoxina* cultures. This is the first report of siderophore formation by ectendomycorrhizal fungi.

Keywords: ectendomycorrhizae, E-Strain fungi, *Wilcoxina* siderophore, ferricrocin

Introduction

Mycorrhizal fungi are important in the mineral nutrition of their plant hosts. The fungal mycelium acts as a functional extension of the root system, forming a nutrient absorbing web that extends far beyond the root itself. Mycorrhizal fungi can utilize organic nitrogen sources that may be unavailable to their host plants (Read *et al.* 1989). They increase the uptake of phosphorus, storing it as polyphosphate granules to be released to the plant during times of phosphate deficiency (Bolan 1991). Mycorrhizal fungi may also be important in the iron nutrition of the plant. Whole plant studies have shown that mycorrhizal plants have greater rates of iron uptake than their non-mycorrhizal counterparts (Cress *et al.* 1986, Leake *et al.* 1990), and the production of siderophores has recently been reported for iron-limited cultures of ericoid and ectomycorrhizal fungi (Szaniuslo *et al.* 1981, Haselwandter *et al.* 1992, Haselwandter 1995). For the infection of the ericaceous shrub *Calluna vulgaris* by *Hymenoscyphus ericae*, a direct involvement of a hydroxamate siderophore produced by the fungus was postulated (Shaw *et al.* 1990). Siderophore formation could solubilize iron previously unavailable to the host plant.

However, it may also be a mechanism to compete with the host for available iron. Infection of *Pinus elleottii* with the ectomycorrhizal fungus *Pisolithus tinctorius* decreased iron uptake by roots from the synthetic siderophore desferrioxamine B (Leyval & Reid 1991).

The genus *Wilcoxina* comprises a specific group of ascomyceteous fungi that form a distinct mycorrhizal association. They commonly infect a variety of conifers and deciduous trees including *Pinus*, *Betula* and *Quercus* (Wilcox 1990), and are noted for their ectendomycorrhizal condition, forming both a Hartig net and a prominent intracellular infection in the cortex of the root (Wilcox 1991). They are peculiar in that they may also form ectomycorrhizae with only an intercellular infection. These fungi, initially named E-strain, have been segregated into the genus *Wilcoxina* with the majority of isolates classified as *W. mikolae* or *W. rehmsii* (Yang & Korf 1985, Egger *et al.* 1991). *Wilcoxina* species are extremely cosmopolitan. They have been isolated from plant hosts growing in widely contrasting ecological conditions including nursery soils of high pH and good fertility, mine spoils characterized by low pH and high heavy metal contamination, natural forests and plantations, urban areas, and peat soils (Mikola 1988).

In this paper, we describe the isolation and identification of a siderophore produced by three different isolates of *Wilcoxina* species grown in culture. Using chromatographic techniques and electrospray mass spectroscopy, we show that all three isolates produce the hydroxamate siderophore ferricrocin (Figure 1) under iron-limiting, but not iron-

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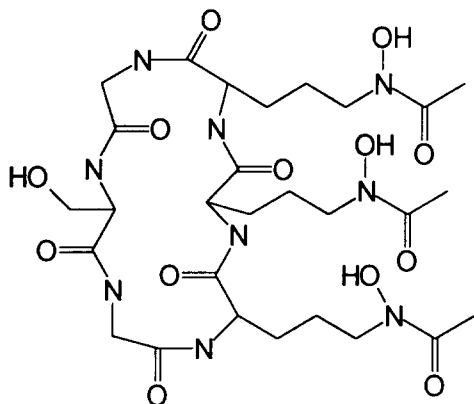


Figure 1. The structure of deferriferrirocrocic acid.

replete, conditions. This is the first report of siderophore production by ectendomycorrhizal fungi.

