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Sequestering of Iron as a Significant Virulence Factor of *Verticillium Dahliae* and it's Manipulation as a Potential Method for the Control of *Verticillium* Diseases

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Title

SEQUESTRING OF IRON AS A SIGNIFICANT VIRULENCE FACTOR OF VERTICILLIUM DAHLIAE AND ITS MANIPULATION AS A POTENTIAL METHOD FOR THE CONTROL OF VERTICILLIUM DISEASES.

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A. ABSTRACT

The objectives of the present project were: a) to evaluate the effect of iron applications on host tolerance towards Verticillium dahliae. b) to gain an insight into the role of iron in virulence and c) to characterize iron sequestration systems by V. dahliae and other fungal pathogens. Results presented indicate that when either FeEDDHA or foliar microelement applications containing iron were made to peanuts grown under field conditions in soil infested with the pathogen, a highly beneficial influence on plant vigor, green color of foliage and yield was obtained. No such effects were noted using the same treatments on plants grown in non-infested soil. Iron application may therefore be recommended for controlling verticillium wilt in peanuts. Iron limiting conditions increased the sensitivity of peanut, tomato, eggplant, potato and cannola to V. dahliae. Supplement of iron significanlty alleviated disease symtpoms in all the above mentioned crops.

The advancement and distribution of V. dehlia in iron-deficient and iron-sufficient peanut or tomato plants was similar. Iron deficiency as well as infection significantly induced phytoalexins production in peanuts. Medicarpin, the major phytoalexin detected, was 18.6, 23.8 and 43.4 times higher in iron-deficient healthy, iron-sufficient infected and iron-deficient infected peanuts than the healthy control. Nevertheless, phytoalexins production was not correlated with retardation of the pathogen. On the basis of the foregoing results, it was hypothesized that iron may exert its influence by rendering plants more tolerant towards virulence factors of the pathogen such as pectic enzymes, toxins or others.

Extensive studies on siderophores production and characterization of iron uptake systems was carried out with several phytopathogenic fungi. V. dahliae, Stemphylium botryosum (tomato pathogen) and Gaeumannomyces tritici produce dimerum acid and coprogen B as major hydroxamate siderophores whereas Geotrichum candidum was identified as a non-siderophore producer. All the 4 fungi appeared to possess receptors for hydroxamate siderophores of foreign origin. The kinetics specificity and mechanisms of the siderophore-mediated systems as well as iron uptake systems which do not involve siderophores (in S. botryosum and G. candidum) were characterized. Peanut and wheat plants could utilize iron from dimerum acid and coprogen B.

B. OBJECTIVES OF ORIGINAL RESEARCH PROPOSAL

Iron is an essential nutrient for most living cells and Fe^{3+} is the predominant oxidation state prevailing in aerobic environment. In spite of its abundance in nature, the availability of iron is extremely low owing to the profound insolubility of the ferric ion. Therefore, microbes, plants and animals have developed sequestering systems for the ferric ion to ensure its utilization. In bacterial and mycotic diseases of animals, iron acquisition has been established as a critical element of virulence.

The ultimate goal of the present proposal was to gain a deeper insight into the role of iron in virulence of plant pathogens and to demonstrate that iron can be manipulated for inducing tolerance towards verticillium wilt. The following points were investigated: a) The effect of iron applications on tolerance towards verticillium wilt in the field, b) mechanisms involved in the reponse of the verticillium-infected plants to iron and c) characterization of different systems present in V. dahliae and other fungal pathogens for competition of iron. Although we originally proposed to use potato as the major host for investigation in Israel, peanut was selected instead. The latter exhibits higher sensitivity to iron deficiency and was easier to grow and analyze in various field and greenhouse experiments.

C. BODY OF THE REPORT

The body of the report is presented as individual and independent manuscripts, some of which have already been published.

JOURNAL OF PLANT NUTRIITON, 11(6-11), 893-905 (1988)

EFFECT OF IRON STATUS ON VERTICILLIUM WILT DISEASE AND ON IN VITRO PRODUCTION OF SIDEROPHORES BY VERTICILLIUM DAHLIAE

KEY WORDS: Verticillium dahliae, iron deficiency, peanut, eggplant, siderophores.

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ABSTRACT

In peanuts cultivated in plots that were either artificially infested with Verticillium dahliae or non-infested, soil application of FeEDDHA or foliar application of Ryplex (Fe, Zn, Cu and Mn) significantly increased yield only in plots where the fungus was present. Addition of FeEDDHA to eggplants infected with V. dahliae significantly reduced disease severity in calcareous soil.

Coprogen B was the predominant siderophore produced by seven different strains of V. dahliae during a growth period of up to 18 days. Low amounts of dimerum acid and an additional unidentified siderophore were also detected. However, a decrease in coprogen B with a concomitant increase in dimerum acid and pH were measured in culture filtrates during a longer incubation period. A correlation was established between the pH value of the medium and the relative concentrations of the two siderophores. The magnitude of siderophore repression by iron varied among different isolates.

INTRODUCTION

Verticillium dahliae Kleb. is a destructive and widespread soilborne pathogen which infects numerous crop plants (Pegg, 1974). The disease is expressed by stunting, wilting and premature senescence. The fungus survives in the soil as microsclerotia which are associated with infected plant debris. As the crop residues decompose, the fungal propagules are released into the soil (Pegg, 1974).

Verticillium dahliae retards the development of peanut plants and causes irregular chlorosis and wilt. Krikun and Frank (1975) have reported that some cultivars and experimental breeder lines of peanut which has previously been found to be highly tolerant to the disease in sandy soils, succumbed to V. dahliae in calcareous loess, except when Sequestrene Fe 138 (FeEDDHA) was added into the soil close to the plants at the age of approximately 7 weeks. These results indicated that iron deficiency may increase the sensitivity of peanuts to Verticillium wilt.

Sesquestration of iron via siderophores has been established as an important determinant of virulence in bacterial and mycotic diseases of animals (Weinberg, 1984). A similar function could be assumed for siderophores in plant diseases (Neilands and Leong, 1986). A recent study with Erwina chrysanthemi supports the latter hypothesis (Expert and Toussaint, 1985). Dimerum acid has been reported to be the sole siderophore produced by V. dahliae (Harrington and Neilands, 1982). The present study was undertaken to examine the capability of V. dahliae to sequester iron under natural conditions and further characterize the production of siderophores by this pathogen.

MATERIALS AND METHODS

Plant Materials

Peanut plants (Arachis hypogaea L. cv. Shulamit) were cultivated in infested and non-infested plots in which potatoes had been grown previously. In the infested plots, the potatoes were infected by V. dahliae. Test plots were 5 m long and consisted of three beds (3 rows/bed). The experiment was arranged in a randomized complete block design with five replications per treatment. The soil was sandy loam. The treatment with Sequistrene Fe 138 (FeEDDHA, Ciba Geigy) was applied one month after the sowing date (May 15). Foliar application of Ryplex were sprayed 2, 2.5 and 3 months after sowing. Ryplex concentrations were

0.5% for the Fe and 0.2% for Mn, Zn and Cu. Ryplex is a lignosulfonate natural chelate containing approximately 6%* w/w of either Fe, Zn, Cu or Mn. It is sprayed with urea (1%w/v) to facilitate penetration. Three meters of the center bed were hand dug at maturity for yield determination.

Eggplant seeds (Solanum melongena L. cv. Black Beauty) were planted in pots containing washed sand and irrigated with half Hoagland solution without iron. Roots of seedlings approximately 3 cm high were immersed in a mycelial and spore suspension for 1 h and transferred to pots (15 x 12 cm) containing mountain rendzina light clay soils with a 63% $CaCO_3$ content. FeEDDHA was added at the rate of 50 mg Fe/pot with the irrigation water on the 7th and 14th days after seeding. The experiment was terminated after 35 days. Shoots were oven dried at 70°C for 48 h and weighed. Chlorophyll content of leaf samples was analyzed and calculated by the method of Mackinney (1941) as described by Barak and Chen (1982). There were three replications of 7 plants for each treatment (Table 2).

Isolates and culture media

The following isolates of V. dahliae were obtained from infected potatoes of different countries: dv-1 (Israel), p1, pb, sb (Canada), pk (England) and C-3, C-6 (USA). The fungi were maintained on potato dextrose agar or grown in liquid medium as described previously (Manulis et al., 1984). The iron limited medium for siderophores production contained the following compounds: glucose, 20g; asparagine, 2 g; KH_2PO_4, 1.5 g; $MgSO_4.7H_2O$, 100 mg; $ZnSO_4$, $7H_2O$, 20 mg; $MnSO_4$, 20 mg; pyridoxin, 5 mg; thiamine Hcl, 10 mg and double glass distilled water (pH 4.8).

Isolation and identification of siderophores

At the end of the growth period the siderophores were extracted from the culture filtrates by benzyl alcohol and purified to homogeneity as described previously (Manulis et al., 1987). The amount of total siderophores was measured according to Subramanian et al. (1965). To facilitate structure determination, the siderophores were deferrated with 8-hydroxyquinoline (Weibe and Winkelmann, 1975). ^{1}HNMR spectra were recorded in D_2O on a Brucker AM360WB spectrometer and IR spectra were obtained as described previously (Manulis et al., 1984). Authentic sample of dimerum acid was obtained from D. J. B. Neilands and coprogen B was isolated from Stemphylium botryosum (Manulis et al., 1987). Results presented are averages of at least three different experiments.

TABLE 1
Effect of Soil Application of FeEDDHA and Foliar Applications of Minor Elements on Yield of Peanuts Growing in Verticillium dahliae-Infested and Non-infested Soil.

Treatment	Yield per plant (g)	
	Infested Soil	Non-infested soil
Ryplex* (1 spray)	76.0^{b}**	106.8^{a}
Ryplex (2 sprays)	88.2^{ab}	107.2^{a}
Ryplex (3 sprays)	79.6^{b}	91.8^{a}
FeEDDHA (10 kg ha^{-1})	104.8^{a}	109.2^{a}
Control	50.6^{c}	96.0^{a}

*Ryplex contained Fe, Zn, Mn and Cu
**Means within columns followed by the same letter are not significantly different (LSD=0.05)

RESULTS

Effect of iron on tolerance to V. dahliae

The presence of V. dahliae in the soil caused a reduction of approximately 50% in the yield of peanut plants (Table 1). Soil application of FeEDDHA in infested soil one month after sowing significantly increased the yield, which reached a level close to that in the non-infested soil. A significant but smaller increase in yield was obtained by one to three foliar applications of Ryplex which were applied 2 months after seeding. No significant effect of any of these treatments on yield was recorded in non-infested soil. No visible disease symptoms could be detected in the plants during the experimental period.

The effect of iron on the severity of Verticillium wilt in eggplants grown under iron limited conditions is shown in Table 2. Addition of FeEDDHA significantly increased the height and dry weight of shoots in infected plants, as well as the chlorophyll content. Addition of iron to healthy eggplants did not induce a significant effect on the height or dry weight of shoots, but increased significantly the chlorophyll content. The dry weight of shoots from infected plants to which iron was added was significantly higher than that of healthy plants which did not receive additional iron.

TABLE 2
Effect of FeEDDHA on Disease Severity Caused by Verticillium dahliae in Eggplant Growing in Calcareous Soil.

Treatment	Shoot height (cm)	Shoot weight (mg dry wt)	Chlorophyll content (mg/1)
Healthy plants	6.7[a]*	150[bc]	76[b]
Healthy plants +FeEDDHA	6.6[a]	180[ab]	102[a]
Infected plants	5.3[b]	120[c]	54[c]
Infected plants +FeEDDHA	7.2[a]	220[a]	81[b]

*Within columns, means followed by a common letter are not significantly different (LSD=0.05).

Identification and production pattern of siderophores

Siderophores were extracted from filtrates of 18-day-old cultures and initially purified by gel filtration through a column of Bio-Gel P-2 (2.5 x 115 cm). Three major components which eluted with double distilled water from the column were designated as A, B and C according to their migration rate towards the cathode in paper electrophoresis. Siderophore C was a highly cationic compound, whereas B and A were only slightly cationic in pyridine-acetate buffer, pH 5.2. Further purification was achieved by chromatography on Cellex CM column which exhibited differential adsorption towards the three siderophores. Each siderophore was brought to homogeniety by employing preparative paper electrophoresis and Sephadex LH-20 column, as described by Manulis et al. (1987). Initial identification of siderophore B as dimerum acid and C as coprogen B was demonstrated by obtaining identical Rfs following co-chromatography with authentic samples in different solvents (Manulis et al., 1987). Identical migration rates of the two siderophores with authentic samples were also obtained with high voltage electrophoresis (2000 V) in pyridine-acetate buffer pH 5.2. Comparative ^{1}HNMR and IR spectra of the deferrated siderophores with deferrated authentic samples supported the identity of siderophore B and siderophore C as dimerum acid and coprogen B, respectively. ^{1}NMR

Growth, pH changes and production of siderophores as a function of incubation period are shown in Fig. 2. Maximum total siderophore production was observed on the 24th day after the organism reached

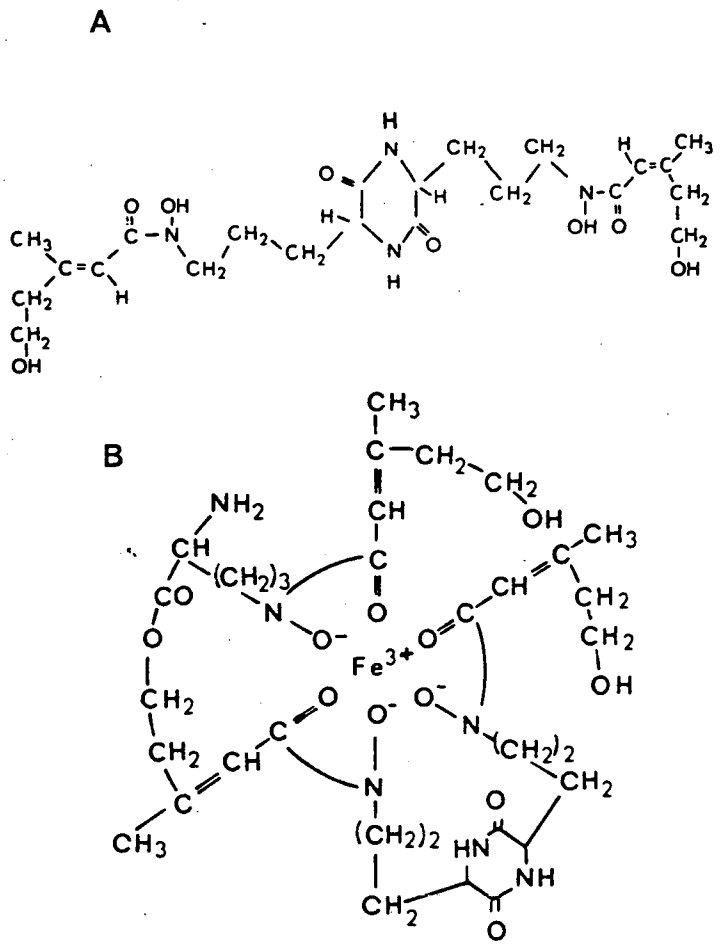

Fig. 1. Dimerum acid (A) Coprogen B

spectra of these siderophores were identical with previously reported spectra (Zahner et al., 1963; Diekmann, 1970; Harrington and Nielands, 1982; and Manulis et al., 1987).

the stationary phase. The concentration of total siderophores in the medium decreased sharply as the culture aged. Initial growth was followed by a decrease in pH from 4.8 to approximately 3.8 after 11 days, with a subsequent increase to 6.9 at the end of the growth period. Coprogen B was the predominant siderophore (>60%) produced by different strains of V. dahliae during a growth period of 18 days, whereas the amount of dimerum acid or siderophore A was minor (<20%) (Fig. 3).

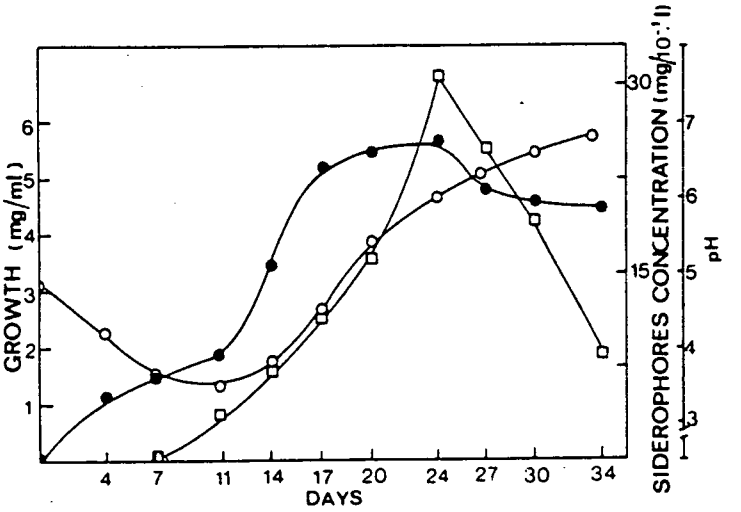

FIG. 2. Production of siderophores as function of growth and pH siderophores (□); pH (○) and growth (●).

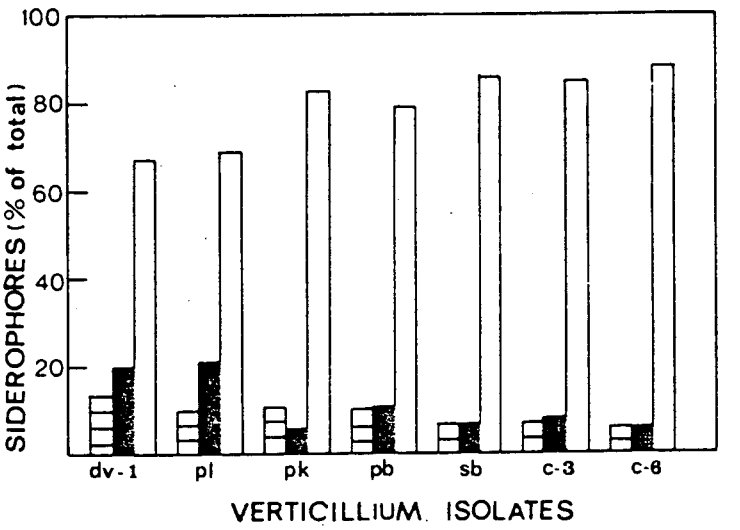

FIG. 3. Production of siderophores by different isolates after 18-day-growth period. Dimerum acid (⊟); siderophore A (■) and coprogen B (□).

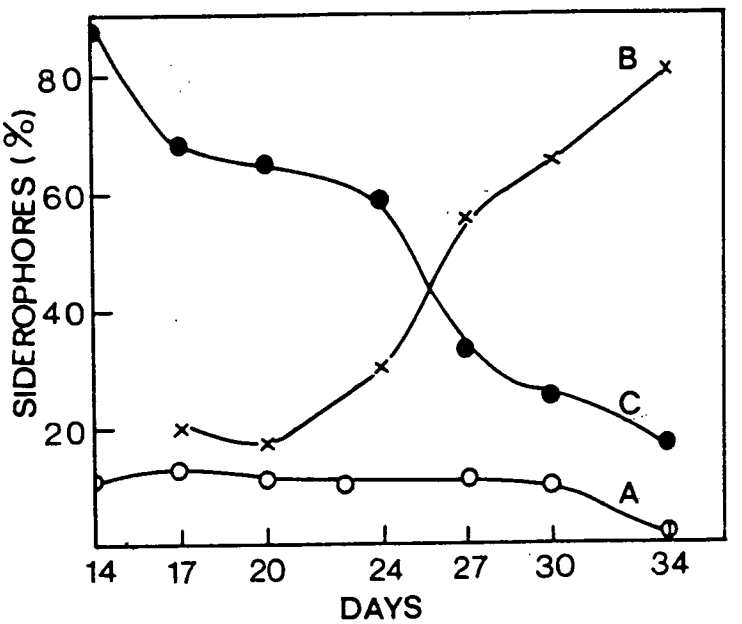

FIG. 4. Production of individual siderophores as a function of time. Siderophore A (A); dimerum acid (B) and coprogen B (C).