Materials and methods

Culture conditions

Wilcoxina mikolae var. *mikolae*, isolate CSY-14, *W. mikolae* isolate RMD-947 and *W. rehmsii* isolate CSY-85 (Egger *et al.* 1991) were grown in pH 5.5 media containing 55.5 mM glucose, 5.8 mM KH_2PO_4 , 0.6 mM MgSO_4 , 1.8 μM MnCl_2 , 10 μM FeNa_2EDTA , 0.69 μM CaCl_2 , 0.36 μM ZnSO_4 , 0.32 μM CuSO_4 , 0.41 μM Na_2MoO_4 and 4 mM nitrogen as urea or nitrate. Maintenance cultures were grown in disposable Petri dishes on cellophane over 1% agar. To induce iron limitation, triplicate cultures were transferred to liquid medium in acid-washed polycarbonate flasks and grown at 26°C in the dark with or without added iron. These low iron cultures were then used as the inoculum for later experiments. To determine the dry weight growth curve, 15 replicate flasks with and without iron were inoculated with *W. mikolae*, isolate CSY-14. At 1–2 week intervals, three separate flasks for each treatment were filtered through individual tarred Whatman 934 AH glass fiber filters, the filters dried at 80°C and weighed.

Siderophore assays

Culture filtrates were concentrated to dryness by lyophilization, resuspended in distilled water and assayed for siderophore production using the CAS assay without shuttle (Schwyn & Neilands 1987), the ferric perchlorate assay (Atkins *et al.* 1970) or the HPLC iron-binding assay (Speirs & Boyer 1991). For the HPLC assay, the crude siderophore was mixed with an equal volume of ^{55}Fe (3.45 GBq/mmol, 0.21 mM total Fe; Amersham, Arlington Heights, IL) chelated with 5 mM nitrilotriacetic acid. Samples were allowed to incubate for 30–60 min and the labeled siderophores separated by HPLC using a 4.6 × 150 mm Hamilton PRP-1 column (Hamilton, Reno, NV) and a 20 min gradient of 5–50% aqueous acetonitrile acidified with 1% acetic acid

at 0.8 ml min⁻¹. Peaks were detected using a flow-through scintillation counter (Radiomatic Instrument and Chemical, Tampa, FL) in the tritium counting window with a 3 s update time. In some cases, purified samples were deferrated and analyzed using a second column (YMC Basic) as described below. Siderophore concentrations were calculated from the counts in the siderophore peak using specific activity of the radioactive iron and assuming a 1:1 iron to siderophore binding ratio.

Purification of ferricrocin

Preliminary studies indicated *W. rehmsii* CSY-85 produced slightly more siderophore than the other isolates. Therefore this isolate was used to isolate and purify the fungal siderophore for structural studies. Filtrate from cultures grown under –Fe conditions was concentrated by lyophilization and applied to a C-18 solid phase extraction cartridge (Sep Pac; Waters Associates, Milford MA) in about 2 ml distilled water. After washing with about 5 ml water, the siderophore was eluted with 2–5 ml methanol followed by an equal volume of methanol containing 1% acetic acid. Both methanol fractions tested positive for siderophore activity by HPLC. These fractions were pooled together and concentrated under a stream of nitrogen. The siderophore was saturated with freshly dissolved ferric chloride and purified by reverse phase HPLC using a Hamilton PRP-1 column (4.6 × 150 mm) and a gradient of 5–50% acetonitrile containing 1% acetic acid in water. Fractions were collected and those containing a visible absorbance maximum in the 425–440 nm region were pooled and lyophilized. Ferricrocin eluted at 12.3 min.

Characterization of ferricrocin

UV and visible absorbance spectra were taken in water or methanol using an Aminco MR3000 photodiode array spectrometer. Approximately 200 nmol of the CSY-85 siderophore purified by HPLC was used for electrospray mass spectroscopy and amino acid analysis. Amino acids were analyzed after hydrolysis using a Waters Associates Pico-tag system. For electrospray mass spectroscopy, samples were directly infused into a Sciex API-3 triple-quad operating at 5.2 keV with an orifice of 35 V using ammonium acetate as the counter ion.

Isolation of siderophore standards

Ferrichrome, ferrichrome A and ferricrocin were isolated from low iron cultures of *Ustilago sphaerogena* and *Aspergillus fumigatus* for comparative purposes. *U. sphaerogena* ATCC 12421, purchased from the ATCC (Rockville, MD) and *A. fumigatus*, obtained as a gift from Dr June Wang (State University New York, College of Environmental Science and Forestry, Syracuse, NY), were maintained on potato dextrose broth (Difco, Detroit, MI). To induce siderophore formation, a small amount of this culture was transferred to 300–400 ml modified Grimm–Allen media (Garibaldi & Neilands 1955) lacking iron.

Ornithine (1.6 g l^{-1}) and acetate (2.1 g l^{-1}) were added to the *A. fumigatus* medium to increase siderophore production. Cultures were grown for 1 week, transferred to fresh iron-free media and incubated at 27°C with shaking (120 r.p.m.). Siderophore production was monitored weekly using the ferric perchlorate and HPLC assays. After approximately 3 weeks, these cultures reached stationary phase. The cells were removed by centrifugation, ferrous sulfate added to the culture medium and the pH adjusted to 3.0. The medium was saturated with ammonium sulfate, the siderophores extracted into benzyl alcohol and then back extracted into water by the addition of diethyl ether as described in Garibaldi & Neilands (1955). The aqueous phase was dried by lyophilization, redissolved in a minimal volume and applied to a $2.5 \times 90 \text{ cm}$ BioGel P-2 column in 10% aqueous methanol. Fractions were collected and monitored at 425 nm. This column readily separated the ferrichrome from ferrichrome A produced by *U. sphaerogena*. Fractions with the individual siderophores were concentrated by lyophilization and purified to homogeneity using a preparative Hamilton PRP-1 column ($7 \times 305 \text{ mm}$) and a 20 min linear gradient of 15–60% acetonitrile with 1% acetic acid:water at 1.5 ml min^{-1} . Approximately 250 mg ferrichrome, 200 mg ferrichrome A and trace amounts of ferrichrome C were isolated per liter culture of *U. sphaerogena* and 16 mg ferricrocin isolated from a liter culture of *A. fumigatus*. The structure of these standards was verified using a combination of amino acid analysis, electrospray mass spectroscopy and high resolution (300 MHz) proton NMR spectroscopy.

HPLC chromatography

Authentic ferricrocin was obtained as a gift from Professor G. Winkelmann (University of Tübingen, Tübingen, Germany) and compared with the siderophores isolated from *Ustilago*, *Aspergillus* and *Wilcoxina* using HPLC with both UV and radioactive detection. For radioactive detection, the siderophores were first deferrated by mixing overnight with excess 8-hydroxyquinoline previously purified by sublimation. The ferric 8-hydroxyquinoline complex was removed by repeated extraction with chloroform. The siderophore in the aqueous layer was then labeled with radioactive iron as described in the section on siderophore assays and analyzed by HPLC using the two different conditions. (i) Hamilton PRP-1 column ($4.6 \times 150 \text{ mm}$) with a 20 min linear gradient of 5–50% acetonitrile acidified with 1% acetic acid in water. The retention times for the different siderophores were: ferricrocin (12.6 min), ferrichrome and ferrichrome C (12.8 min), and ferrichrome A (15.5 min). (ii) YMC basic column ($4.6 \times 250 \text{ mm}$; YMC, Wilmington, NC) using a 30 min gradient of acetonitrile in 10 mM phosphate, pH 3.0, as described by Konetschny-Rapp *et al.* (1988). This consists of a 15 min linear gradient from 10 to 20% acetonitrile followed by a 15 min concave gradient from 20 to 50% prepared using a Waters Gradient Maker (Water Associates) curve 9. The YMC column contains a mixture of C1 through C8 hydrocarbons but functionally behaves as a totally endcapped C8 column. The retention times for the different

siderophores were: ferricrocin (11.1 min), ferrichrome (11.5 min), ferrichrome C (12.3 min) and ferrichrome A (23.1 min). Both columns were run at 0.8 ml min^{-1} and monitored at 434 nm or by detecting ^{55}Fe with the flow-through scintillation counter as described above.