Results in Fig. 4 illustrate that the amount of coprogen B is significantly reduced after 14 days with a corresponding increase in dimerum acid. After 30 days, dimerum acid became the major siderophore produced by V. dahliae. Because a significant elevation in pH was recorded after 14 days (Fig. 2), it was assumed that the changes in the relative amounts of the two siderophores after this period might be influenced by pH. A sequential decrease in coprogen B and an increase in dimerum acid were correlated with a rise of the pH (Fig. 5a). A correlation was also established between an increase in total siderophores production and elevation in pH to 6.5 (Fig. 5b). The response of different isolates to repression of siderophore production by iron varied among different isolates (Fig. 6). The concentration of iron required for total repression of siderophores was in the range of 0.2 to 3 mg/l.

DISCUSSION

The development of non-infected peanut plants in plots heavily infested with V. dahliae (Table 1)

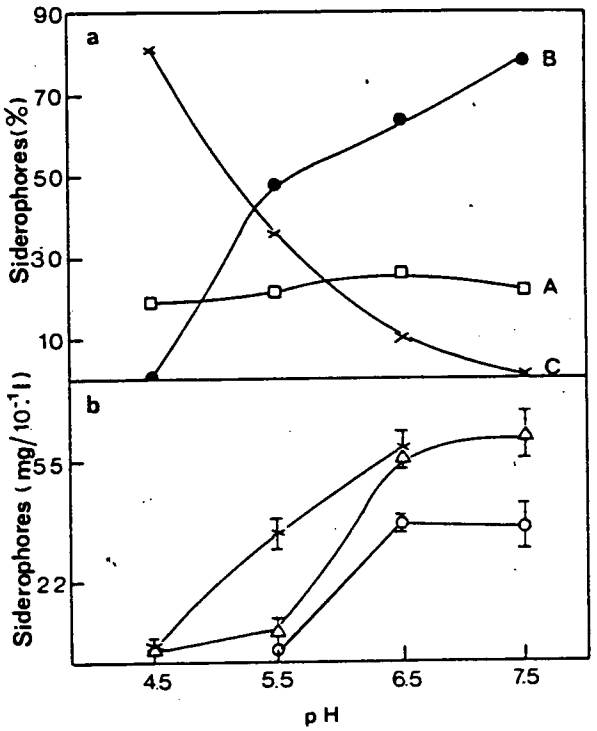

FIG. 5. Production of total and individual siderophores as a function of pH. Siderophores were determined after 20 days growth. Top: Production of individual siderophores: Coprogen B (C); dimerum acid (B) and siderophore A (A). Bottom: Production of total siderophores in various buffers at (0.1 M); citrate (×); phosphate (O); citrate-phosphate (△).

permitted study of the capability of the pathogen to deprive the plant of iron even when the former was present ex planta. Peanuts are highly sensitive to iron deficiency and have been used for evaluation of iron status in the soil (Barak and Chen, 1982). The dramatic increase in yield following FeEDDHA application to infested, but not to non-infested soil (Table 1) indicates that this fungus can efficiently sequester iron from the environment and compete for iron with plants and presumably with common microorganisms as well. Although peanut may serve as a host to V. dahliae (Krikun and Frank, 1975), the absence of Verticillium wilt in spite of the presence of the pathogen in the soil could result from differential host specificity. As indicated earlier,

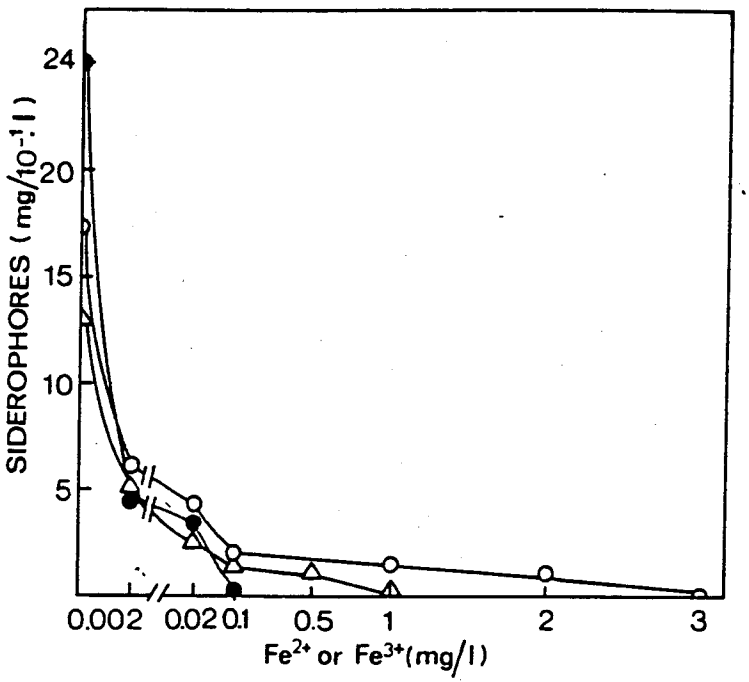

FIG. 6 Effect of iron concentration on siderophore production by various isolates. dv-1 (○); pl (△); sb (●).

the pathogenic isolate originated from infected potatoes and was presumably avirulent to peanut. Nevertheless, the possibility that the root system of the peanut plants was infected cannot be entirely excluded.

Iron deficiency caused by calcareous soil significantly increased disease severity in eggplants as expressed by growth retardation and chlorophyll content (Table 2). Addition of FeEDDHA completely restored normal growth and partially restored chlorophyll content. Aggravation of disease symptoms due to iron limitation was also demonstrated during the infection of bean by Fusarium solani (Guerra and Anderson, 1985). Botrytis cinerea, for example, produced lesions on leaves of broad bean more rapidly and many more spreading lesions developed under conditions of iron deficiency (Brown and Swinburne, 1982). In the latter case the withdrawal of iron by chelates was responsible for a decrease in phytoalexins production and consequently impaired the defense response. Reduction in lignin formation in beans

infected with F. solani under iron limiting conditions (Guerra and Anderson, 1985) could facilitate penetration and colonization by the pathogen. The mechanism for increases susceptibility of eggplants to V. dahliae due to iron deficiency is not yet clear.

Dimerum acid has been previously reported as the sole siderophore produced by V. dahliae (Harrington and Nielands, 1982). In the present work, coprogen B seemed the major siderophore produced by seven different isolates of this pathogen, whereas dimerum acid and an additional unidentified hydroxamate siderophore were present in minor quantities (Fig. 3). The discrepancy between the two studies is most likely due to variations in cultural conditions. The initial pH of the culture medium used by Harrington and Neilands (1982) for example, was 6.3 as opposed to 4.8 in the present work. As illustrated in Fig. 5a, the increase in production of dimerum acid with a corresponding decrease in coprogen B was correlated with elevation of the pH to 6.3. The pattern of siderophore production as a function of growth (Fig. 2) strongly resembles the kinetics of siderophore secretion in Fusarium dimerum which also forms dimerum acid and coprogen B (Diekmann, 1977). The inverse correlation in the production of the two siderophores (Figs. 4, 5) could imply that dimerum acid is formed as a breakdown product of coprogen B. The latter phenomenon could occur by enzymatic cleavage of the ester bond which links a fusarinine unit and dimerum acid to form coprogen B (Fig. 1).

The ability of microbial pathogens to compete successfully for a vital nutrient such as iron is considered a prerequisite for infection (Neilands, 1981). In animal systems, there is a substantial body of evidence correlating siderophore production with virulence (Weinberg, 1984). The role of siderophores in the pathogenicity of V. dahliae is still unclear. The capability of the pathogen to withdraw iron from the rhizosphere and induce iron deficiency (Table 1) may occur via siderophores.

Hydroxamate siderophores have been isolated from soil (Powell et al., 1980) and it is quite reasonable to assume that they are being secreted by V. dahliae for its survival ex planta. Whether conditions exist for the production of siderophores in planta is still an unresolved question. Harrington and Neilands (1982) failed to detect siderophores in cotton plants infected with V. dahliae. Moreover, iron depletion of the host plant during infection (Table 2) may be caused by

mechanisms other than siderophores. Thus, citrate, endogenous phenolic compounds, heme or iron proteins, rather than siderophores, may be the major iron source for V. dahliae within the host plant.

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REFERENCES

1. Barak, P., and Y. Chen. 1982. The evaluation of iron deficiency using a bioassay-type test. Soil Sci. Soc. Am. J. 46: 1019-1022.

2. Brown, A. E., and T. R. Swinburne. 1982. Iron chelating agents and lesion development by Botrytis cinerea on leaves of Vicia faba. Physiol. Pl. Path. 21: 13-21.

3. Diekmann, H. 1970. Vorkommen und strukturen von coprogen B und Dimerumsaure. Arch. Mikrobiol. 73: 76-76.

4. Expert, D., and A. Toussaint. 1985. Bacteriocin-resistant mutants of Erwinia chrysanthemi: Possible involvement of iron acquisition in phytopathogenicity. J. Bact. 163: 221-227.

5. Guerra, D., and A. J. Anderson. 1985. The effect of iron and boron amendments on infection of bean by Fusarium solani. Phytopathology 75: 989-991.

6. Harrington, G. L., and J. B. Neilands. 1982. Isolation and characterization of dimerum acid from Verticillium dahliae. J. Pl. Nutr. 5: 675-682.

7. Krikun, J., and Z. R. Frank. 1975. Effect of Sequestrene on the reaction to Verticillium dahliae of peanuts growing in calcareous loess soil. Phytoparasitica 3: 77-78.

8. Mackinney, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140: 315-322.

9. Manulis, S., Y. Kashman, and I. Barash. 1987. Identification of siderophores and siderophore-mediated uptake of iron in Stemphylium botryosum. Phytochemistry (in press).

10. Manulis, S., Y. Kashman, D. Netzer, and I. Barash. 1984. Phytotoxins from Stemphylium botryosum: structural determination of stemplyloxin II, production in culture and interaction with iron. Phytochemistry 23: 2193-2198.

11. Neilands, J. B. 1981. Iron absorption and transport in microorganisms. Ann. Rev. Biochem. 1: 27-46.

12. Neilands, J. B., and S. A. Leong. 1986. Siderophores in relation to plant growth and disease. Ann. Rev. Plant Physiol. 37: 187-208.

13. Pegg, G. F. 1974. Verticillium diseases. Rev. Pl. Path. 69: 378-384.

14. Powell, P. E., G. R. Cline, C. P. P. Reid, and P. J. Szaniszlo. 1980. Occurence of hydroxamate siderophore iron chelators in soils. Nature, Lond. 287: 833-834.

15. Subramanian, K. N., G. Padmanban, and P. S. Sarma. 1965. Folinciocalteus reagent for the estimation of siderochromes. Anal. Biochem. 12: 106-112.

16. Weibe, C., and G. Winkelmann. 1975. Kinetic studies on the specificity of chelate iron uptake in Aspergillus. J. Bacteriol. 123: 837-842.

17. Weinberg, E. D. 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. 64: 65-102.

18. Zahner, H., W. Keller-Schierlein, R. Hutter, K. Hessleisinger, and A. Deer. 1963. Stoffweehsel produkte von microorganismen. Arch. Mikrobiol. 45: 119-135.

Effect of FeEDDHA soil additions on yield of peanuts grown under field conditions in plots free and plots infested with V. dahliae

J. Krikun, R. Barak, L. Livescu, A. Nachmias

Previous studies conducted at the Gilat Experiment Station, Negev, Israel showed that when either foliar microelement applications or FeEDDHA soil applications were made to peanuts grown in soil which was infested with V. dahliae, such treatments had a highly beneficial influence on plant vigor, green color of foliage and yield. No such effects were noted using the same treatments on plants grown in non-infested soils. In order to confirm the results of the Fe EDDHA treatments, a further experiment was conducted where four rates of Fe EDDHA applications were tested.

Materials and methods

The experiment was conducted in a loessial soil, pH. 7.9; $CaCO_3$, 18%. Two plots, adjacent to each other, were used. Four years previously one of the plots was artificially infested with V. dahliae by incorporating chopped potato stems containing microsclerotia of the fungus into the soil. Both plots were identical with regard to previous cultural practices. After inoculum incorporation the plots were used for various Verticillium studies, comparing the effects of the fungus in infested and, as a check, in non-infested soil.

Prior to sowing peanuts, potato was grown on both plots. In the infested plot, Verticillium infection of potato was 100%; in the non-infested plot, no infection was observed.

The plots were prepared for peanut culture as for commercial growing. Sowing was on raised beds, two rows per bed. The cv. was Shulamit. Sowing date was 24 April. Six weeks after sowing differential treatments using Sequestrine 138 (FeEDDHA) were conducted by manually opening small furrows near the plants and applying 0, 50, 100 or 200 kg ha^{-1} of the chelated iron. After application the furrows were covered with soil and 300 m^3 ha^{-1} of irrigation water applied. There were six replications. Plots were 5 meters long and consisted of three beds. At time of planting Bradyrhizobium was injected into the soil near the seeds.

Observations were made during the growing season for disease symptom development. To test the possibility that plants may be infected but not showing typical disease symptoms, isolations were made from the vascular bundles of symptomless plants.

In order to determine if infected plants supplied with Fe EDDHA had different V. dahliae colonization levels as compared with plants growing with no fe EDDHA added, a study was made which compared numbers of propagule forming units in stems of infected plants growing in 0 and 100 kg ha^{-1} Fe EDDHA amended plots. This was done by blenderizing in water 1 cm stem sections and after proper dilution, distributing 0.1 ml aliquots on sorbose agar. After two weeks incubation at 25°C V.dahliae colonies could easily be counted by noting those colonies which produced microsclerotia on this medium.

At the end of the growing season, three meters from the center bed of each plot were dug up and the yield weighed. Yield data is provided on a plant basis.

Results and discussion

Germination and growth during the first six weeks were similar in both plots. At this time, chlorosis symptoms and a slowdown of growth of the plants in the infested plots began to appear. With the application of the chelate treatments these symptoms disappeared in the treated plots in the infested plot.

Typical V. dahliae symptoms did not begin to appear until 2.5 months after sowing. They were never greater than 10% in any one treatment in the infested plot. When typical leaf symptoms appeared, consisting of intervernal chlorosis and usually marginal necrosis, vascular discoloration of the stem was always present, and V. dahliae could always be isolated. The pathogen was never isolated from the above ground portion of plants which showed no symptoms.

Counts of Verticillium colony forming units from stems from infected plants growing in 0 and 100 kg ha^{-1} Fe EDDHA treated plots suggested no difference in colonization due to treatment; counts from 0 Fe EDDHA plots were 126 per plate as compared to 171 per plate from chelate treated plots (differences not significant).

In the infested plots, plants in the 0 Fe EDDHA plot remained chlorotic, whereas those receiving the chelate recovered from the chlorosis and developed normally. No chlorosis or stunting was observed in the 0 Fe EDDHA plots in the non-infested plots.

The yield data is presented in Table 1. As can be seen from the results, Fe EDDHA treatments greatly affected yield in the infested plots, but not in the non-infested one.

These results, which have also been obtained previously, are difficult to explain. We can at present not put forth a hypothesis

which can explain the above results. From our data it would seem that either the previous presence of a heavily infected crop (potato) influenced the soil Fe status in the following crop (peanut) or that the very presence of the pathogen in the soil and/or the rhizosphere limits Fe uptake by peanut.

In discussions with Dr. R. Chenney from the A.R.S., Beltsville, Md. the possibility was raised that saprophytic colonization of the potato stems by V. dahliae after incorporation into soil may have an affect of immobilizing soil Fe. Very preliminary laboratory results indicate that when V. dahliae produced microsclerotia there is much greater siderophore production than when the fungus is not producing microsclerotia. It may be that, if in fact this occurs under field conditions, a partial explanation of the above results may be hypothesized.

Table 1. Effect of FeEDDHA soil applications on yield of peanut growing in Verticillium dahliae infested and non-infested soil (gr. plant^{-1})

Treatment	Infested	Non-infested
FeEDDHA kg ha^{-1}		
0	31 a[1] A[2]	70 b B
50	61 a B	85 a B
100	72 a B	81 a B
200	82 a B	93 a B

1 - numbers followed by lower case letters different between columns at 0.05% level.

2 - numbers followed by upper case letters different within columns at 0.05% level.

Effect of nutrient supplements in Verticillium wilt of potato

J. Krikun, S. Shabtai, R. Barak, L. Livescu, A. Nachmias

As shown in the previous report, a combination of Fe, Zn, Cu and Mn as foliar applications to potato foliage of Verticillium infected plants reduced yield loss in such plants, while no effect was noted in control (non-infected) plants. This research was continued in the spring season of 1989 with the following modifications:

1. Two cvs. were tested: Blanca and Nicola
2. Rather than using a combination of minor element sulfates, a commercial foliar nutrient preparation which contains the minor elements in chelated form was used for foliar applications.
3. Iron, supplied as Fe EDDHA was used as a soil treatment.

As in previous years, the experiments were conducted in infested and non-infested plots at Gilat Experiment Station, in the Negev Region.

Materials and Methods

Cvs. used: Blanca and Nicola

Dates of sowing: Control plot 22 February 1989

Verticillium plot 24 February 1989

Seedbed Preparation: Pre-irrigation with 600 m^3 ha^{-1}, plowing to 35 cm, disced and beds prepared.

Fertilization: 1,200 kg double superphosphate ha^{-1}

300 kg KCl ha^{-1}

400 kg NH_4SO_4 ha^{-1}

incorporated into soil prior to sowing.

During the growing season 270 kg ha^{-1} N, as NH_4NO_3 supplied via the irrigation system.

Plots were 10 meters long; 4 rows. There were four replications.

Supplemental nutrition:

1) Sequestrine 138 - Fe EDDHA applied at the rate of 10 kg ha^{-1} on April 23, 1989, by diluting in sand, and hand spread between the rows. After spreading, an irrigation of 300 m^3 ha^{-1} was used to infiltrate the chelated Fe into the root zone.

2. Microelement sprays: Wuxal preparation 7: obtained from Aglukon, Dusseldorf, West Germany, at rates of 1.0 and 1.5% of the formulated material. The sprays on both cvs. were conducted on 25 April and 8 and 28 of May. A further spray was conducted on the cv. Nicola on 8 June.

Disease ratings

Vine vigor ratings as related to Verticillium symptoms were conducted on 28 April and on 16 and 28 May; ratings were from 0-5; 0=completely healthy; 5 - all plants dead.

Yield analysis

The plots were dug up after complete drying in the non-infested plots, in this case for Blanca on 25 June and for Nicola, on 3 July.

Five meters of row from the two center rows of the plots were dug up; total 10 meters row. Rows were 0.9 meters apart; total 9 m^2. The yield was separated into three size classes: less than 100 gr.;

100-200 gr. and over 200 gr. per tuber.

At harvest haulms were examined for vascular discoloration. Twenty tubers of over 200 grams in weight from each replication were examined for stolon end vascular discoloration.

Results and discussion

The results of Verticillium ratings are presented in Table 1. The effects of the treatments on the yield of the cvs. Blanca and Nicola are presented in Tables 2 and 3.

As can be seen from the results presented in Tables 1, 2, and 3, all three treatments reduced Verticillium symptom expression in both cvs. Yield improvement due to the treatments was noted only in the cv. Blanca. It is interesting to note that the yield improvement was apparent in the over 200 gr. category of tuber size. It is accepted that Verticillium usually causes a reduction in tuber size, therefore the results obtained with the cv. Blanca are in keeping with the proposed hypothesis that the supplemental fertilization treatments maintained the plants in a better physiological state and permitted greater translocation of photosynthate to the tubers than in untreated plants. That the cv. was not lacking any of the usual soil supplied nutrients when non-infected can be seen from the results in the non-infested plots; and from the fertilizers added before sowing.

The added yield effect due to the treatments is in the order of 10 metric tons ha ; and particularly as it is with large tubers, quite meaningful. As can be seen from Table 2, the yield in the non-infested plots was higher than in the infested ones; although strict comparisons cannot be made as the two plots were ca 1.0 km apart.

It should be pointed out that in the infested plots there was 100% plant infection; there was 0% infection in the non-infested plots.

As can be seen from Table 3, the treatments had no effect on yield of Nicola under both disease levels. In contrast to Blanca most of the yield was in the 100-200 gr. range. Nicola has a longer growing period than Blanca, and as is apparent from the results Blanca is a heavier yielder. It may be that the rate of bulking is important in determining the effect of the nutrient treatments. That the treatments were partially effective can be seen from the results in Table 1, with regard to symptom expression. It can also be observed that symptom expression in Nicola at the earlier stages of growth was less severe.

Our results, and those of previous years, suggest that certain nutritional treatments can greatly alleviate yield loss in at least certain cvs. of potato. These treatments are very inexpensive, and ecologically sound. More needs to be understood about the possible mechanisms. In last year's report we showed lowered K levels in infected plants, and that foliar microelement treatments increased the K level.