Results and discussion

Growth of *W. mikolae* under iron-limiting conditions

Wilcoxina species grow extremely slow in culture, often taking 30 or more days to reach stationary phase even under nutrient complete conditions. To investigate the effects of iron on growth and siderophore production, replicated flasks of *W. mikolae* CSY-14 were inoculated and grown in media with and without added iron. *Wilcoxina* forms clumped hyphal balls in solution, making it difficult to remove a representative subsample from any given culture. For this reason, entire flasks were harvested for dry weight determinations. Cultures grown with and without iron showed similar growth up through day 20. After this point, cultures with added iron grew significantly better than cultures without added iron (Figure 2a). Siderophore production was monitored in these cultures using both the CAS and HPLC iron-binding assay. The presence of a single, strong iron-binding compound eluting at the same retention time as ferricrocin was detected in iron-deficient cultures harvested after day 20 but not in the iron-replete cultures (Figure 2b). Maximum siderophore concentrations reached an average of $28 \mu\text{M}$ as measured using the ferric perchlorate or the HPLC iron-binding assays. In contrast, anomalous results were obtained when the CAS assay was used to analyze crude culture media. CAS-positive activity was present in both iron-replete and iron-deficient cultures of *W. mikolae* starting as early as day 15 and generally mirrored the increase in dry weight (data not shown). We do not think this reflects siderophore production in these cultures. Other work in our laboratory has shown that the CAS assay is extremely sensitive to interferences by reducing compounds (B. Airel and G. L. Boyer, unpublished) and may detect compounds other than siderophores when used with unpurified culture supernatants (Aronson & Boyer 1994).

Characterization of ferricrocin

The iron-binding peak in the HPLC assay eluted very close to our ferrichrome standard using a Hamilton PRP-1 column. To confirm its structure, the siderophore was isolated from low-iron cultures of *W. rehmii* CSY-85 and *W. mikolae* CSY-14. The UV-vis spectrum of the ferric siderophore complex showed an absorbance maximum between 420 and 430 nm (Figure 3), strongly suggesting a hydroxamate siderophore. Amino acid analysis of the siderophore isolated from *W. rehmii* CSY-85 gave predominantly the amino acids serine, glycine and ornithine, in a 1.0:2.5:3.0 ratio. Glycine is a common contaminant found in amino acid analysis. A more probable ratio of 1:2:3

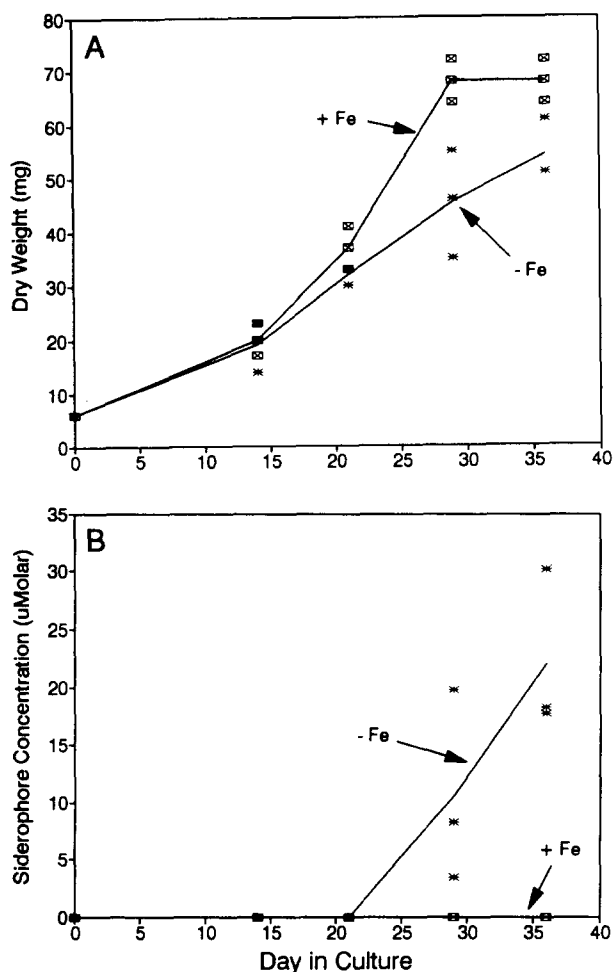


Figure 2. Increase in (a) dry weight and (b) media siderophore concentration as measured by the HPLC iron-binding assay for cultures of *W. mikolae* CSY-14 grown under iron-replete and iron-limiting conditions. Each point represents an individual flask. The lines are the average of the three replicates.

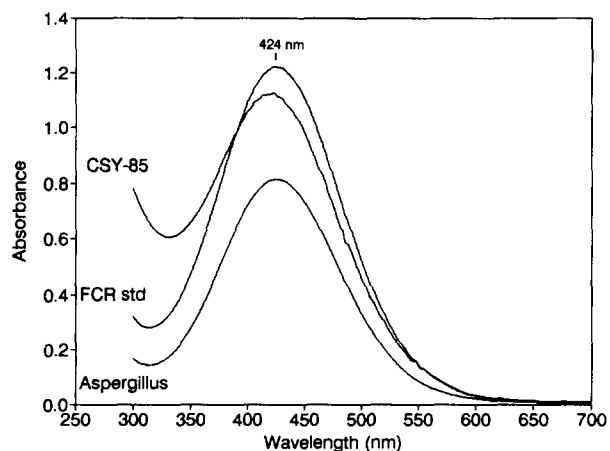


Figure 3. The visible absorbance trace of ferricrocin isolated from *W. rehmsii* CSY-85, *A. fumigatus* and ferricrocin obtained as a gift from Professor G. Winkelmann.

would suggest the siderophore from *W. rehmsii* was the hexapeptide ferricrocin (Figure 1). Electrospray mass spectroscopy, run using ammonium acetate as the counter ion, showed a strong molecular ion at $m/z = 788$ (100% base peak) $[\text{FeMNH}_4]^+$ and $m/z = 793$ (35%) $[\text{FeMNa}]^+$. An observed fragment ion at $m/z = 772$ (35%) can be assigned to the loss of oxygen from the hydroxamate group in the molecular ion $[\text{FeMNH}_4-\text{O}]^+$ (Dell *et al.* 1982). Thus the molecular weight of the ferric siderophore complex was 770 a.m.u., in agreement with that expected for ferricrocin. As an additional confirmation of structure, the siderophores isolated from *W. rehmsii* CSY-85, *W. mikolae* CSY-14 and *W. mikolae* RMD-947 were chromatographed against ferrichrome, ferrichrome A, ferrichrome C and ferricrocin using two different HPLC solvent systems. In both solvent systems, the siderophore peak from the three *Wilcoxina* species co-eluted with ferricrocin isolated from *A. fumigatus* and an authentic ferricrocin standard.

There is disagreement in the literature on the visible absorbance maxima for ferricrocin. Initial results in our laboratory gave an absorbance maximum in the visible region of 436 nm, close to the value of 434 nm reported in several recent reviews (Matzanke 1991, Winkelmann 1993). Subsequent traces, taken in water or methanol after extensive HPLC purification, centered around 423–426 nm (Figure 3). Authentic ferricrocin obtained as a gift from G. Winkelmann, and ferricrocin isolated in our laboratory from *A. fumigatus* gave absorbance maxima of 424 and 423 nm, respectively. Wong *et al.* (1983) determined the absorbance maximum for ferricrocin as part of their examination of the physical properties of this siderophore. They report a value of 424 nm in the text but list 434 nm in their summary table. It now appears this latter value was a misprint that is being perpetuated through the literature.

HPLC is widely used to detect fungal siderophores (Konetschny-Rapp *et al.* 1988). Labeling the siderophore with ^{55}Fe coupled with radiochemical detection offers a simple and rapid method for the detection of siderophores in culture supernatants (Boyer *et al.*, in preparation). This method is selective for compounds with a high affinity for iron and resistant to many of the chemical interferences that affect other assays such as the CAS assay. It readily detected ferricrocin production in *Wilcoxina* cultures at concentrations below that which could be detected using other techniques. Similar to HPLC coupled with visible absorbance at 435 nm, it also provides information on the number and identity of siderophores that are present in the sample. Production of hydroxamate siderophores under low iron conditions is common in fungi. While this is the first report of siderophore formation in ectendomycorrhizal fungi, ferricrocin and related compounds have been reported in ericoid mycorrhizal species (Schuler & Haselwandter 1988, Federspiel *et al.* 1991, Haselwandter *et al.* 1992). Both *Wilcoxina* and the ericoid fungi *Hymenoscyphus* are ascomycetes, as are *Aspergillus* and *Neurospora*. Siderophores are also produced by basidiomycetous ectomycorrhizal fungi (Szanijszlo *et al.* 1981); however, their structure is currently unknown. It is somewhat unusual in that only a single siderophore was detected in all three *Wilcoxina* isolates examined. Other