Bolle-Jones showed that an interaction existed between K and Fe in potato. Increasing Fe greatly increased yield at three out of four levels of K tested, the differences being 11 fold at the lowest K level. Thus it is possible that in Verticillium infected plants, besides the positive effects of Fe in itself, side effects pertaining to K uptake and translocation may be operative.

Jolley et al have also shown an interaction of K and Fe in tomato and soybean, which may have a bearing on the work reported here.

References

Bolle-Jones, E.W. 1955.
Plant and Soil 6:129-173.

Jolley, V.D., J.C. Brown, M.J. Blaylock and S.D. Camp. 1988.
Jl. of Plant Nutrition 11:1159-1175.

Table 1. Effect of foliar or soil treatments on Verticillium symptoms of potato; cvs. Blanca and Nicola

	Blanca		Nicola	
	days after sowing		days after sowing	
	82	94	82	94
Treatment				
Control	2.13* a	2.88 a	1.00 a	2.13 a
Wuxal				
Type 7				
1.0%	1.38 b	1.75 b	1.00 a	1.50 b
1.5%	1.25 b	1.63 b	0.25 ab	1.63 b
FeEDDHA				
10 kg ha^{-1}	1.13 b	1.50 b	0.0 b	1.38 b

* - Verticillium ratings: 0-5, 0 = no symptoms; 5 = all plants dead

Mean separation in columns by Duncan's multiple range test, 5% level

Table 2. Effect of foliar or soil treatments on yield (kg $9m^{-2}$) of potato, cv. Blanca grown in infested and non-infested plots

	Infested Plot			Non-infested Plot		
	up to 100 g	100-200 g	>200 g	up to 100 g	100-200 g	>200 g
Treatment						
Control	6.24 a	16.47 a	11.38 a	11.25 a	19.78 a	23.63 a
Wuxal						
Type 7						
1.0%	5.06 a	19.47 a	20.03 b	9.38 a	21.50 a	29.38 a
1.5%	5.63 a	19.01 a	22.00 b	9.50 a	19.88 a	26.88 a
FeEDDHA	5.30 a	20.06 a	21.43 b	9.18 a	20.95 a	30.25 a
10 kg ha^{-1}						

Mean separation in columns by Duncan's multiple range test, 5% level

Table 3. Effect of foliar or soil treatments on yield (kg $9m^{-2}$) of potato, cv. Nicola grown in infested and non-infested plots

	Infested Plot			Non-infested Plot		
	up to 100 g	100-200 g	>200 g	up to 100 g	100-200 g	>200 g
Treatment						
Control	14.78 a	21.83 a	4.48 a	20.36 a	28.03 a	5.48 a
Wuxal						
Type 7						
1.0%	13.28 a	21.59 a	5.06 b	19.28 a	26.88 a	6.85 a
1.5%	14.80 a	20.98 a	3.30 b	20.28 a	25.73 a	7.43 a
FeEDDHA	17.70 a	20.75 a	3.81 b	19.83 a	28.10 a	7.10 a
10 kg ha^{-1}						

Mean separation in columns by Duncan's multiple range test, 5% level

The Relationship Between Iron Nutrition and Verticillium Wilt Resistance in Tomato

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ABSTRACT

The effect of iron nutritional status on Verticillium Wilt disease in tomato possessing single gene resistance to Race 1 of Verticillium dahliae was investigated using hydroponic culture media. Iron limiting conditions increased the sensitivity of resistant tomatoes to the pathogen as expressed by wilting. Distance of longitudinal invasion was approximately the same in both iron replete and iron limited treatments. However, the magnitude of wilting in resistant near-isoline Pixie II was considerably less than that expressed by the susceptible Pixie variety. Infection of tomato did not enhance iron stress severity as quantified by root peroxidase activity and chlorophyll content of young leaves.

INTRODUCTION

Nearly all living cells require iron for their survival. Though relatively abundant in nature, the low solubility of iron under aerobic conditions limits its availability to organisms. Thus, plants and microbes utilize iron sequestering systems to maintain a well regulated supply of this essential element.

Acquisition of iron has been reported to be a critical virulence factor in a number of plant-pathogen interactions (Kloepper et al., 1980; Expert and Toussaint, 1985). The influence of iron nutritional status on Verticillium wilt severity has been observed in peanut and eggplant (Krikun and Frank, 1975; Barash et al., 1988). Krikun and Frank (1975) reported that peanut varieties which were highly tolerant to Verticillium Wilt in sandy soils, succumbed to the disease when grown in iron limiting, calcareous loess soil. The addition of an iron amendment to the calcareous soil allowed the plants to retain tolerance to the disease. A similar study by Barash et al. (1988) utilizing eggplant revealed comparable results. Enhancement of disease expression through iron limitation has also been observed during infection of bean by Fusarium solani (Guerra and Anderson, 1985) and Botrytis cinerea (Brown and Swinburne, 1982). Reduction in lignin formation and decreased phytoalexin production were cited as reasons for the aggravation of disease symptoms under iron deficient conditions. The mechanisms involved in iron's effect on sensitivity of peanut and eggplant to Verticillium dahliae are not yet clear.

Plants exhibit complex physiological and anatomical responses to various forms of stress induced by different biotic and abiotic factors. A considerable amount of information is known about iron stress response

mechanisms in tomatoes (Bienfait, 1985) and tomato response to infection by Verticillium spp (Pegg, 1981). The present study was undertaken to examine the interaction and combined effect of these two stress inducing conditions in tomato.

MATERIALS AND METHODS

Plants, Pathogen, and Inoculation

Pixie II-resistant and Pixie-susceptible tomato varieties were used as near-isolines differing in the presence of the Ve gene for resistance to race 1 of V. dahliae. Ace VF (resistant) and Marglobe (susceptible) were also utilized in experiments. Seeds purchased from W. Atlee Burpee Co. (Warminster, PA) were surface sterilized for three minutes in 0.5% sodium hypochlorite and germinated on stainless steel screens covered with moist cheesecloth. The tomato seedlings were transferred to opaque 10L polyethylene tubs (24 seedlings per tub) containing 8L of standard nutrient solution plus 36μM FeEDTA. The standard nutrient solution was composed of 1.90 mM $Ca(NO_3)_2$, .47 mM $Mg(NO_3)_2$, .24 mM KCl, .61 mM KNP_3, .60 mM K_2HPO_4, .32 m M $(NH_4)_2$ SO_4, 7.4 μM $MnCl_2$, 41.3 μM H_3BO_4, 1.9 μM $ZnSO_4$, 0.5 μM $CuSO_4$, and 0.4 μM Na_2MoO_4 with a pH of approximately 7.0. All nutrient solutions in the experiments were continuously aerated and were changed weekly. At the late 3-leaf stage, plants were inoculated by submergence of the roots into an aerated conidial suspension (1.0×10^6 conidia/ml) of V. dahliae for 24 hours. After inoculation, individual seedlings were placed into opaque polyethylene bottles containing 1L of standard nutrient solution. To allow induction of iron deficiency, 5.0 mM $NaHCO_3$, 0.25 g $CaCO_3$, and 54 μM EDTA were added to all treatments. Iron replete treatments also contained 54 μM $FeCL_3$. The growth environment consisted of

16 hours light with an approximate energy level of 210 $\mu E\ m^{-2}\ s^{-1}$ followed by 8 hours of darkness. The day and night temperatures were 22°C ± 0.7 and 21°C ± 0.7, respectively, while the average relative humidity was about 70%. Analysis of specific physical and biochemical parameters was initiated after 20 additional days of growth.

Evaluation of Disease

Wilting was used as the criterion for disease expression in the tomato plants (Visser and Hattingh, 1980). Wilt symptoms were quantified by measurement of leaf stomatal resistance with a MK3 automatic porometer (Delta - T devices LTD.). Measurements were taken on terminal leaflets of lower leaves.

Fungal Infection

Longitudinal fungal progression through the vascular system was measured 23 days after inoculation. Leaves and lateral roots were removed and the main axis was dipped into 95% ethanol for 5 seconds followed by submergence in 0.5% sodium hypochlorite for 3 minutes. Segments 0.5 cm in length were cut and placed on czapeks agar media containing 100 ppm kanamycin monosulfate. After 7 days of incubation, the segments were visually inspected for outgrowth of V. dahliae.

Evaluation of Iron Deficiency

Iron deficiency was quantified by a visual chlorosis rating (1 = no chlorosis, 5 = severe chlorosis) and determination of chlorophyll content.

Chlorophyll content was measured in a manner described by Inskeep and Bloom (1985).

Peroxidase Activity

Root samples of Pixie and Pixie II were assayed for peroxidase activity using the technique modified from Reuveni and Ferreira (1985). Immediately following excision, 0.3 g root samples were placed in dry ice. The frozen tissues were homogenized with a chilled mortar and pestle. The pulverized material was added to 3.0 ml of cold 15 mM sodium phosphate buffer, pH 6.0, and the homogenate was centrifuged at 10,000 g for 10 min at 4°C.

Aliquots of the enzyme extract (50 µL) were added to 3.0 ml of the assay solution consisting of 15 mM sodium phosphate buffer, pH 6.0, 1.0 mM H_2O_2, and 0.1 mM O-methoxyphenol (guaiacol). Enzyme activity was expressed as change in absorbance (470 nm) min^{-1} g^{-1} fresh weight.

Total Iron

Total iron analysis of leaf and petiolar tissue was conducted on the susceptible Marglobe variety. Perchloric and nitric acid tissue digests were analyzed for total iron using atomic absorption spectrophotometry (Perkin-Elmer Model 560 atomic absorption spectrophotometer).

O-dihydroxyphenols

The method utilized for analysis of total O-dihydroxyphenols was modified from Collier (1979). Root material (0.25 g) was added to 3.0 ml 95% ETOH and homogenized for 10 minutes (Virtis 23 homogenizer). The homogenate was filtered (0.45 µm) and loaded on 4.0 x 0.8 cm acid alumina

columns (Activity Grade 1). The columns were rinsed with two bed volumes of 95% enthanol followed by diazotozation of the O-dihydroxyphenols with two bed volumes of a 5% $NaNO_2$ plus 5% acetic acid solution. The diazotized compounds were eluted with two bed volumes of 5.0 N NaOH and assayed spectrophotometrically at 520 nm. Caffeic acid was used as the standard.

Siderophore Assay

Siderophore assay agar media was prepared according to the method outlined by Schwyn and Neilands (1987). This assay is non-specific and is very sensitive to molecules possessing iron chelating properties. The assay was conducted to test for the presence of iron chelates in the vascular system. Xylem fluid was squeezed out of stem cuttings and placed on the assay media. After 24 hours the media was visually inspected for color change.

RESULTS

Measurements of stomatal resistance provide evidence indicating that iron deficiency of tomato plants bearing major gene resistance to V. dahliae increases their sensitivity to the pathogen (Fig. 1). Iron deficient resistant plants exhibited significant wilting while iron replete plants showed little wilting. However, visual observation revealed slight stunting of iron replete Pixie II lower leaves. Though sensitivity of resistant varieties was significantly increased by iron deificiency, the magnitude of wilting was considerably less than that expressed by the susceptible Pixie variety. The iron status of infected Pixie had no significant effect on stomatal resistance.

The assay used to determine extent of longitudinal invasion indicated the pathogen was able to advance a significantly greater distance in Pixie versus Pixie II (Table 1). The distance invaded in the resistant varieties was approximately the same for both iron replete and iron limited treatments. Nearly all petiole segments excised from lower leaves of inoculated treatments tested positive for presence of the pathogen.

Infection of either resistant or susceptible tomato did not aggravate iron stress in the host as quantified by root peroxidase activity, visual chlorosis rating and determination of chlorophyll content (Table 1). However, iron stress of diseased Pixie was considerably less severe than any other iron limited treatment. In addition, peroxidase activity in the infected, iron deficient Pixie treatment was significantly higher than other treatments (Fig. 2). All other iron limited treatments demonstrated reduced activity. Root peroxidase is a sensitive indicator of iron stress and an increase in iron stress is correlated with a decrease in enzyme activity (Sijmons, 1985).

In contrast, infection by vascular wilt pathogens is correlated to elevation in peroxidase activity (Pegg, 1981). Under the present conditions, elevation of peroxidase activity due to infection was only observed in conjunction with iron stress. Reduced activity, which would be expected under iron limitation, was apparently delayed or masked by the additional effect of infection. In a similar experiment conducted with the susceptible Marglobe variety, total iron analysis of leaf and petiole tissue indicated that infected plants subjected to iron limitation contained 31% more iron than non-infected plants. No difference was observed in the iron replete treatment.

Analysis of O-dihydroxyphenols yielded results too variable for discernment of significant differences between treatments. All iron deficient treatments tested positive for the presence of siderophores and/or chelates in the xylem fluid. The iron replete treatments tested negative. These results may be attributed to high concentration of iron free EDTA in the nutrient solution of iron deficient treatments.

DISCUSSION

The use of hydroponic culture media permitted study of the interaction between iron deficiency and pathogenicity of V. dahliae in tomato. Incorporation of near-isolines Pixie (susceptible) and Pixie II (resistant) into the experiments allowed for a viable comparison between resistant and susceptible cultivars. The results showed that infected tomatoes possessing major gene resistance expressed an increase in wilting upon iron limitation. However, the level of wilting expressed by iron stressed Pixie II was considerably less than that expressed by the susceptible Pixie variety.

In addition, longitudinal progression of the pathogen in stems of Pixie II and Ace VF was not significantly different between iron replete and iron deficient treatments. These results suggest that enhanced expression of disease upon iron limitation is due to an increase in sensitivity of resistant plants to the pathogen rather than restriction of fungal invasion.

Iron is directly involved in many enzymatic and electron transport reactions in cells. Thus, its limitation can severely impact plant metabolism and subsequently increase a plant's sensitivity to virulence factors such as toxins, hydrolytic enzymes, and/or hormones. Iron

limitation may also cause impairment of one or more of the resistance factors regulated by the Ve gene. Although many of these factors are associated with restriction of fungal invasion, i.e., tylose formation (Sinha and Wood, 1958), hypersensitivity, phytoalexin accumulation (Tjamos and Smith, 1975), and secretion of vascular coatings (Robb et al., 1987), physiological mechanisms have also been identified as critical to resistance (Dixon and Pegg, 1969). The specific Ve gene regulated physiological mechanisms are currently unknown.

Stunting induced by iron limitation may be directly responsible for enhancement of wilt symptoms in resistant varieties. A reduction in total plant volume (quantified by height, Table 1) may have allowed for a more concentrated build-up of symptom inducing compounds as compared with non-stunted plants.

A positive correlation between elevated peroxidase activity and presence of the Ve gene has been observed in tomato (Reuveni and Ferreira, 1985). Guerra and Anderson (1985) have suggested that peroxidases involved in resistance to disease are directly impacted by iron deficiency. Peroxidases are iron containing enzymes especially sensitive to iron deficiency (Sijmons et al., 1985). They catalyze redox reactions with H_2O_2 and phenols. One of the mechanisms associated with iron stress response in tomato is accumulation and subsequent root exudation of phenolics resulting from reduced peroxidase activity. Phenolics, in turn, are known to play a complex role in pathogenesis of wilt diseases, i.e., metabolism of IAA and other growth substances and regulation of enzyme activity including polygalacturonase (Pegg, 1981). The complex interaction between phenols and resistance has yet to be fully understood.

Research conducted by Barash et al. (1988) suggested that V. dahliae was able to induce iron deficiency in peanut plants. They proposed that sequestration of iron may be a significant virulence factor in the disease. In the current study, the pathogen demonstrated no significant ability to withhold iron from plants although iron did play a role in expression of disease. However, the use of peroxidase activity and chlorophyll content as parameters for quantification of iron stress may not be valid in this plant-pathogen interaction. Nevertheless, the results imply that iron sequestration may not be a significant virulence factor in this system.

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REFERENCES

Barash, I., R. Zion, J. Krijun, and A. Nachmeas. 1988. Effect of iron status on Verticillium wilt disease and on in vitro production of siderophores by Verticillium dahliae. J. Plant Nut.

Bienfait, H. F. 1985. Regulated redox processes at the plesmalemma of plant root cells and their function in iron uptake. J. Bioenerg. Biomem. 17:73-83.

Brown, A. E. and T. R. Swinburne. 1982. Iron chelating agents and lesion development by Botrytis cinerea on leaves of vicia faba. Physiol. Pl. Path. 21:13-21.

Collier, G. F., V. C. Huntington, and E. F. Cox. 1979. A possible role for chlorogenic acid in calcium-related disorders of vegetable crops with particular reference to lettuce tipburn. Commun. Soil Sci. and Plant Anal. 10:481-490.

Dixon, G. R. and G. F. Pegg. 1969. Hyphal lysis and tylose formation in tomato cultivars infected by Verticillium albo-atrum. Trans. Brit. Myco. Soc. 53:109-118.

Expert, D. and A. Toussaint. 1985. Bacteriocin-resistant mutants or Erwinia chrysanthemi: Possible involvement of iron acquisition in phytopathogenicity. J. Bact. 163:221-227.

Guerra, C. and A. J. Anderson. 1985. The effect of iron and boron amendments on infection of bean by Fusarium solani. Phytopathology 75:989-991.

Inskeep, W. P. and P. R. Bloom. 1985. Extinction coefficients of chlorophyll a and b in N,N-Dimethylfomamide and 80% acetone. Plant Physiol. 77:483-485.

Kloepper, J. W., J. Leong, M. Teintze, and M. N. Schroth. 1980. Pseudomonas siderophores: A mechanism explaining disease-suppressive soils. Cur. Microbio. 4:317-320.

Krikun, J. and Z. R. Frank. 1975. Effect of sequestrene on the reaction to Verticillium dahliae of peanuts growing in calcareous loess soil. Phytoparasitica 3:77-78.

Pegg, G. F. 1981. Biochemistry and physiology of pathogenesis. In Fungal Wilt Disease of Plants, Ed. by Mace, M. E., A. A. Bell, and C. H. Beckman, pp 193-253. Academic Press, New York.

Reuveni, R. and J. F. Ferreira. 1985. The relationship between peroxidase activity and the resistance of tomatoes (Lycopersicum esculentum) to Verticillium dahliae. Phytopath. Z. 112:193-197.

Robb, J., D. A. Powell, and P. F. S. Street. 1987. Time course of wall-coating secretion in Verticillium-infected tomatoes. Physiol. and Mole. Path. 31:217-226.

Schwyn, C. and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160:47-56.

Sijmons, P. C., P. E. Kolattukudy, and H. F. Bienfait. 1985. Iron deficiency decreases suberization in bean roots through a decrease in suberin specific peroxidase activity. Plant Physiol. 78:115-120.

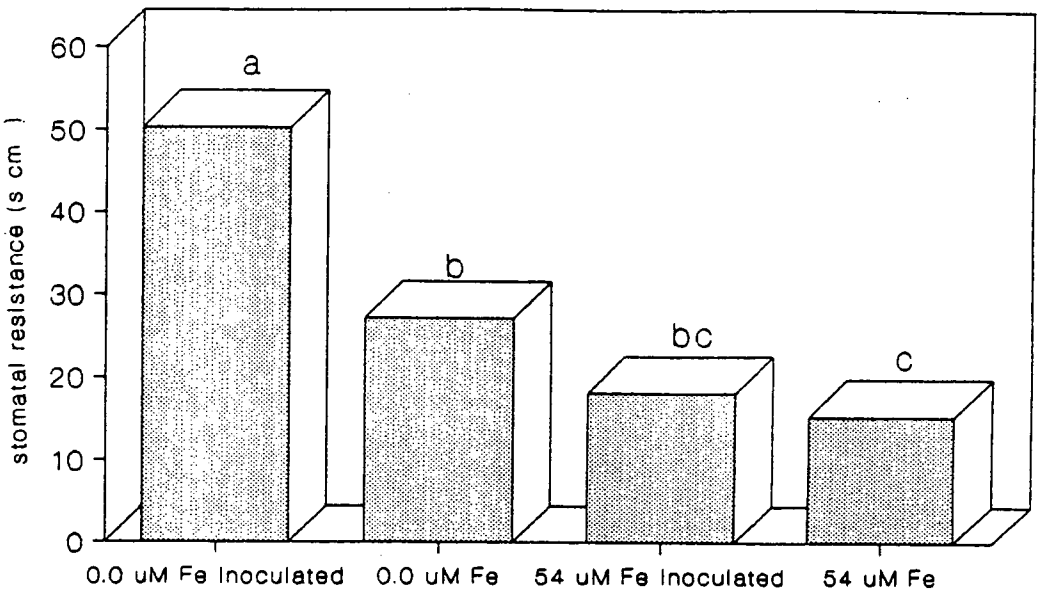
Sinha, A. K. and R. K. S. Wood. 1968. Studies on the nature of resistance in tomato plants to Verticillium albo-atrum. Ann. Applied Bio. 62:319-327.