fungi such as *Aspergillus* (Zähner *et al.* 1963), *Neurospora* (Wong *et al.* 1983) and *Microsporum* species (Mor *et al.* 1992) also produce ferricrocin; however, in most cases, ferricrocin is only one of a suite of siderophores produced under iron-limiting conditions. The ericoid mycorrhizal fungus *Hymenoscyphus ericae* produces ferricrocin as its major siderophore, but also lesser amounts of ferrichrome and ferrichrome C. Ferricrocin and ferrichrome C were also reported in media from several species of *Microsporum* (Mor *et al.* 1992) and the ericoid fungus *Olidiodendron griseum* (Haselwandter *et al.* 1992). The highly phytopathogenic fungi *Alternaria longipes* produces at least 10 siderophores of the coprogen family in addition to ferricrocin (Jalal & Van der Helm 1989). In contrast, cultures of *W. mikolae* showed only the presence of ferricrocin. One possibility is that these compounds, if present, were below our detection limits (about 2 µM). Alternately, our HPLC system may have failed to resolve the different compounds. Ferrichrome, ferrichrome C and ferricrocin all elute very closely together in reverse-phase HPLC (Konetschny-Rapp *et al.* 1988, Haselwandter *et al.* 1992). It is likely that they would not be resolved using the polymeric Hamilton PRP-1 column. However, these compounds can be readily separated using the higher efficiency silica columns. We utilized a YMC Basic column to differentiate the individual compounds (see Materials and methods). Even using these techniques, the three *Wilcoxina* isolates tested showed only a single siderophore peak (Figure 4). In *Neurospora crassa*, coprogen is excreted from the mycelium whereas ferricrocin serves primarily as an intracellular storage compound for iron (Matzanke *et al.* 1988). We do not know at this time if ferricrocin serves both as an intracellular and extracellular siderophore in *Wilcoxina*. Intracellular siderophores may already be in the ferri form and thus difficult to detect using the ⁵⁵Fe HPLC binding assay. This would require any cold iron in the siderophore to be quantitatively exchanged with radioactive ⁵⁵Fe. This localization of ferricrocin in *Wilcoxina*

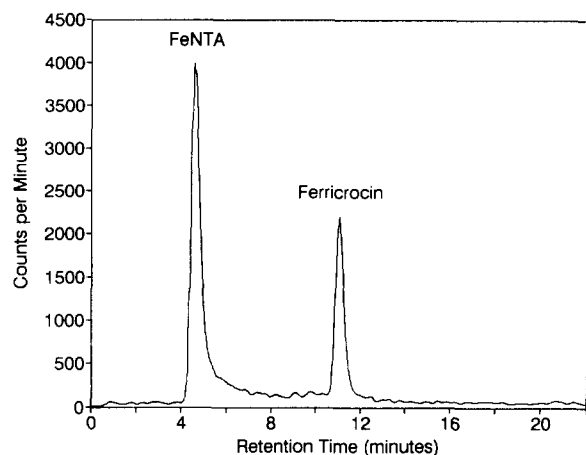


Figure 4. HPLC chromatograph of culture supernatant from low iron cultures of *W. mikolae* RMD-947 after labeling with ⁵⁵Fe and run using the YMC basic column and a 10 mM phosphate buffer:acetonitrile concave gradient (see Materials and methods).

is of interest since E-strain fungi commonly form mycorrhizal associations with conifers grown in mine spoils contaminated with heavy metals. In at least one case (Fekete & Barton 1992), siderophores have been shown to exert a protective function under these circumstances. Mycorrhizal infection has been reported to increase heavy metal tolerance in some seedlings (Bradley *et al.* 1982, Jones & Hutchinson 1986). Thus ferricrocin production could be beneficial to the plant for more reasons than just increased iron uptake.

Acknowledgements

This research was supported by the McIntire Stennis Cooperative Research Program in Forestry. We would like to thank Professors H. Wilcox and J. Wang at the State University of New York, College of Environmental Science and Forestry, Syracuse NY USA for the cultures of *Wilcoxina* species and *A. fumigatus*, and Professor G. Winkelmann at the University of Tübingen, Tübingen, Germany for the kind gift of a ferricrocin standard.

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