Tjamos, E. C. and I. M. Smith. 1975. The expression of resistance to Verticillium albo-atrum in monogenically resistant tomato varieties. Physiol. Plant Path. 6:215-225.

Visser, S. and M. J. Hattingh. 1980. Criteria for quantal host response of tomato plants to infection by Verticillium dahliae. Plant Dis. 64:207-208.

Fig. 1. Disease symptoms were measured by stomatal resistance (1/transpiration) 20 and 21 days after inoculation. A: Ace VF (resistant). B: Near-isolines Pixie (susceptible) and Pixie II (resistant) (P = 0.05).

Fig. 2. All 0.0 μM iron treatments exhibited depressed peroxidase activity with the exception of infected Pixie. Infection of iron replete treatments showed no influence on enzyme activity (P = 0.05).


60
50
40
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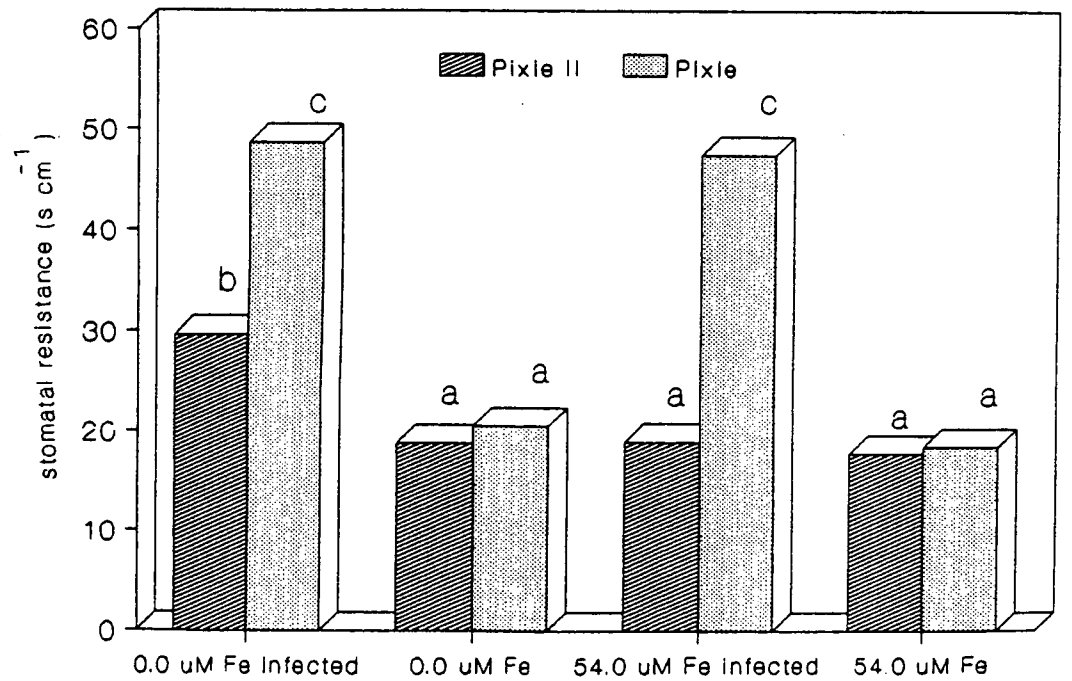
20
10
0
stomatal resistance (s cm -1)
a
b
bc
c
0.0 uM Fe Inoculated
0.0 uM Fe
54 uM Fe Inoculated
54 uM Fe


60
50
40
30
20
10
0
stomatal resistance (s cm -1)
Pixie II
Pixie
b
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a
a
a
c
a
a
0.0 uM Fe Infected
0.0 uM Fe
54.0 uM Fe Infected
54.0 uM Fe

fig. 1

Table 1. Comparison of stunting, distance of longitudinal fungal invasion, and quantified iron stress (1 = no stress, 5 = severe stress) in near-isolines (P = 0.05).

variety	treatment		plant height (cm)	fungal invasion (cm)	chlorosis rating	chlorophyll content (mg/cm^{-2})
Pixie II	0.0 uM iron	inoculated	13.7 ^{a}	8.2 ^{a}	3.3 ^{b}	1.0 ^{a}
		control	13.7 ^{a}	--	3.3 ^{b}	0.6 ^{a}
	54.0 uM iron	inoculated	20.5 ^{b}	9.0 ^{ab}	1.0 ^{a}	14.5 ^{d}
		control	21.9 ^{b}	--	1.0 ^{a}	14.6 ^{d}
Pixie	0.0 uM iron	inoculated	10.9 ^{c}	10.9 ^{b}	2.0 ^{c}	4.3 ^{b}
		control	16.8 ^{d}	--	3.5 ^{b}	0.8 ^{a}
	54.0 uM iron	inoculated	18.3 ^{d}	14.5 ^{c}	1.0 ^{a}	12.4 ^{c}
		control	24.4 ^{e}	--	1.0 ^{a}	16.2 ^{e}

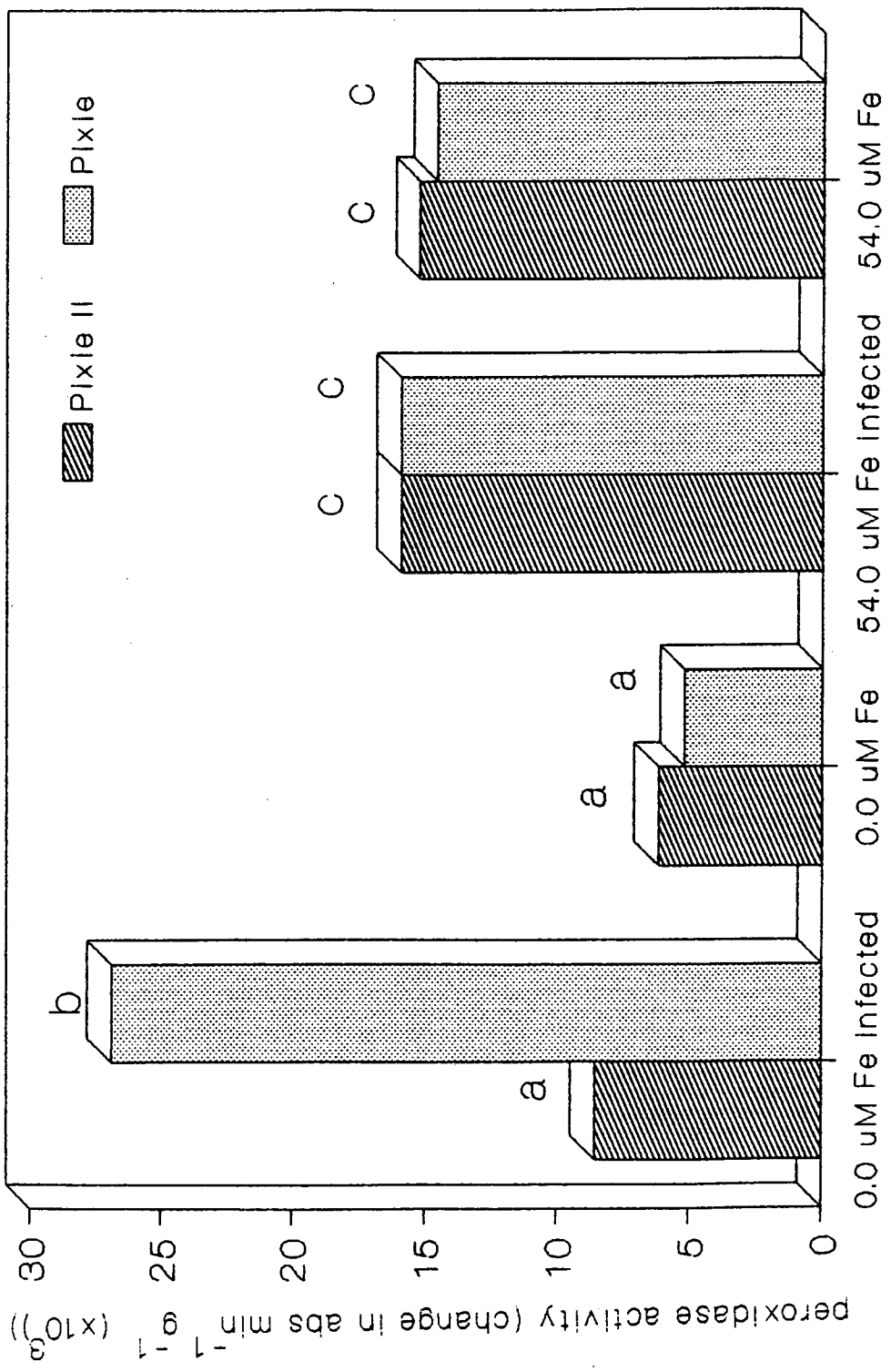
peroxidase activity (change in abs min^{-1} g^{-1} (x10^3))
0
5
10
15
20
25
30
Pixie II
Pixie
b
a
a
a
C
C
C
C
0.0 uM Fe Infected
0.0 uM Fe
54.0 uM Fe Infected
54.0 uM Fe

Fig. 2

EFFECT OF IRON ON THE RESPONSE OF PEANUTS TO VERTICILLIUM DAHLIAE

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Abstract

Soil application of Fe-EDDHA suppressed virulence of Verticillium dahliae in peantus culltivated in calcareous soil. Reduction in virulence was expressed by inhibition of characteristic disease symptoms such as plant stunting, reduced leaf size and chlorosis. The phytoalexins medicarpin, demethylmedicarpin, vestiton and formononetin were detected in stems of both infected as well as noninfected peanuts. However, the total amount of these phytalexins in infected plants was abouta 4.8 times higher than in noninfected plants which were supplied with iron. The amount of medicarpin, the major phytoalexin that was detected, was higher x18.6 in uninfected plants grown under iron stress and x23.8 and x43.4 in infected plants grown with and without iron. Since iron application did not influence the advancement and distribution of the pathogen within the plant it was concluded that phytoalexins may not be involved in the suppression of virulence in this system.

Introduction

Previous studies (2) have shown that in peanuts cultivated in plots that were either artificially infested with Verticillium dahliae or non-infested, soil application of Fe-EDDHA significantly increased yield only in plots where the fungus was present. The present study was undertaken to further characterize the effect of iron on the development of the pathogen within the host plant and on the defense mechanisms of peanuts to verticillium wilt disease.

Materials and Methods

Peanut plantas (Arachis hypogea L. cv. Shulamit) were germinated in vermiculite for 8 days before being transferred to pots containing rendzina calcarious soil (pH 7.6). The plants were placed in a growth chamber at 25°C under fluorescence and incadescence illumination (100 wm^{-2}) and a photoperiod of 12h. The plants were regularly irrigated with double distilled water and once per week with 0.5 x Hoagland solution. The growth period was 22-24 days.

The plants were separated into the following 4 different treatments (10 plants per each treatment): a) plants infected with V. dahliae and grown without iron b) infected plants grown with iron. In the latter case 15 mg Fe-EDDHA dissolved in 50 ml 0.5 x Hoagland was added aftger 2 days c) healthy plants grown without iron and d) healthy plants grown with iron.

The pathogen (isolate Dvir) was kept on PDA plate for inoculation, the fungus was grown in liquid medium as previously described (1). Prior to inoculation the mycelium was filtered through 4 layers of cheesecloth and macerated with Waring Blendor for 30 seconds. Six grams of wet weight mycelia was used per 1 liter of distilled water. Eight-day-old peanut seedlings were removed from the vermiculite and rinsed with water. The roots were immersed inthe mycelial suspension for 1 h and then were planted in the pots. Control plants were immersed in sterile water instead of mycelial suspension.

The amount of the pathogen within the plant was estimated by colony forming units (cfu). Stem or roots of each plant were separated and surface sterilized by 1% sodium hypochloride for 2 min following by excessive washings with sterile water. They were then extracted with 10 ml of sterile water with Polytron in maximal speed for 1 min. The extract (0.2 ml) was uniformly distributed on PDA plates containing chloramephenicol (250 ug/ml) and streptomycin (200 ug/ml). Colonies were counted after 5 days incubation at 25oC.

Phytoalexins were extracted from stems of 6 plants of each treatment as described by Keen (4). The obtained dry material was dissolved in acetonitril (0.5 ml per g. fresh wt.). The phytoalexins in 0.2 ml were then determined by calibrated and computerized HPLC (Perp LC/system 500 Waters Associates). Peroxidase and chlorophyll was determined as described by Guidotti et al. (3) and Barak and Chen (1) respectively.

Results and Discussion

The effect of Fe-EDDHA on Verticillium wheat development in peanuts grown under calcarious soil is described in Table 1. It can be observed that addition of iron to the healthy plants did not show any significant effect on light, shoot weight or leaf area but significantly increased the chlorophyll content of the chlorotic leaves. On the other hand, addition of iron to infected plants significantly increased the height and shoot weight of the plants as well as leaf area and chlorophyll to the level of the healthy control. Thus iron virtually neutralized most of the major symptoms of verticillium wilt during early development of peanut plants.

The next question which was addressed is whether the defense mecahisms of the plants, as far as it expressed by phytoalexins production, was affected. Major phytoalexins which were previously identified in peanuts include the isoflavanones vestitone and formononetin and the pterocarpans, demethyl medicarpin and medicarpin. The content of these phytoalexins in infected and healthy peanuts with and without iron is given in Table 2. The level of total phytoalexins was induced by either iron deficiency and/or by infection. Thus healthy plants which were grown under iron deficiency contained 2.8 times more phytoalexins than healthy plants with iron. Infected peanut plants with iron contained 3.6 times higher phytoalexins and infected plants without iron 4.8

fold than the healthy control. Medicarpin was the major phytoalexin detected and its concentrtation as compared to healthy plants with iron was 18.6, 23.8 and 43.4 higher in healthy plants without iron, infected with iron and infected without iron stress respectively.

The foregoing results indicate that iron stress is capable of inducing phytoalexins production but at the same time aggravates the symptoms of Verticillium wilt in peanuts. Thus the phytoalexins detected may not be involved in the defense mechanism. Since peroxidase activity is known to be associated with plant response to infection, the activity of the enzyme in roots was monitored (Table 3). Resuls indicate that the level of peroxidase was significantly increased by infection but reduced under iron deficiency.

Further experiments were aimed at elucidating the effect of iron on the advancement of the pathogen within the host. The pathogen could be isolated from the roots and stem up to the 5th leaf in both iron-treated and non-treated infected plants. Results in Table 4 indicate that the CFU in plants with iron was even slightly higher than plants grown under iron deficiency. These and previous results clearly indicate that the advancement of the pathogen within the host and the defense mechanisms that might inhibit its progress are not significantly affected by the iron status of the plant. We therefore, hypothetise that iron may exert an effect on expression of virulence mechanisms such as toxins, pectic enzymes or hormones released by the pathogen or alternatively, render the plant more tolerant towards these virulence determinants.

References

1. Barak, P. and chen, Y. 1982. The evaluation of iron deficiency using bioassay-type test. Soil. Sci. Soc. Am. J. 46:1019-1022.

2. Barash, I., Zion, R., Krikun, J. and Nachmias, A. 1988. Effect of iron status on Verticillium wilt diseases and on in vitro production of siderophores by Verticillium dahliae. J. of Plant Nutrition 11:893-905.

3. Guidotti, G., Colombo, J. and Poa, P.U. 1961. Enzymatic determination of glucose. Anal. Chem. 33:151-153.

4. Keen, N.T. 1978. Phytoalexins efficient extraction from leaves by a facilitated diffusion technique. Phytopathology 68:1237-1239.

Table 1

Effect of Fe-EDDHA on disease severity caused by Verticillium dahliae in peanuts growing in calcareous soil.

Treatment	Shoot height (cm)	Shoot weight (d.f.wt.)	Leaf area (cm)	Chlorophyll content ($mg.l^{-1}$/mg f.wt.)
Healthy plants	19^a	11.6^a	23^a	18^b
Healthy plants + Fe-EDDHA	21^a	12^a	23^a	48^a
Infected plants	13^b	8^b	19^b	12^b
Infected plants + Fe-EDDHA	18^a	11^a	25^a	44^a

Means within columns followed by the same letter are not significantly different (LSD=0.05).

Table 2

Effect of Fe-EDDHA on phytoalexins production in stems of peanut plants infected and non-infected with Verticillium dahliae

Phytoalexins	Non-infected		Infected	
	+Fe	-Fe	+Fe	-Fe
	mg/gr.F.wt.			
(-) Demethylmedicarpin	56.8	81.8	75.2	28.9
Vestitone	5.29	2.17	37.8	11.07
(-) Formonoretin	4.86	28.19	31.6	61.49
Medicarpin	4.94	92.09	117.8	214.62
Total	71.89	204.28	262.4	315.44

Table 3

Effect of Fe-EDDHA on peroxidase activity in peanut roots of healthy and infected plants with Verticillium dahliae[1]

Treatment	Peroxidase activity (units/ml)
Healthy plants	34[a]
Healthy plants + Fe-EDDHA	48[b]
Infected plants	69[c]
Infected plants + Fe-EDDHA	87[d]

[1]Plants were grown in calcarious soil as described in Table 1. Each treatment contained 10 plants. Means within columns followed by different letters are significantly different.

Table 4

Effect of Fe-EDDHA on colonization of Verticillium dahliae in peanut plants as expressed by colony forming units.

Plant Part	Colony forming units	
	with iron	without iron
Lower root	650	350
Upper root	2540	600
Lower stem	1150	1800
Upper stem	3250	2250
Total	6590	5000

Results are an average of 5 different experiments.

The effect of Verticillium dahliae infection on the nutrient status of two crop species

J. Krikun

As part of the ongoing study of mineral nutrition effects on Verticillium wilt, an opportunity was afforded to conduct tissue analysis in two crops which were grown under controlled conditions and inoculated or non-inoculated with V. dahliae. The two crop species were eggplant (Solanum melongonea) and four cvs. of canola (1 mustard, 3 rapeseed).

The analyses were conducted in an attempt to relate infection effects on plant nutrient composition under conditions of full nutrient availability, in plants grown under controlled (greenhouse) conditions and under controlled pathogen levels.

Materials and methods

The plants were grown in peat-vermicullite mix containing added nutrients. During the growing period additional nutrients were supplied with the irrigation water, the fertilizer being 20-20-20.

The experiments were conducted in a greenhouse, with added illumination as needed. Temperature range was 20-27^{o}C.

Substrate infestation was carried out by adding ca. 50 microsclerotia per cc. substrate mix in the case of canola. In eggplant, young seedlings growing in jiffy pots were inoculated and tranferred to large pots.

At the end of each experiment, the portions to be analyzed were

prepared for tissue analysis by washing, oven drying and weighing. Analysis was conducted by the techniques employed in the Plant Testing Laboratory at Volcani Center.

For eggplant, the plant was divided into upper and lower sections and analyzed separately. For canola, because of the normal leaf shed, only the stem was analyzed.

In eggplant the plants were harvested 24 days after inoculation; in canola 50 or 63 days after seeding.

Symptom onset and expression were monitored every two days.

At the conclusion of the experiment portions of the stem were used to confirm infection by isolation techniques.

In eggplant two isolates were used; one from potato and one from pea. In canola only the potato isolate was used.

Results and discussion

The results of the effects of infection on dry weight and nutrient composition of eggplant are presented in Table 1.

The results of the effect of infection on the dry weight of the stem, including pods, and on selected nutrients in canola are presented in Table 2.

Infection in eggplant was 100% with both isolates; in canola it varied from 67-100% depending on the cultivar.

In eggplant symptom onset was eight days after inoculation; in canola symptom onset was 29 days after sowing. In canola the symptoms appeared earlier in the cvs. Tobin and Domo, which flowered earlier than Karat and Westar.

As can be seen from Table 1, the most obvious symptoms of inoculation with both isolates of Verticillium dahliae in eggplant is a

decrease in dry weight of the upper half of the plant. Examination of the nutrient analysis results shows that when the upper half of the plant is considered there is a marked decrease in the K content; there are no differences in the N and P contents. There is a higher Ca level in the lower half of infected plants, there are no differences in Mg, Mn, Cu and Zn. There is a lower Fe content in both lower and upper portions of infected as compared to non-infected plants. The differences with regard to K and Fe would become even more striking when we consider that the upper portion of healthy plants was 130% and 90% greater in dry weight than in the pea and potato isolate infected plants. Thus total uptake of K and Fe is greatly reduced in eggplant infected with V. dahliae. In contrast N and P per-cent values of the tissues are not affected.

Table 2 presents the results of infection on stem and pod weight; and on K, Ca, Fe and Mn content of these tissues. Examination of the results shows that infection significantly affected plant dry weight, and lowered the K, Ca and Fe content in the cv. Domo. In the cv. Karat there was also less Ca and Fe. The most striking feature, however, with regard to nutrient composition in infected as contrasted to non-infected plants was their lowered Mn content; there being only half the concentration in infected plants, in all four cvs. tested. The results per se of the Mn contents may also be of interest as there seem to be large differences in uptake between cvs., without any regard to infection; the same applies to Ca.

The above results and those presented in Table 3 from a previous study for tomato grown under hydroponic conditions demonstrate that rather profound changes in the mineral nutrition of V. dahliae infected

plants occurs. The major differences occur with regard to K and Fe. Increasing Fe availability by the addition of chelated Fe in potato and eggplant (Barash et al.) decreases symptom expression and increases plant size or yield. It remains to be seen if infection effects are due to uptake, translocation or both. In cotton, the suggestion has been made that decreased K content found in plants infected with Verticillium is due to decreased uptake (Hafez et al.)

In this regard it may be useful to point out that Mikkelson has shown that highly Verticillium resistant cultivars of cotton take up significantly more K from nutrient solutions low in K, and produce higher dry weights, than more susceptible cvs. (Kerby & Adams).

The results of the K analysis, conducted in plants grown under controlled nutrient conditions clearly demonstrate a Verticillium induced effect, and not a K effect on infection as suggested for pistachio (Ashworth et al.).

Recognizing that there are interactions between nutrient should perhaps be of priority in attempting to understand the effects between nutrition, nutrient composition and Verticillium infection. It is obvious that further research is needed to better understand the above. Such studies may suggest avenues of alleviating disease losses caused by Verticillium dahliae, and perhaps other pathogens as well.

References

Barash, I., R. Zion, J. Krikun and A. Nachmias. 1988. Jl. of Plant Nutrition 11:893-905.

Hafez, A.A.R., P.R. Stout and J.E. de Vay. 1975. Agron. J. 67:359-361.

Kerby, T.A. and F. Adams. 1985. K nutrition in cotton. In: K in Agriculture. Proc. PPI, ASA, CSSA, SSSA Symp. Atlanta, GA.

Ashworth, L.J., S.A. Gaona and E. Surber. 1985. Phytopathology 75:1091-1093.

Table 1. Effect of *V. dahliae* infection of eggplant on plant dry weight (gr) and mineral components of the tissues

Treatment	Dry wt.	Upper half of plant								
		% dry matter					PPM			
		N	P	K	Ca	Mg	Mn	Cu	Zn	Fe
Control	2.98	5.5	1.0	6.5	2.18	0.64	242	14.3	29	158
V. dahliae potato isolate	1.27	5.3	0.8	5.1	2.04	0.61	228	12.4	34	133
V. dahliae pea isolate	1.52	5.0	0.8	5.1 *1	1.93	0.63	203	17.5	32	126 *

	Dry wt.	Lower half of plant								
		% dry matter					PPM			
Control	1.45	6.8	1.0	5.4	0.46	0.36	132	9.8	42	159
V. dahliae potato isolate	1.15	6.8	1.0	4.8	0.87	0.34	129	18.6	47	127
V. dahliae pea isolate	1.48	6.8	1.0	4.3	0.93 *	0.39	129	16.4	43	124 *

1Denotes significant differences between control and *V. dahliae* infected plants at 0.05% level.

Table 2. Effect of V. dahliae infection on dry weight (gr.) of stem and pods and on selected mineral components of these tissues of four cvs. of canola.

Cultivar	Dry weight	% K	% Ca	PPM Fe	PPM Mn
Domo					
control	10.6	3.13	1.26	38.7	74.3
infected	7.5	2.17	0.92	26.8	35.0
	*[1]	**[2]	**	**	**
Tobin					
control	11.4	2.68	0.53	21.8	34.0
infected	8.5	3.10	0.72	30.5	14.5
	**			**	**
Karat					
control	16.4	2.00	1.63	32.3	61.0
infected	11.6	1.87	1.16	25.0	31.2
	**		**	**	**
Wester					
control	17.0	2.50	0.48	20.0	34.0
infected	15.4	2.97	0.71	21.7	15.8
			*		*

1. Denotes sig. diff. between control and inoculated at 0.05% level.

2. Denotes sig. diff. between control and inoculated at 0.01% level.

Table 3. The effect of V. dahliae infection of tomato grown in half-Hoagland's nutrient solution on N, P and K content in the lower, middle and upper portions of the foliage (percent of dry matter).

Nutrient	Plant portion	Control plants	Infected plants
N	U	6.00	6.25
	M	5.25 a[1]	6.50 b
	L	5.75	5.25
P	U	0.88	1.05
	M	0.70	0.80
	L	0.63	0.60
K	U	4.75	4.54
	M	4.17 a	3.81 b
	L	4.09 a	2.38 b

U = Upper, M = middle, L = lower, portions.

1. Numbers followed by different letters between columns are sig. different at the 0.05% level.

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IDENTIFICATION OF SIDEROPHORES AND SIDEROPHORE-MEDIATED UPTAKE OF IRON IN *STEMPHYLIUM BOTRYOSUM*

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Key Word Index—*Stemphylium botryosum*; siderophores; iron uptake.

Abstract—Under iron-deficient conditions *Stemphylium botryosum* f. sp. *lycopersici* produces three major siderophores; dimerum acid, coprogen B and an unidentified monohydroxamate siderophore designated as A. The system of siderophores mediating uptake of iron was characterized. It exhibits active transport, saturation kinetics and an optimum at pH 6 and 30°. The rate of iron uptake via dimerum acid and coprogen B was four times higher than siderophore A. *S. botryosum* was capable of taking up iron from hydroxamate siderophores produced by other fungi, e.g. ferrichrome, fusigen, rhodotorulic acid but not ferrioxamine B. Double labelling experiments suggest that ferric coprogen B accumulates in mycelial cells as an intact chelate.

INTRODUCTION

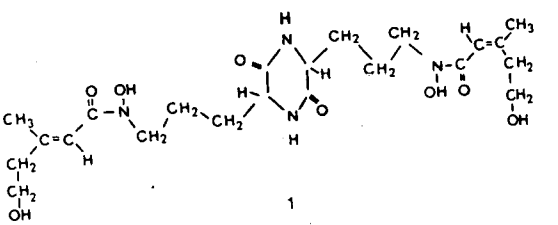
The uptake of iron in fungi and other microorganisms under iron deficient conditions, is generally mediated by siderophores [1, 2]. Siderophores are low-molecular-weight ferric chelating agents which supply the metal to the cell by binding to specific receptors in the cell membrane. An array of hydroxamate siderophores have been isolated from a small number of fungi belonging to the Basidiomycetes, Ascomycetes and Deuteromycetes [2, 3].

Stemphylium botryosum Walr. f. sp. *lycopersici* is the causal agent of leaf spot and foliage blight disease of tomato [4]. The capacity of an invading pathogen to obtain iron from living tissue may emerge as a critical element in the host–pathogen interaction. Evidence in support of the latter hypothesis has been adduced in bacterial and fungal diseases of animals [1] and could be involved in virulence of plant pathogens as well. *S. botryosum* was reported to secrete two novel phytotoxic chelates of ferric ion designated as stemphyloxin I and stemphyloxin II [4–6]. Stemphyloxins differ from siderophore compounds in their dependence on iron for optimal biosynthesis and a distinctively lower affinity for ferric ion [6]. They have been recently implicated in the acquisition of iron by *S. botryosum* under conditions of iron sufficiency [7]. The present study was undertaken to characterize the siderophore mediated absorption of iron by the phytopathogenic fungus *S. botryosum*.

RESULTS AND DISCUSSION

Isolation and identification of siderophores

Siderophores were extracted from filtrates of 16-day-old cultures and purified as described in the Experimental. Three major components eluting from the Bio-Gel P-2 column were designated as A, B and C according to their migration towards the cathode on paper electrophoresis. Siderophore C was a highly cationic compound whereas B and A were only slightly cationic in pyridine–acetate buffer, pH 5.2. Further purification was achieved by chromatography on a Cellex CM column which exhibited differential adsorption towards the three siderophores. Thus siderophores A and B were eluted with distilled water whereas siderophore C was removed only with pyridine–acetate buffer, pH 5.2. Each siderophore was finally brought to homogeneity by employing preparative



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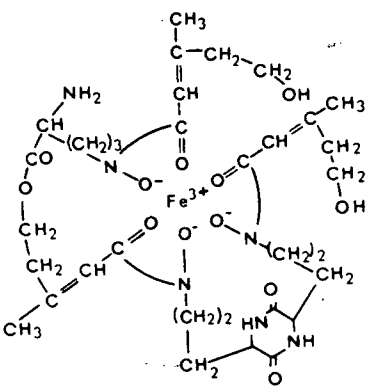

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paper electrophoresis and separation on Sephadex LH-20 (see Experimental).

Initial identification of siderophore B as dimerum acid (1) and C as coprogen B (2) was carried out by cochromatography with authentic samples in silica gel TLC (Table 1). Identical migration rates of siderophore B with dimerum acid and siderophore C with coprogen B were also demonstrated with high voltage electrophoresis. Comparative ^{1}H NMR spectra and IR spectra, with literature values [8–10] also supported the identity of siderophore B as dimerum acid and siderophore C as coprogen B. The structure of siderophore A has not yet been determined. However, based on its stability towards dissociation in acid solution and decolourization in the presence of 0.02 M EDTA it could be identified as a monohydroxamate [11]. Thus the maximal absorbance of siderophore A was shifted from 440 nm at pH 7 to 460 nm at pH 4.6 and was associated with a decrease in absorbance. The production of dimerum acid has previously been reported in *Fusarium dimerum* [8] and *Verticillium dahliae* [9] whereas coprogen B has been found in *Fusarium* sp. and *Myrothecium* sp. [8].

Production of siderophores

The secretion of siderophores by *S. botryosum* was dependent on iron deficiency and was completely repressed in the presence of 2 μM iron (Fig. 1). Production of siderophores was correlated with mycelial growth and reached maxima during the stationary growth phase after approximately 16 days. A sharp decrease in siderophore concentration, presumably due to degradation, was observed after 20 days. The total amount of siderophores secreted by *S. botryosum* was approximately 125 mg/l. The relative amounts of siderophore A, dimerum acid and coprogen B after 16 days were 2.2:1:1.5, respectively.

Characterization of siderophore-iron uptake by S. botryosum

Uptake of ^{59}Fe-labelled coprogen B, dimerum acid and siderophore A was found to be linear within 2 hr (Fig. 2). The transport rate of ^{59}Fe-siderophore A was four times lower than with the two other siderophores. Iron uptake was completely inhibited by 1 mM sodium azide. All three siderophores exhibited pH optima at *ca* pH 6 and temperature optima at 30°. Kinetic studies of initial uptake versus siderophore concentration exhibited saturation kinetic with apparent K_m of 2.8 μM for coprogen B and 2.2 μM for dimerum acid. The foregoing results indicate that the siderophore-mediated iron transport in *S. botryosum* exhibits many features similar to those found in other fungal systems [1, 3].

The specificity of ferric ion transport system by various ligands in *S. botryosum* is shown in Table 2. In addition to its own siderophores, this fungus was capable of utilizing effectively exogenous siderophores which are naturally produced by other fungi, such as rhodotorulic acid, fusigen and ferrichrome. On the other hand, ferrioxamine B was ineffective. Rhodotorulic acid is structurally related to dimerum acid but fusigen and ferrichrome represent different structural classes of hydroxamate siderophore

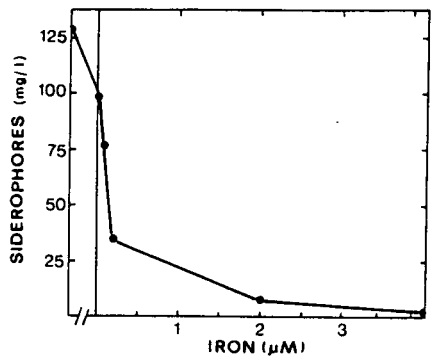

Fig. 1. Production of siderophores by *S. botryosum* as a function of iron concentration. Siderophore concentration was determined after 16 days. Value below zero was obtained by growing the fungus in a medium from which traces of iron were removed with 8-hydroxyquinoline [18].

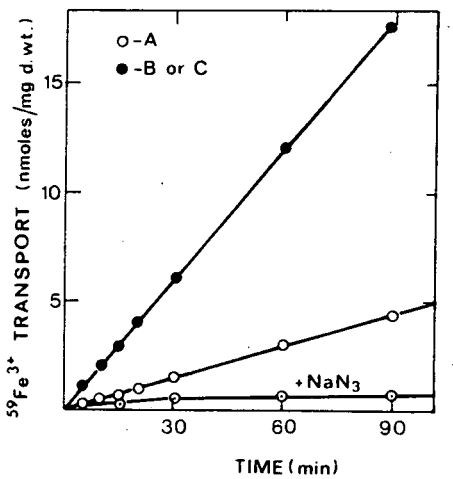

Fig. 2. Effect of siderophores and sodium azide on iron uptake. Initial uptake was determined as described in the Experimental. The labelled chelate was added to mycelium grown in an iron-deficient culture. The final iron concentration was 1 μM (0.2 μCi/ml). Sodium azide concentration was 1 mM. A—Siderophore A, B—dimerum acid and C—coprogen B.

Table 1. R_f values of siderophores in four solvents

Siderophore*	R_f in solvent†			
	1	2	3	4
A	0.68	0.19	0.25	0.37
B	0.72	0.10	0.13	0.34
C	0.54	0.07	0.11	0.49
Dimerum acid	0.72	0.10	0.13	0.34
Coprogen B	0.54	0.07	0.11	0.49
Rhodotorulic acid	0.61	0.05	0.07	—
Fusigen	0.66	0.03	0.06	—

*The siderophores were taken to dryness and dissolved in MeOH prior to their application on TLC.

†TLC in: (1) dioxan–0.33 N HOAc (2:1); (2) *n*-BuOH–HOAc–H_2O (4:1:1); (3) *n*-PrOH–HOAc–H_2O (4:1:1); (4) *n*-PrOH–NH_4OH (14:1).

Table 2. Relative uptake of chelated siderophores by *S. botryosum**

Siderophore	Relative uptake (%)
Coprogen B	100
Dimerum acid	100
Siderophore A	25
Rhodotorulic acid	100
Ferrichrome	75
Fusigen	83
Ferrioxamine B	8

*Conditions were the same as those described for Fig. 2.

[2, 11]. Various species of *Aspergillus* were able to utilize iron from siderophores of the ferrichrome-type family irrespective of the type of ferrichrome which these species normally produced. However, they were not able to take iron from an exogenous chelate of a different class, such as coprogen [12]. *Neurospora crassa* was reported to accumulate iron by its own chelate coprogen as well as by structurally unrelated siderophores such as ferricrocin and ferrichrysin [13].

Results shown in Fig. 3 indicate that iron uptake via the siderophores was four times higher with siderophore A and eight times higher with both coprogen B and dimerum acid in mycelium grown under iron starvation as compared to mycelium grown under iron-sufficient conditions. These results together with those illustrated in Fig. 1 suggest simultaneous regulation of the biosynthesis of siderophores and their transport processes by iron.

The fate of the ferric-coprogen B complex during uptake was followed by comparing the kinetics of the tritiated labelled ligand (Fig. 4) with that of the ^{59}Fe-labelled siderophore (Fig. 2). In both cases the iron complex was taken up quantitatively for at least 90 min through an energy dependent transport system as indicated by the sensitivity to sodium azide. Double labelled experiments with both the iron and the ligand revealed two basic mechanisms for iron uptake from ferric siderophore [14]. Mechanism 1 known as the iron shuttle mechanism consists of uptake of the iron and the ligand at identical rates. After internal release of the iron the free ligand reappears in the medium and may serve for another round of iron transport. The latter mechanism is exemplified by ferrichrome uptake in *Ustilago sphaerogena* [15]. Mechanism 2, known as the iron taxi mechanism [14] involves the donation of iron to the cell without penetration of the complex or the ligand. Iron uptake from rhodotorulic acid in *Rhodotorula pilimanae* [16] or ferrichrome A in *U. sphaerogena* [14] occurs by this mechanism. An additional mode of uptake was demonstrated in *Neurospora crassa* where ferric coprogen was taken up as an intact chelate molecule without being decomplexed in appreciable amounts [17]. Results presented for ferric-coprogen B in *S. botryosum* are in accordance with the latter pattern, namely the uptake and accumulation of the iron–ligand complex without subsequent release of the ligand back into the medium. These results support the view that siderophores such as coprogen or coprogen B may have a storage function for iron

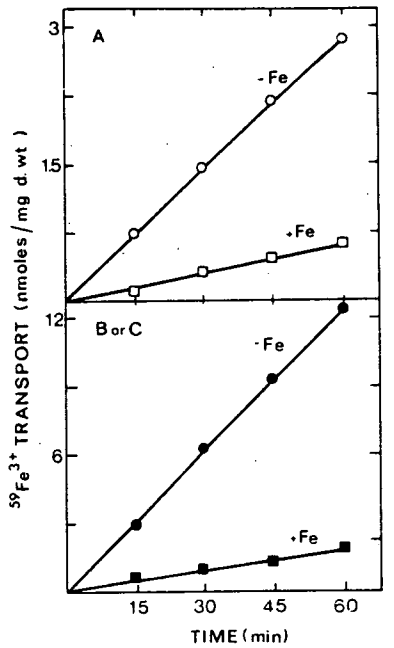

Fig. 3. Comparative uptake of ^{59}Fe-siderophores by iron-sufficient and iron-deficient mycelium. Uptake conditions are as described in Fig. 2. A—iderophore A, B—dimerum acid, C—coprogen B.

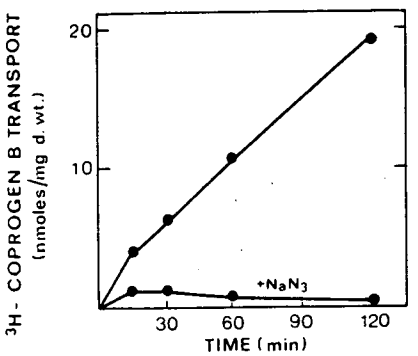

Fig. 4. Uptake of tritiated ferric coprogen B. Transport conditions were as described in Fig. 2.

in addition to their role in solubilization and transport [17].

Results presented here and our previous study [7] indicate that *S. botryosum* possesses mechanisms for iron acquisition under iron-sufficient and iron-deficient conditions. The role of these systems in pathogenicity of this fungus remain to be investigated.

EXPERIMENTAL

Culturing. The fungal strain of *S. botryosum* f. sp. *lycopersici* used in this study and culturing conditions have been previously described [6]. The fungus was grown for 16 days in 1-l. Roux bottles containing 100 ml of a defined sucrose–glutamate medium, composed of the following compounds (g/l. of double

distilled water): sucrose, 20; monosodium glutamate, 9; $MgCl_2 \cdot 6H_2O$, 0.5; KH_2PO_4, 0.3; thiamine HCl, 0.005; and pyridoxine, 0.005. All the chemicals were of analytical grade. This low-iron medium contains *ca* 0.015 mg Fe/l. (*ca* 3×10^{-7} M), as contamination from other ingredients. In some experiments the medium was treated with 8-hydroxyquinoline [18] to remove any traces of iron. Measurement of growth was performed by harvesting the mycelia by filtration through Whatman no. 1 paper and drying to constant weight at 60°.

Extraction and purification of siderophores. At the end of the growth period the mycelia were removed by filtration through Whatman no. 1 paper and the culture filtrates (1500 ml) were collected. The filtrate was evaporated under vacuum at 40–45° to about 1/5 of the original volume and excess $FeCl_3$ was added to convert siderophores to iron chelates. The reddish solution was saturated with $(NH_4)_2SO_4$ and left overnight at 4°. The filtrate was then centrifuged at 10000 *g* for 10 min and the clear supernatant was extracted with benzoyl alcohol according to the method of Neilands [19]. The dark-reddish organic layer was mixed with 2 vol. Et_2O and extracted with 0.1 vol. distilled water. The coloured aq. layer was washed with Et_2O (0.5 vol × 3) and reduced to dryness *in vacuo*.

The siderophores were dissolved in 5 ml water and initially purified by gel filtration through a column of Bio-Gel P-2 (2.5 × 115 cm). Fractions of 5 ml were collected and the amount of siderophores in each fraction was determined by absorbance at 440 nm. Individual components of the extract were then separated, concentrated *in vacuo* and chromatographed on Cellex CM (Bio-Rad) column (3 × 5 cm). The column was rinsed with water to remove neutral siderophores and the cationic siderophores were eluted with pyridine–HOAc–H_2O (14:10:930) pH 5.2. Fractions containing individual components were concentrated and applied to preparative paper electrophoresis (17 × 23 cm Whatman 3 MM) with pyridine buffer, pH 5.2 at 200 V for 2 hr. The siderophores were detected by their reddish colour and were eluted from the paper with water. Final purification was carried out with Sephadex LH-20 (Pharmacia) column (2.5 × 25 cm) using MeOH as an eluting solvent.

Analytical methods. The amount of total siderophores was measured according to Subramanian *et al.* [20]. Purity of the compounds was established by high voltage electrophoresis [21] and silica gel thin layer chromatography [4, 7]. For facilitation of structure determination the siderophores were deferrated with 8-hydroxyquinoline according to Wiebe and Winkelmann [12]. ^1H NMR spectra were recorded on Bruker WH-270 spectrometer and IR spectra as previously described [6].

^{59}Fe *uptake assay conditions.* Five-day-old mycelium was removed from the growth medium by suction filtration with an Ederol filter paper (no. 15) and washed several times with deionized water. The mycelium was then resuspended (4 g fr. wt per 100 ml) in 0.05 M phosphate buffer, pH 6 for 30 sec in a blender. The homogenous suspension of the fungal cells obtained by the latter procedure contained negligible amounts of broken cells which did not interfere with the uptake assay.

The standard procedure for iron uptake was carried out in 100 ml flasks, containing 20 ml of cell suspension. The flasks were shaken for 10 min prior to and after addition of the siderophore on a reciprocal shaker at 30°. The reaction was started by a rapid addition of the ^{59}Fe-chelated siderophore solution. Samples of 3 ml were removed at various time intervals and immediately filtered through GF/C Whatman filter paper (2.5 cm diameter) and rinsed × 3 with 5 ml 50 mM EDTA (ethylene diaminetetraacetic acid). The filters with the mycelial pads were transferred into 5 ml polyethylene vials, dried for 1 hr at 70° and counted in a Packard gamma counter C (model 5166). Counts were corrected for the readings obtained at zero time.

The ^{59}Fe-labelled siderophores were prepared by addition of $^{59}FeCl_3$ (specific activity 3–20 mCi/mg Fe in 0.05 N HCl) to an approximately 10% excess of ligand in water to give a stock solution of 0.5 mM (0.1 mCi/ml) in complex. The molar ratio between the ferric ion and the ligand was 2:3 for dimerum acid, 1:1 for coprogen B and 1:3 for siderophore A. The ligand was preincubated for at least 1 hr at 25° prior to the experiment to allow complete formation of the complex.

Tritiated coprogen B was prepared at the Radiochemistry department of the Institute for Atomic Research, Negev. The tritiated coprogen B was repurified by paper electrophoresis and Sephadex LH-20 column. High voltage electrophoresis showed that approximately 90% of the radioactivity was associated with the spot of coprogen B. Stock solutions (0.5 mM) of the ^3H-labelled ferric coprogen B (specific activity 0.1 mCi/ml siderophore) were prepared as above, using unlabelled ferric chloride. The uptake experiments were as described earlier except that the filter papers containing the mycelial pads were transferred into counting vials containing 0.5 ml of 60% perchloric acid–30% H_2O_2, 2:1) and incubated at 70° for 1 hr. Ten millilitres of hydroluma scintilation counting solution was then added and the vials were counted in Packard liquid scintilation counter model 3380.

Chemicals. Rhodotorulic acid was obtained from Dr. B. Hemming, Monsanto Co. St. Louis; dimerum acid, from Dr. J. B. Neilands, University of California, Berkeley, fusigen and coprogen B, from Dr. G. Winkelmann, University of Tubingen, Tubingen. Isotopic ferric chloride was purchased from the Radiochemical Centre, Amersham.

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REFERENCES

1. Neilands, J. B. (1981) *Annu. Rev. Nutr.* **1**, 27.
2. Neilands, J. B. (1981) *Annu. Rev. Biochem.* **50**, 715.
3. Hider, R. C. (1984) *Structure Bonding* **58**, 25.
4. Barash, I., Pupkin, G., Netzer, D. and Kashman, Y. (1982) *Plant Physiol.* **69**, 23.
5. Barash, I., Manulis, S., Kashman, Y., Springer, J. P., Chen, M. H. M., Clardy, J. and Strobel, G. A. (1983) *Science* **220**, 1065.
6. Manulis, S., Barash, I., Kashman, Y. and Netzer, D. (1984) *Phytochemistry* **23**, 2193.
7. Manulis, S., Netzer, D. and Barash, I. (1987) *Can. J. Microbiol.* (submitted).
8. Diekmann, H. (1970) *Arch. Mikrobiol.* **73**, 65.
9. Harrington, G. J. and Neilands, J. B. (1982) *J. Plant Nutr.* **5**, 675.
10. Zahner, H., Keller-Schierlein, W., Hutter, R., Hess-Leisinger, K. and Deer, A. (1963) *Arch. Mikrobiol.* **45**, 119.
11. Emery, T. (1971) *Adv. Enzymol.* **35**, 135.
12. Weibe, C. and Winkelmann, G. (1975) *J. Bacteriol.* **123**, 837.
13. Winkelmann, G. (1974) *Arch. Microbiol.* **98**, 39.
14. Ecker, D. J. and Emery, T. (1983) *J. Bacteriol.* **155**, 616.
15. Emery, T. (1971) *Biochemistry* **10**, 1483.
16. Carrano, C. J. and Raymond, K. N. (1978) *J. Bacteriol.* **136**, 69.
17. Matzanke, B. and Winkelmann, G. (1981) *FEBS Letters* **130**, 50.
18. Donald, C., Passey, B. I. and Swaby, R. J. (1952) *J. Gen. Microbiol.* **7**, 211.
19. Neilands, J. B. (1952) *J. Am. Chem. Soc.* **74**, 4846.
20. Subramanian, K. N., Padmanaban, G. and Sarma, P. S. (1965) *Analyt. Biochem.* **12**, 106.
21. Emery, T. (1965) *Biochemistry* **4**, 1410.

Acquisition of iron by *Stemphylium botryosum* under iron-replete conditions

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The assimilation of iron by *Stemphylium botryosum* under iron-replete conditions involved mainly three steps: (*i*) binding of iron to the cell surface, (*ii*) reduction of Fe^{3+} into Fe^{2+}, and (*iii*) transport of the ferrous iron across the cell membrane. However, the possibility that the fungus is capable of transporting ferric iron also has not been excluded. Adsorption was responsible for up to 75% of the iron accumulated by the cells. Mycelial cells exhibited a high capacity for Fe^{3+} reduction. Approximately 70% of the reductive activity was assigned to cell surface bound reducing compounds which were also released to the medium. Iron transport by *S. botryosum* was an energy-dependent saturable process that required sulfhydryl groups. Uptake was significantly inhibited by Cu^{2+} but not by various other cations. On the basis of inhibition studies with chelates specific for Fe^{3+} or Fe^{2+} and on the high capacity of the mycelium to reduce external ferric iron, it was suggested that uptake of iron into the cell occurred predominantly in the ferrous form. The phytotoxic iron chelate stemphyloxin I induced iron adsorption on the mycelial surface but did not appear to exert a direct effect on the membrane-mediated transport of iron.

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Trois étapes sont impliquées lors de l'assimilation du fer par le *Stemphylium botryosum*, sous conditions de plénitude en fer: (*i*) l'attachement du fer à la surface de la cellule, (*ii*) la réduction de Fe^{3+} et (*iii*) le transport du fer à l'état ferreux au travers de la membrane. Toutefois, la possibilité que le champignon puisse aussi transporter l'ion ferrique n'a pas été exclue. Au niveau cellulaire, jusqu'à 75% de l'accumulation du fer fut redevable à l'adsorption. Les cellules mycéliennes ont montré une haute capacité de réduction de Fe^{3+}. Environ 70% de l'activité réductrice a été attribuée à la présence de composés réducteurs liés à la surface des cellules; ces composés ont aussi été libérés dans les milieux de croissance. Chez le *S. botryosum*, le transport du fer est un processus saturable qui requiert de l'énergie et la présence de groupements sulfhydryles. L'absorption fut inhibée de façon significative par le cuivre, mais non par la présence de divers autres cations. Les études sur l'inhibition à l'aide de chélats spécifiques pour le Fe^{3+} et le Fe^{2+}, ainsi que la capacité élevée du mycélium de réduire l'ion ferrique externe, suggèrent que l'absorption du fer dans la cellule se fait sous forme d'ion ferreux de façon prédominante. La stemphyloxine I, un chélat de fer phytotoxique, a induit l'adsorption du fer à la surface mycélienne, mais elle n'a pas semblé exercer un effet direct sur le transport du fer par la membrane.

[Traduit par la revue]

Introduction

In many microorganisms the uptake of iron under iron-deficient conditions is mediated by siderophores (Neilands 1981*a*, 1981*b*). Siderophores are low molecular weight ferric-chelating agents which supply the metal to the cell by binding to specific receptors in the cell membrane. Although the systems for iron uptake via siderophores in bacteria and fungi are now well documented (Hider 1984), the mechanisms for iron utilization in the absence of siderophores has received little attention (Neilands 1981*a*; Winkelmann 1979; Rodriquez et al. 1984). Such systems may be of special significance under iron-replete conditions which generally repress the biosynthesis of siderophores (Neilands 1981*a*).

Stemphylium botryosum Wallr. f. sp. *lycopersici* is the causal agent of leaf spot and foliage blight disease of tomato. This fungus secretes three major hydroxamate siderophores only under extreme iron deficiency (Manulis et al. 1987). Two other chelates of ferric ion, designated as stemphyloxin I and stemphyloxin II, have been identified in the culture filtrates of *S. botryosum* under iron-replete conditions (Barash et al. 1983; Manulis et al. 1984). Stemphyloxins, which are novel phytotoxic compounds, are highly functionalized β-ketoaldehyde *trans* decalone. These chelates require iron for optimal secretion (Manulis et al. 1984). Stemphyloxins share some properties with siderophores, namely, regulation by iron and preferred binding of ferric ion. However, they differ from siderophore compounds in their dependence on iron for optimal biosynthesis and a distinctly lower affinity for ferric ion (Manulis et al. 1984). It was, therefore, postulated that stemphyloxins may function in the uptake of iron under conditions of iron sufficiency, as opposed to hydroxamate siderophores, whose function is aimed at extreme conditions of iron deficiency (Manulis et al. 1987). The present investigation was intended to characterize the mechanism responsible for iron assimilation in *S. botryosum* under nonlimiting iron supply, and to clarify whether stemphyloxins contribute to acquisition of iron under such conditions.

Methods

Organism and growth conditions

The culture of *S. botryosum* f. sp. *lycopersici* used in this study has been described (Barash et al. 1982). The fungus was grown in 1-L Roux bottles, containing 100 mL of a defined sucrose–glutamate

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medium composed according to Manulis et al. (1984), but with the addition of 40 μM ferric chloride. The liquid cultures were incubated for 4 days at 25°C under continuous illumination of 4 $W \cdot m^{-2}$.

Assay of ^{59}Fe uptake

The mycelia were removed from the growth medium by suction filtration on a Buchner funnel, with an Ederol filter paper (no. 15), and washed several times with deionized water. The mycelia were then resuspended (4g fresh weight per 100 mL) in 0.05 M phosphate buffer, pH 6, for 30 s with the lower speed of a Waring blender. The homogeneous suspension of the fungal cells obtained by the latter procedure contained negligible amounts of broken cells, which did not interfere with the uptake assay. Dry weight was determined by drying the cells for 20 h at 50°C.

The standard procedure for iron uptake was carried out in 100-mL Erlenmeyer flasks, which contained 20 mL of cell suspension. The flasks were shaken for 10 min prior to the addition of the labelled iron and during the experiment on a reciprocal shaker at 30°C. The reaction was started by a rapid addition of 0.1 ml of the iron (^{59}Fe) solution. Samples of 3 mL were removed at various time intervals and immediately filtered through GF/C Whatman filter paper (2.5 cm diameter) and rinsed 3 times with 5 mL of 50 mM EDTA (ethylenediaminetetraacetic acid). The filters with the mycelial pads were transferred into 5-mL polyethylene vials, dried for 1 h at 70°C, and counted in a Packard gamma counter C (model 5166). Counts were corrected for the readings obtained at zero time. Radioactive ferric ion (^{59}Fe) was given as either $FeCl_3$ or in a chelated form with stemphyloxin I, EDTA, or citrate at a final iron concentration of 1 μM (0.2 μCi/mL; 1 Ci = 37 GBq). The iron chelates were freshly prepared 1 h before the experiment. The molar ratios of ferric ion and the chelates were 1:3 with stemphyloxin I, 1:2 with EDTA, and 1:20 with citrate. Results presented are the means of at least three different experiments.

Results obtained by the foregoing procedure included iron transported into the cell as well as iron bound to the cell's surface. To measure iron transport the surface-bound iron was removed as follows. After incubation with labelled iron, the cells (3 mL) were promptly transferred into a vial (10 mL) containing 1 mL solution of 100 mM EDTA and incubated for 1 h on a reciprocal shaker at 30°C. The cells were then fltered, washed with distilled water, and counted as previously described.

Determination of Fe^{3+} reduction

Experiments with mycelial cells were performed in 100-mL flasks on a shaker at 30°C. The reaction mixture contained 8 mL of cell suspension (2 g fresh weight per 100 mL) and 1 mL of either BPDS (4,7-di(4-phenylsulphonate)-1,10-phenantroline) or ferrozine (disodium salt of 3-(-2-pyridyl)5,6-(4-phenylsulfonic acid-1,3,triazine) at a final concentration of 0.3 mM. The reaction was started by adding 1 mL of either freshly prepared 0.1 mM $FeCl_3$ or 0.1 mM ferric ion chelated to stemphyloxin I (1:3, mol/mol), ethylenediaminedi-*o*-hydroxyphenyl acetic acid (EDDHA) (1:2), EDTA (1:2), citrate (1:20), and coprogen B (1:1). At various time intervals samples of 1 mL were quickly removed and filtered through CF/C filters by suction with vacuum. The absorbance of the chelates formed by ferrous ion with either BPDS or ferrozine was immediately measured spectrophotometrically at 530 and 562 nm, respectively. Concentrations of Fe^{2+} were calculated assuming molar absorptive coefficients of 22 000 and 22 600 for BPDS and ferrozine, respectively. Reaction mixtures lacking cell suspension or ferric ion were used as blanks.

Reduction of ferric ion was also performed by filtrates from 10-day-old culture. The filtrate (200 mL) was concentrated 10 times under reduced pressure. Proteins were precipitated by adding 2 volumes cold acetone (−18°C) and incubating overnight prior to centrifugation. The supernatant was taken to dryness under reduced pressure and redissolved in 20 mL of distilled water. The procedure for measuring reduction activity was as described earlier, except that the reaction mixture contained 0.5 mL concentrated filtrate, 3.5 mL distilled water, 0.5 mL BPDS or ferrozine at a final concentration of 0.3 mM, and 0.5 mL of 0.1 mM ferric chloride.

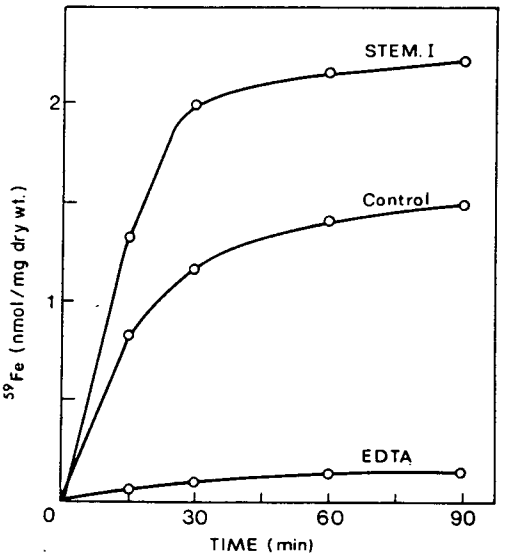

FIG. 1. Accumulation of iron by mycelial cells. Iron (1 μM) was given as $^{59}FeCl_3$ or as a chelate with citrate (20 μm), stemphyloxin I (3 μm), and EDTA (2 μm). Preparation of chelates and experimental conditions were described in Methods. Control, unchelated Fe^{3+} or ferric ion chelated to citrate; Stem. I, Fe^{3+} chelated to stemphyloxin I.

Chemicals

Stemphyloxin I was obtained according to Manulis et al. (1984). $^{59}FeCl_3$ (3–20 mCi/mg) was purchased from Radiochemical Centre, Amersham. A complete reduction of $^{59}FeCl_3$ (1 μM) to ferrous ion was obtained by incubation with 10 μM ascorbic acid for 30 min. Ferrozine, BPDS, NTA (nitrilotriacetic acid), EDTA, and EDDHA were purchased from Sigma Chemical Co.

Results

Iron accumulation by mycelial cells

As shown in Fig. 1, $^{59}FeCl_3$ accumulation by cells of *S. botryosum* was linear up to 15 min and reached saturation at approximately 60 min. Identical kinetics pattern was obtained with ^{59}Fe–citrate. However, when iron was supplied as ^{59}Fe – stemphyloxin I, linearity was retained up to 30 min and iron accumulation increased by approximately 70%. EDTA exhibited an inhibitory effect.

To distinguish between iron transported into the cells from iron bound to the cell's surface, the cell surface bound iron was removed by an excessive amount of EDTA as illustrated in Fig. 2. EDTA is a metabolically inert chelating agent and is assumed not to enter fungal cells under physiological conditions (Cochrane 1958). A decrease of 50% in bound iron was observed within 10 min in cells suppled with $^{59}FeCl_3$ for 15 or 30 min as compared with 80% decrease in cells which were momentarily suspended in $^{59}FeCl_3$ (zero time). Maximal removal of iron reached 75 and 90%, respectively with the former and latter cells after 1 h. Thus, about 25% of the iron was taken into the cells. It should be noted that although the percentage of iron removal from cells incubated with $^{59}FeCl_3$ for various periods was similar, the absolute values obtained were different. In spite of the rapid removal of surface bound iron at zero time, 10% of the labelled iron was retained by the cells, presumably owing to irreversible adsorption. The latter value was always subtracted from the counts obtained during iron uptake. Results in Fig. 3 indicate that after the cell surface bound iron was removed by excessive EDTA a linear transport

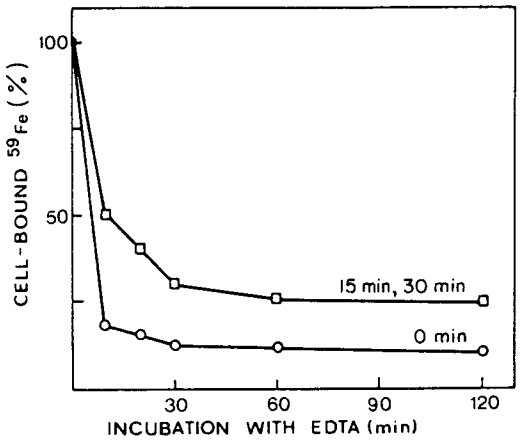

FIG. 2. Removal of cell surface bound iron by EDTA as a function of time. Iron was given as $^{59}FeCl_3$ under conditions as in Fig. 1. At various time intervals (0, 15, and 30 min), the cells (3 mL) were promptly transferred into vials containing 1 mL of 100 m*M* EDTA. The vials were incubated in a shaker at 30°C and then filtered, washed, and counted as described in Methods.

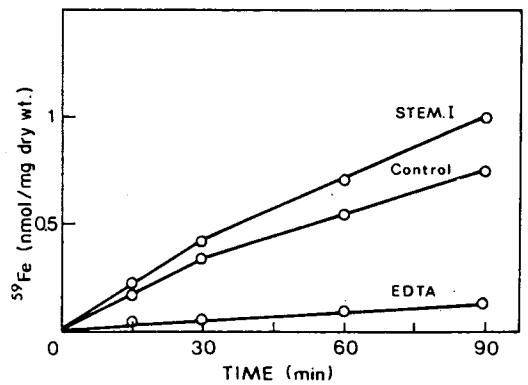

FIG. 3. Effect of stemphyloxin I and EDTA on iron uptake. Conditions were as in Fig. 1, except that the cell surface bound iron was removed by incubation for 1 h with excessive EDTA, as illustrated in Fig. 2.

could be observed at least up to 90 min. Continued exposure to EDTA caused about 90% inhibition of iron transport, whereas stemphyloxin stimulated transport by approximately 30%.

Properties of the iron transport system

Iron transport under conditions described in Fig. 3 showed pH dependence with a broad optimum at pH 5.5–7.5 and optimal temperature between 30 and 37°C. The requirement for functional membranes and energy is demonstrated by the inhibitory effects of diethylstilbestrol and sodium azide (Table 1). Sulfhydryl groups were important in this system since inhibition was evident with iodoacetamide. Iron adsorption was not affected by these inhibitors. The presence of 1 m*M* thioglycollate increased iron uptake by 65%. A plot of transport rate versus Fe^{3+} concentration yielded saturation kinetics with a half-saturation concentration of about 50 μM and a maximum rate of 0.07 nmol $Fe^{3+} \cdot min^{-1} \cdot mg$ dry wt.$^{-1}$ (data not shown). However, simple Michaelis–Menten kinetics were not followed.

Specificity of the iron transport system

The specificity of iron transport was studied first by adding chloride salts of various cations (Table 1). Zn^{2+}, Ni^{2+}, and

TABLE 1. Effect of various inhibitors and cations on Fe^{3+} transport

Inhibitor or cation (1 m*M*)	Inhibition (%)
Iodoacetamide	76
Diethylstilbestrol	60
Sodium azide	56
Zn^{2+}	30
Ni^{2+}	10
Mn^{2+}	10
Fe^{3+}	90
Fe^{3+} (10 μM)	44
Cu^{2+}	90
Cu^{2+} (10 μM)	30

NOTE: Transport conditions are as described in Methods.

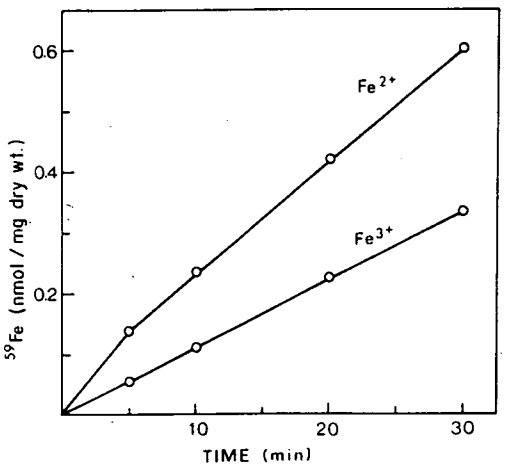

FIG. 4. Relative rates of ferric and ferrous ions uptake. Conditions were as in Fig. 3. Concentration of Fe^{3+} or Fe^{2+} was 1 μM.

Mn^{2+} at concentration of 1 m*M* inhibited the $^{59}FeCl_3$ transport by 10 to 30%, whereas Cu^{2+} and nonlabelled Fe^{3+} at the same concentration exhibited 90% inhibition and at a concentration of 10 μM caused 44 and 30% inhibition, respectively. In other experiments (results not shown), stimulation of iron transport by 20 to 40% was observed with chloride salts of 1 m*M* K^+, Na^+, Mg^{2+}, and Ca^{2+}.

The valence form in which iron is transported into the cells was investigated. The rate of iron uptake in the ferrous form was higher by approximately 80% than in the ferric form (Fig. 4). Further experiments were designed to determine the effect of either ferrous- or ferric-specific chelators on iron uptake. Results given in Fig. 5A indicate that the Fe^{2+} trapping reagent BPDS caused 70% inhibition of ferrous ion transport, whereas the ferric-specific chelators EDDHA and NTA were ineffective. On the other hand, when iron was given in the ferric form (Fig. 5B), EDDHA, NTA, and BPDS caused after 20 min inhibition of 83, 61, and 38%, respectively. Significant inhibition by BPDS could be detected only after 5 min. The observation that BPDS inhibits Fe^{3+} uptake, albeit at a later stage, suggests that ferric reduction occurs prior to its transport into the cells. This assumption was further tested by addition of EDDHA at various time intervals during transport of ferric ion. Results in Fig. 6 demonstrate that the magnitude of inhibition was inversely proportional to the time at which EDDHA was added. Prior incubation with EDDHA for 30 min resulted in no uptake of

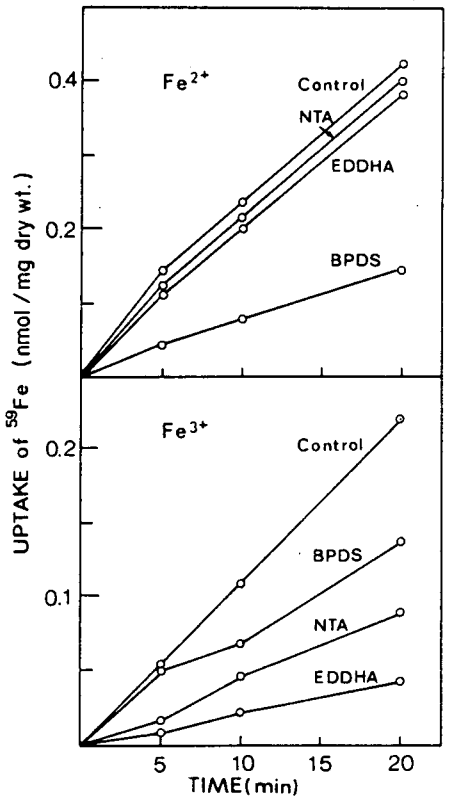

FIG. 5. Effect of various chelates on ferric and ferrous ions uptake. Concentrations of NTA, EDDHA, and BPDS were 2, 2, and 6 μM, respectively. Uptake conditions were as in Fig. 4. Inhibitors were added at zero time.

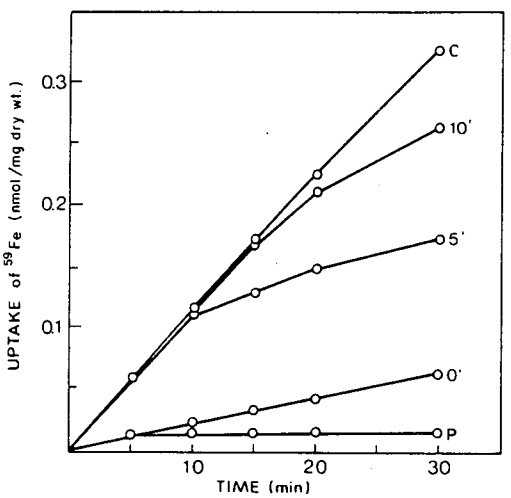

FIG. 6. Kinetics of Fe^{3+} inhibition by EDDHA. EDDHA concentration was 10 μM. P, preincubation of Fe^{3+} with EDDHA for 30 min prior to starting transport. The time in which EDDHA was added is designated by 0, 5, and 10 min. C, control.

iron. However, the onset of inhibition following introduction of EDDHA was directly proportional to the addition time of the chelate. Thus, inhibition was immediately expressed when EDDHA was given at zero time, whereas 5 and 10 min, respectively, were necessary for detecting inhibition when it was introduced after 5 and 10 min.

The observed delay in inhibition by EDDHA could result

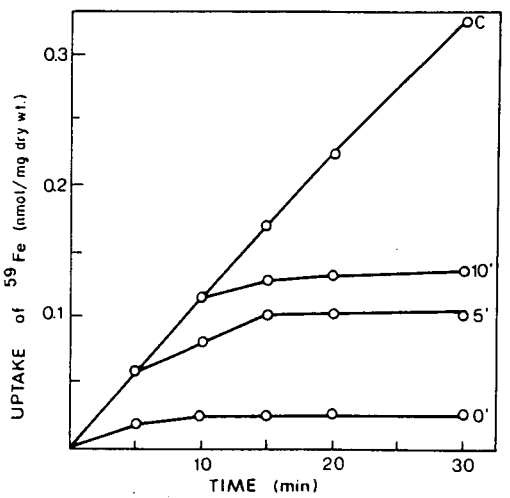

FIG. 7. Kinetics of Fe^{3+} inhibition by EDTA. EDTA concentration was 10 μM. Symbols and conditions are as in Fig. 6.

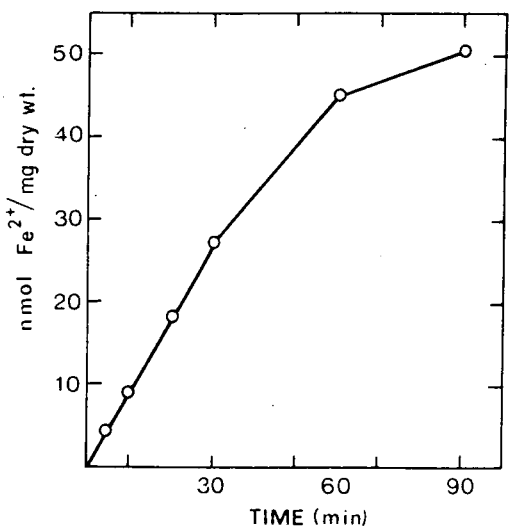

FIG. 8. Time course of reduction of $FeCl_3$ by mycelial cells. Preparation of cell suspension and reaction mixture is described in Methods.

from a rapid reduction of ferric ion and its uptake in the ferrous form. Therefore, inhibition became effective only after depletion of the ferrous ion pool, which increased proportionally to the transport period. In contrast to EDDHA, no lag period for the onset of inhibition was observed following introduction of EDTA which chelates ferrous and ferric ion (Fig. 7).

Reduction of Fe^{3+} by cell suspension and culture filtrate

Addition of ferric ion to mycelial cell suspensions resulted in its rapid and linear reduction at least up to 60 min (Fig. 8). To determine whether the reduction was carried out by enzymatic reaction or by means of reducing compounds, heat-treated cells were compared with untreated cells and culture filtrates for their capacity to reduce ferric ion bound to various chelates. Results shown in Table 2 indicate that living mycelial cells, as well as concentrated culture filtrates, could readily reduce $FeCl_3$, Fe^{3+}–citrate and Fe^{3+} – stemphyloxin I. A decrease of approximately threefold in reduction capacity was observed with Fe^{3+}–EDTA, whereas Fe^{3+}–EDDHA and Fe^{3+} – coprogen B (an hydroxamate siderophore produced by this fungus)

TABLE 2. Reduction of Fe^{3+} and various ferric chelates by mycelium and culture filtrate of *S. botryosum*

Ferric chelate	Mycelium	Heat-treated mycelium[a]	Culture filtrate[b]
$FeCl_3$	26.7	24.8	43
Fe^{3+}–citrate	28.6	19.1	41.6
Fe^{3+}–stemphyloxin I	29.7	20.4	37.6
Fe^{3+}–EDTA	8.8	5.6	13.2
Fe^{3+}–EDDHA	0.5	—	0.6
Fe^{3+}–coprogen B	1.2	—	1.1

NOTE: Reduction was carried out as described in Methods. Concentration of $FeCl_3$ and ferric chelates was 0.1 m*M*. Results are given in nmol $Fe^{2+} \cdot 30\ min^{-1} \cdot mg\ dry\ wt.^{-1}$ and are an average of three different experiments.

[a]Mycelial suspension was heated for 10 min at 100°C.

[b]Culture filtrates were prepared as described in Methods.

were only slightly reduced (Table 2). At least 70% of the reduction capacity was retained also by cells which were heated for 10 min at 100°C. Similarly, heat-treated filtrate retained approximately 90% of its reduction capacity (data not shown here). The foregoing results may indicate that reducing compounds present on the cell surface, rather than enzymatic means, were mainly responsible for the reduction process. These compounds appeared also to be released into the filtrates. The chemical nature of the reducing compounds has not yet been identified.

Approximately 30% in the reduction capacity of the cells which was lost by heat treatment (Table 2) could be attributed either to enzymatic reduction or to the breakdown of reducing compounds. The presence of enzymatic reduction was supported by the ability of the viable cells, but not the heat-treated, to reduce ferricyanide and 2,6-dichlorophenol indophenol (results not shown).

Discussion

Results of the present study suggest that acquisition of Fe^{3+} by *S. botryosum* under conditions of iron sufficiency involved three steps: (*i*) binding of iron to the cell surface; (*ii*) reduction of Fe^{3+} into Fe^{2+}, and (*iii*) active transport of the ferrous ion across the cell membrane. Up to 75% of the iron accumulated by the cells was adsorbed (Fig. 2). Iron in the ferric form is the predominant oxidation state prevailing under aerobic conditions (Neilands 1981*a*). Ferric iron exhibits an exceedingly high affinity for hydroxy ions with which it forms insoluble oxyhydroxide polymers (Neilands 1981*a*; Spiro et al. 1966). Adsorption of iron hydroxide polymers to cell surfaces of chelator-free organisms occurs between a physiological pH range of 4–8 (Winkelmann 1979) and is common among many bacteria living in fresh water (van Veen et al. 1978). The unsheathed bacteria of the genus *Sphaerotilus* were reported to accumulate large amounts of iron hydroxide when incubated in the presence of $FeCl_3$ (Rogers and Anderson 1976). Fungal cell surfaces also seem to accumulate iron. Thus, Winkelmann (1979) has demonstrated high adsorption of iron to the cell surface of *Neurospora* mutants defective in siderophore production. It appears that the property to concentrate iron at microbial cell surfaces, presumably by binding to polysaccharides and proteins, may serve as a means for storage of iron prior to its subsequent transport into the cell.

Mycelial cells of *S. botryosum* exhibited high capacity for reduction of free and chelated Fe^{3+} (Table 2), suggesting that the ferrous ion might be the predominant form of the absorbed iron. The reduction seems to be carried out primarily by heat-stable reducing compounds, although some enzymatic reduction cannot be excluded. The location of such reductants may be on or within the cell wall, and they were also released to the external milieu. It is noteworthy that the reducing compounds were capable of reducing Fe^{3+} from chelates with binding constants of 10^{24-25} such as stemphyloxin I, citrate, or EDTA, but not from EDDHA or the hydroxamate siderophore coprogen B with binding constants higher than 10^{30}. A similar nonenzymatic reduction has also been reported with the ectomycorrhizal fungus *Cenococcum graniforme* (Rodriguez et al. 1984). Reduction of Fe^{3+} prior to transport has been established for plant systems (Bienfait 1985). However, in contrast to *Stemphylium* and *Cenococcum*, enzymatic processes rather than reducing substances were claimed to act as the major mechanism for ferric ion reduction in plants. It is noteworthy that a recent study by Olsen and Miller (1986) suggests that Fe^{3+} ion may also be absorbed by barley seedlings.

Our data may suggest that iron is transported by *S. botryosum* via an energy-dependent saturable process that requires an intact membrane and sulfhydril groups (Table 1). The transport system appears to be specific for iron since, with the exception of Cu^{2+}, only slight inhibition or stimulation could be detected when various cations at excessive concentration were present during transport. The significant inhibition by Cu^{2+} should be further investigated in order to determine whether it shares a common transport system with iron or exerts a toxic effect on the latter.

Although the possibility that *S. botryosum* can transport ferric ion in the absence of siderophores cannot be excluded, the following lines of evidence support ferrous ion as the preferred form for iron transport: (*i*) initial uptake was 80% higher when iron was supplied as Fe^{2+} as compared with Fe^{3+} (Fig. 4); (*ii*) the reduction capacity of mycelial cells (per mg dry wt.) for Fe^{3+} was considerably higher than the rate of iron transport (Figs. 3 and 8), and (*iii*) the kinetics of inhibition by ferric- and ferrous-specific chelates on iron transport (Figs. 5–7). Ferrous ion in the present study was obtained by reduction with L-ascorbate. Therefore, it might be difficult to assign the higher transport rate of iron merely to Fe^{2+}, since ascorbate also maintained the sulfhydryl groups of the cell surface in the reduced form. The latter appeared significant for the function of the present transport system. A similar enhancement effect was also obtained with thioglycollate which reduces ferric ion as well as disulfide bonds. However, in addition to the high natural capacity of the mycellium to reduce ferric ion, the transport via the ferrous form was also supported by inhibition studies. Thus, the Fe^{2+}-specific chelate BPDS caused a significant inhibition of iron uptake even when provided in the ferric form (Fig. 5). The 5-min delay in the response of BPDS might be due to the presence of mycelial binding sites which effectively compete with BPDS on Fe^{2+}. These binding sites may retain the excessively produced ferrous ion prior to its transport and account for the lag period observed for inhibition by the ferric specific chelate EDDHA (Fig. 6). On the other hand, EDTA which effectively removed the ferrous as well as the ferric ion from the mycelial surface caused immediate inhibition (Fig. 7).

On the basis of the chemical properties and the iron-dependent regulation of stemphyloxins (Manulis et al. 1984), as well as their significant stimulatory effect on iron adsorption (Fig. 1), it is suggested that these chelates contribute to iron uptake by mobilizing Fe^{3+} to the cell's surface. The slightly higher transport rate in the presence of stemphyloxin I (Fig. 3)

may reflect an increase in availability of iron rather than a direct interaction of stemphyloxins with the iron transport system.

Acknowledgement

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BARASH, I., PUPKIN, G., NETZER, D., and KASHMAN, Y. 1982. A novel enolic β-ketoaldehyde phytotoxin produced by *Stemphylium botryosum* f. sp. *lycopersici*. Plant Physiol. **69**: 23–27.

BARASH, I., MANULIS, S., KASHMAN, Y., SPRINGER, J. P., CHEN, M. H. M., CLARDY, J., and STROBEL, G. A. 1983. Crystalization and X-ray analysis of stemphyloxin I, a phytotoxin from *Stemphylium botryosum*. Science (Washington, D.C.), **220**: 1065–1066.

BIENFAIT, H. F. 1985. Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. J. Bioenerg. Biomembr. **17**: 73–83.

COCHRANE, V. W. 1958. Physiology of fungi. John Wiley & Sons, New York.

HIDER, R. C. 1984. Siderophore mediated absorption of iron. Struct. Bonding, **58**: 25–87.

MANULIS, S., BARASH, I., KASHMAN, Y., and NETZER, D. 1984. Phytotoxins from *Stemphylium botryosum*: structural determination of stemphyloxin II production in culture and interaction with iron. Phytochemistry, **23**: 2193–2198.

MANULIS, S., KASHMAN, Y., and BARASH, I. 1987. Identification of siderophores and siderophore-mediated uptake of iron in *Stemphylium botryosum*. Phytochemistry, **26**. In press.

NEILANDS, J. B. 1981*a*. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1**: 27–46.

——— 1981*b*. Microbial iron compounds. Annu. Rev. Biochem. **50**: 715–731.

OLSEN, R. A, and MILLER, R. O. 1986. Absorption of ferric iron by plants. J. Plant Nutr. **9**: 751–757.

RODRIGUEZ, R. K., KLEMM, D. J., and BARTON, L. L. 1984. Iron metabolism by an ectomycorrhizal fungus *Cenococcum graminiforme*. J. Plant Nutr. **7** : 459–468.

ROGERS, S. R., and ANDERSON, J. J. 1976. Measurement of growth and iron deposition in *Sphaerotilus discophorus*. J. Bacteriol. **126**: 257–263.

SPIRO, T. G., ALBERTON, S. E., RENNER, J., TERZIS, A., BILS, R., and SALTMAN, P. 1966. The hydrolytic polymerization of iron (III). J. Am. Chem. Soc. **88**: 2721–2726.

VAN VEEN, W. L., MULDER, E. G., and DEINEMA, M. H. 1978. The Sphaerotilus Leptothrix group of bacteria. Microbiol. Rev. **42**: 329–356.

WINKELMANN, G. 1979. Surface iron polymers and hydroxy acids. A model of iron supply in sideramine-free fungi. Arch. Microbiol. **121**: 43–51.

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BIOLOGY OF METALS

Uptake of iron by *Geotrichum candidum*, a non-siderophore producer

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Summary. *Geotrichum candidum* (isolate I-9) pathogenic on citrus fruits, appears to lack siderophore production. Iron uptake by *G. candidum* is mediated by two distinct iron-regulated, energy- and temperature-dependent transport systems that require sulfhydryl groups. One system exhibits specificity for either ferric or ferrous iron, whereas the other exhibits specificity for ferrioxamine-B-mediated iron uptake and presumably other hydroxamate siderophores. Radioactive iron uptake from $^{59}FeCl_3$ showed an optimum at pH 6 and 35° C, and Michaelis-Menten kinetics (apparent $K_m = 3\ \mu M$, $V_{max} = 0.054$ nmol · mg^{-1} · min^{-1}). The maximal rate of Fe^{2+} uptake was higher than Fe^{3+} ($V_{max} = 0.25$ nmol · mg^{-1} · min^{-1}) but the K_m was identical. Reduction of ferric to ferrous iron prior to transport could not be detected. The ferrioxamine B system exhibits an optimum at pH 6 and 40° C and saturation kinetics ($K_m = 2\ \mu M$, $V_{max} = 0.22$ nmol · mg^{-1} · min^{-1}). The two systems were distinguished as two separate entities by negative reciprocal competition, and on the basis of differential response to temperature and phenazine methosulfate. Mössbauer studies revealed that cells fed with either $^{57}FeCl_3$ or $^{57}FeCl_2$ accumulated unknown ferric and ferrous binding metabolites.

Key words: Iron — Ferrioxamine B — Transport — *Geotrichum candidum*

Introduction

In many microorganisms the uptake of iron under iron-deficient conditions is mediated by siderophores (Neilands 1981; Winkelmann et al. 1987). However, since iron is indispensable for life and microorganisms are known to inhabit numerous different microenvironments, it is reasonable to assume that a multitude of mechanisms for iron assimilation may exist, even in a single organism. Indeed, a few species of bacteria, which appear not to form a siderophore system and to utilize means other than siderophores for iron acquisition, have been detected (Norrod and Williams 1978; Reeves et al. 1983; Evans et al. 1986; Neilands et al. 1987). Among the fungi, only the common yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have so far been reported to lack siderophore production (Neilands et al. 1987).

Geotrichum candidum Lk. ex Pers. is a versatile ubiquitous ascomycete fungus of considerable importance to man. It is a causal agent of sour rot, one of the major decays of citrus fruits (Eckert 1978) and responsible for a watery, soft decay in a wide range of fruits and vegetables (Butler 1960). *G. candidum* may also be pathogenic to man and animals (Emmons et al. 1970). It is associated with food spoilage and is found in various milk products (Butler 1960). The fungus is widespread in nature and a common inhabitant in citrus soils (Eckert 1978). Preliminary studies revealed that, although *G. candidum* has adapted to many different habitats, siderophore production was absent in all the citrus-pathogenic isolates which were examined by us. The present investigation was, therefore, intended to characterize mechanisms responsible for iron assimilation in *G. candidum*.

Materials and methods

Organism and growth conditions. The fungal strain of *G. candidum* (I-9) used in this study, was isolated from a lemon fruit

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infected with sour rot. Cultures were maintained on potato dextrose agar at 26° C, or stored at 5° C. For uptake experiments the fungus was grown for 2 days at 26° C on a rotary shaker at 200 rpm in 1-l conical flasks containing 200 ml of an iron-deficient liquid medium. The medium was composed of the following compounds (g/l of double-distilled water): glucose, 20; asparagine 2; K_2HPO_4, 0.5; KH_2PO_4, 1; $MgSO_4$, 1; $ZnSO_4$, 0.02; $MnSO_4$, 0.02; pyridoxin, 0.005 and thiamin · HCl, 0.01. In some experiments, which were aimed at detection of siderophores, the medium was treated with 8-hydroxyquinoline (Donald et al. 1952) to remove traces of iron. Iron-sufficient medium contained up to 20 μM $FeCl_3$.

Assays for detection of siderophores. For detection of siderophores, *G. candidum* was grown on the glucose/asparagine medium, described earlier, or on glucose/glutamate medium (Manulis et al. 1987b). These media were also tested after treatment with 8-hydroxyquinoline for removal of iron traces. The cell-free supernatants obtained after filtration and extracts of the mycelia obtained after mycelial disruption in French press (Mor et al. 1984) were used for searching for the presence of extracellular and intracellular siderophores, respectively. The procedures developed by Schwyn and Neilands (1987) with the complexometric titration dye Chrom Azurol S (CAS) were employed as a major chemical probe for the presence of siderophores. Other procedures for extraction and detection of hydroxamate siderophores were according to Manulis et al. (1987a).

Assay of ^{59}Fe uptake. Mycelial cells and arthrospores were removed from the growth medium by suction-filtration on a Buchner funnel, with an Ederol filter paper (no. 15) and washed several times with deionized water. The cells were then resuspended (1.5 g fresh mass/100 ml) in 0.05 M phosphate buffer, pH 6, for 15 s with the lower speed of a Waring blender. The standard procedure for iron uptake was carried out in 100-ml-conical flasks, containing 10 ml cell suspension. The flasks were shaken at 35° C for 10 min prior to the addition of the radioactive iron, and during the experiment, on a reciprocal shaker. The reaction was started by a rapid addition of 0.1 ml of the radioactive iron solution. Samples of 3 ml were removed at various time intervals and immediately treated, as further described. In experiments aimed at siderophore-mediated iron uptake, samples were filtered through GF/C filter paper (2.5 cm diameter) and rinsed three times with 5 ml of 50 mM EDTA (ethylenediaminetetraacetic acid). The filters with the mycelial pads were tansferred to polyethylene tubes, dried for 1 h at 70° C and counted in a Packard gamma counter C (model 5166). Values were corrected for the measurements obtained at zero time. When the latter procedure was used for measuring uptake of $^{59}FeCl_3$ or $^{59}FeCl_2$, a significant adsorption of labelled iron on the cell surface was observed, in addition to the transported iron, as reported elsewhere (Manulis et al. 1987b). The cell-surface-bound iron was removed as follows. After incubation with labelled iron, the cells (3 ml) were promptly transferred into a vial (20 ml), containing 1 ml 400 mM EDTA and incubated for 1 h on a reciprocal shaker at 30° C. They were then filtered, washed with distilled water and the radioactivity measured, as previously described. Radioactive iron was given as either $^{59}FeCl_3$, $^{59}FeCl_2$ or chelated to desferrioxamine B, as described elsewhere (Manulis et al. 1987b). Ferrous ion was obtained by pre-incubation of $FeCl_3$ in the presence of excessive concentration (× 100) of L-ascorbic acid for at least 30 min and during the uptake experiment (Manulis et al. 1987b).

Mössbauer studies. Iron-uptake procedures for Mössbauer analyses were carried out as described earlier, except that the cells were enriched with $^{57}FeCl_3$ instead of $^{59}FeCl_3$. The $^{57}FeCl_3$ was obtained by dissolving 2 mg $^{57}Fe_2O_3$ in 1 ml 5 M HCl. Following iron uptake, the mycelia were immediately washed with 50 mM EDTA and further incubated in 100 mM EDTA for 30 min to remove cell-surface-bound iron. The mycelial suspension was filtered and rinsed with distilled water, peeled off the filter paper, frozen with liquid nitrogen and stored at −70° C. The mycelial samples were encapsulated in lucite holders. Measurements were carried out with a flow cryostat in the temperature range of 5–300 K. A 25-mCi $^{57}Co(Rh)$ source was used and kept at ambient temperature.

Chemicals. $^{59}FeCl_3$ (3–20 mCi/mg) was purchased from Amersham International (Amersham, UK). $^{57}Fe_2O_3$ was purchased from Oak Ridge National Laboratory; 4,7-bis(4-phenylsulphonate)-1,10-phenanthroline (BPDS), the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid) 1,2,4-triazine (ferrozine), 2,2′-dipyridyl (dipyridyl) and ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) were purchased from Sigma Chemical Co. Desferrioxamine B (desferal) was kindly provided by Ciba Laboratories.

Results

Lack of siderophore production by G. candidum

G. candidum was grown in glucose/asparagine media which were either deferrated with 8-hydroxyquinoline or after addition of various concentrations (1–10 μM) of ferric chloride. Iron-deprived medium could support only a limited growth, whereas the addition of 2 μM iron more than doubled the cell yield after 48 h. All cultures reached the stationary phase of growth within 48 h. The possibility of siderophore excretion was initially investigated by the addition of drops of a 1% ferric chloride solution to supernatants of low- and high-iron cultures. No apparent colour change was produced when the filtrates were examined at different growth periods of up to a week. Nor could we detect any colour changes after the filtrates were evaporated under vacuum at 35° C to about a tenth of the original volume and excess $FeCl_3$ was added. The concentrated filtrates were extracted with benzyl alcohol, subjected to paper electrophoresis and stained with Folin chiocalteus phenol reagent, as described elsewhere (Manulis et al. 1987a). However, the presence of siderophores, even in trace amounts, could not be confirmed.

For determination of siderophores by the CAS assay, the total phosphate concentration in the asparagine/glucose media was reduced to 0.3 g/l (Schwyn and Neilands 1987). Alternatively, benzyl alcohol extracts of the filtrates of the high-phosphate medium or of the glutamate/glucose medium were employed. Although the CAS assay is considered a universal method for detection of

siderophores, all our attempts to detect them by this procedure were unsuccessful. Neither could we detect cell-bound siderophores by any of the foregoing assays when mycelial extracts, obtained as described in Materials and methods were tested. Therefore, it was concluded that *G. candidum* did not form siderophores, at least under the growth conditions employed in this study.

Iron accumulation by mycelial cells

Results shown in Fig. 1 indicate that $^{59}FeCl_3$ accumulation by cells of *G. candidum* grown in iron-deficient medium was linear with time. Linearity was observed up to approximately 15 min (not shown in the plot). To distinguish between iron transported into the cells from iron bound to the cell's surface, the latter was removed by a high concentration of EDTA, as described earlier. The cell-surface-bound radioactive iron could account for about 34% of the total iron accumulated and the linear kinetics were retained after its removal (Fig.1). The EDTA treatment was, therefore, employed in all the uptake experiments with ferric or ferrous ion.

Properties of the Fe^{3+} uptake system

$^{59}FeCl_3$ transport, under conditions described in Fig. 1, showed pH dependence with an optimum at pH 6. The uptake of iron revealed a continuous increase of transport rates from 20° C to 35° C, followed by a loss of transport activity above 35° C (Fig. 2). A Q_{10} value of 2.5 was calculated between 20° C and 30° C, suggesting the involvement of a biological system rather than diffusion. The energy of activation calculated from Arrhenius plot was 84 kJ (20 kcal).

The requirement for metabolic energy and functional membranes by the ferric ion transport system was demonstrated by high inhibitory effects of sodium azide or CCCP and nystatin, respectively (Table 1). Sulfhydryl groups were important in this system since inhibition was evident with *N*-ethylmaleimide and iodoacetamide. A plot

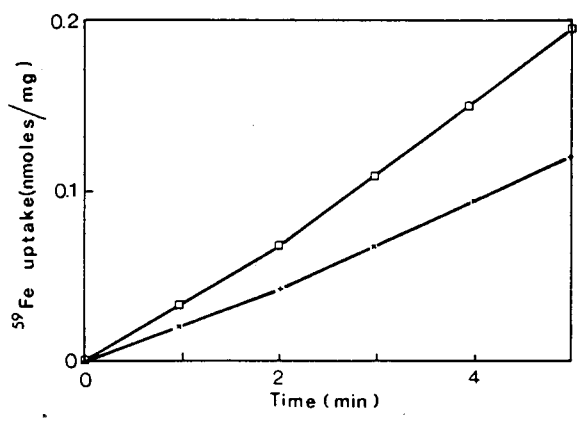

Fig. 1. Accumulation of iron by mycelial cells. Iron (1 μM) was given as $^{59}FeCl_3$. Experimental conditions for iron uptake and removal of cell-surface-bound iron by EDTA are described in Materials and methods. Iron uptake before (□) and after (×) the EDTA treatment

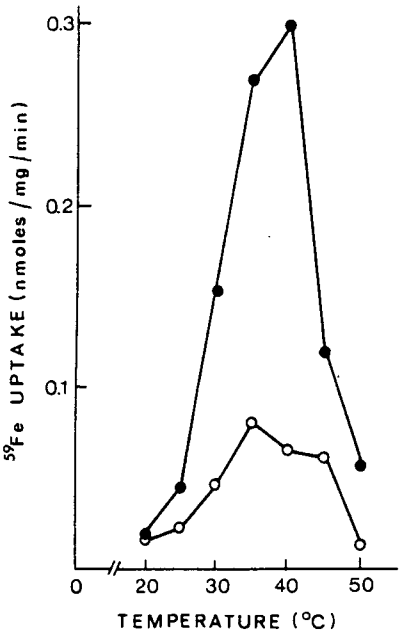

Fig. 2. Effect of temperature on iron uptake. The mycelial suspension was brought to the indicated temperature within 5 min prior to the addition of $^{59}FeCl_3$ (○) or ^{59}Fe-desferrioxamine B (●). Uptake was carried out as described in Materials and methods

Table 1. Effect of inhibitors on $^{59}FeCl_3$ and ^{59}Fe-desferrioxamine uptake

Inhibitor	Inhibition by	
	$^{59}FeCl_3$ (%)	^{59}Fe-ferrioxamine B (%)
Sodium azide (1 mM)	95	99
CCCP (1 mM)	70	99
PMS (1 mM)	30	80
NEM (0.1 mM)	60	60
Iodoacetamide (1 mM)	45	50
Nystatin (0.1 mM)	83	85

CCCP = carbonyl cyanide *m*-chlorophenylhydrazone, PMS = phenazine methosulfate, NEM = *N*-ethylmaleimide

of transport rate versus Fe^{3+} concentration yielded Michaelis-Menten kinetics. The apparent K_m and V_{max} values extrapolated from a Lineweaver-Burk plot were 3 μM and 0.054 nmol · mg^{-1} · min^{-1}, respectively.

The expression of the ferric transport system was dependent on iron deficiency. A reduction of approximately 80% in Fe^{3+} transport was observed when *G. candidum* was grown in the presence of 2 μM $FeCl_3$ (Fig. 3).

Specificity of the Fe^{3+} transport system

The valence form in which iron was transported into the cells was investigated. Results from Lineweaver-Burk plot indicate that reduction of ferric into ferrous ion by L-ascorbate increased the maximal uptake rate by more than four fold (V_{max} = 0.25 nmol · mg^{-1} · min^{-1}), whereas the K_m values for ferric and ferrous ions were identical (3 μM). Thus, both ferric and ferrous ions could be readily transported into the cells of *G. candidum*.

Further experiments were designed to determine whether changes in the valence form of iron occur by mycelial cells prior to its transport. Consequently, the effect of ferric- and ferrous-specific chelators on iron uptake was investigated. Results given in Fig. 4 indicate that the ferric-specific chelator EDDHA caused 60% inhibition of Fe^{3+} transport, whereas the Fe^{2+} trapping reagents,

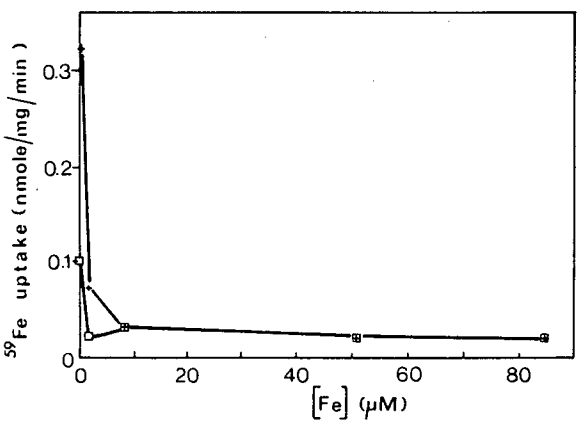

Fig. 3. Uptake of $^{59}FeCl_3$ and ^{59}Fe-desferrioxamine B as a function of iron concentration in the growth medium. *G. candidum* was grown for 48 h in the presence of various $FeCl_3$ concentrations. Transport measurements of $FeCl_3$ (□) and Fe-desferrioxamine B (+) were performed as described in Materials and methods

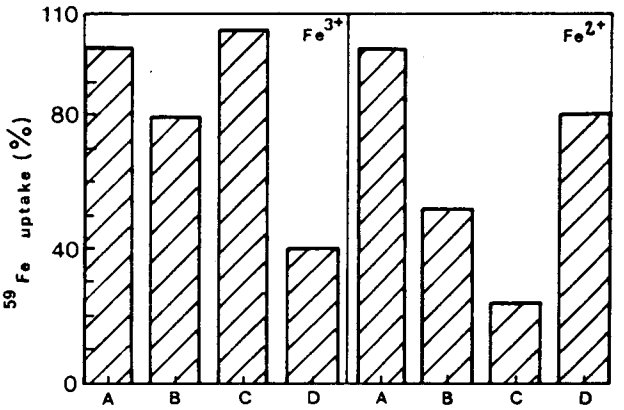

Fig. 4. Effect of various chelates on ferric and ferrous ion uptake. Uptake conditions were as in Fig. 1, except that iron concentration was 6 μM. The concentration of ferrozine, dipyridyl and EDDHA was 60 μM each. Inhibitors were added at zero time. (A) Control; (B) dipyridyl; (C) ferrozine; (D) EDDHA

ferrozine and dipyridyl, were either noneffective or caused only slight inhibition. No inhibition of Fe^{3+} transport could be detected even when ferrozine concentration was increased to 1.2 mM (not shown). When the Fe^{2+} transport was examined (Fig. 4), ferrozine and dipyridyl caused 80% and 50% inhibition respectively, whereas EDDHA was only slightly inhibitory (about 20%). The failure of the ferrous-specific chelators to exert a significant inhibition on the ferric ion transport suggests that the latter may not be reduced prior to its penetration into the cells, at least during the transport period. The latter conclusion was further supproted by our unsuccessful attempts to reduce ferric chloride with cells of *G. candidum* using BPDS as an indicator for ferrous ion formation (Manulis et al. 1987b; Lesuise et al. 1987) and longer incubation periods. The merely partial inhibition of Fe^{3+} by EDDHA or of Fe^{2+} by dipyridyl, could be accounted for by competition between the binding sites on the mycelial surface with the relevant chelators for iron.

The possibility that the iron transport system is shared by other cations was investigated by competition experiments. Ferric ion transport was measured as described in Fig. 1 in the presence of 1 mM Mg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Al^{3+} and Zn^{2+}. Only Zn^{2+} and Al^{3+} caused a significant inhibition (about 60%). However, when the concentration of these cations was reduced to 50 μM, which was still 25-fold higher than the $^{59}FeCl_3$ (2 μM), inhibition could not be detected. It appears that the investigated system is quite specific to ferric and ferrous ions.

Characterization of ferrioxamine B mediated iron uptake G. candidum

Although *G. candidum* is unable to produce any siderophores, it was found capable of utilizing iron effectively from exogenous hydroxamate siderophores of various structural classes (Mor and Barash, unpublished results). The uptake of iron via ferrioxamine B, one of the more efficient siderophores for iron utilization by this fungus, was further characterized. This transport system exhibited a pH optimum at about pH 6 and temperature optima at 40° C (Fig. 2). The calculated Q_{10} between 20° C and 30° C was 6.2 and the energy of activation as calculated from Arrhenius plot was 138 kJ (33 kcal). Kinetic studies of initial uptake versus siderophore concentration exhibited Michaelis-Menten kinetics with apparent K_m of 2 μM and V_{max} of 0.22 nmol · mg^{-1} · min^{-1} (Fig. 5). The requirement for energy, sulfhydryl groups, is evident by the significant inhibitory effect of CCCP, NaN_3 or PMS and *N*-ethylmaleimide or iodoacetamide, respectively (Table 1). The ferrioxamine-mediated iron transport was almost completely repressed in the presence of 2 μM $FeCl_3$ in the medium (Fig. 3).

Since the ferrioxamine and the $FeCl_3$ systems show many similar properties (e.g. Table 1, Fig. 3 and others), it was suspected that the two systems may share a common membrane-mediated carrier. However, a competition experiment described in Table 2 indicates that neither $FeCl_3$ nor Fe-desferrioxamine B could compete with ^{59}Fe-desferrioxamine and $^{59}FeCl_3$ transport, respectively, when given at equimolar concentrations.

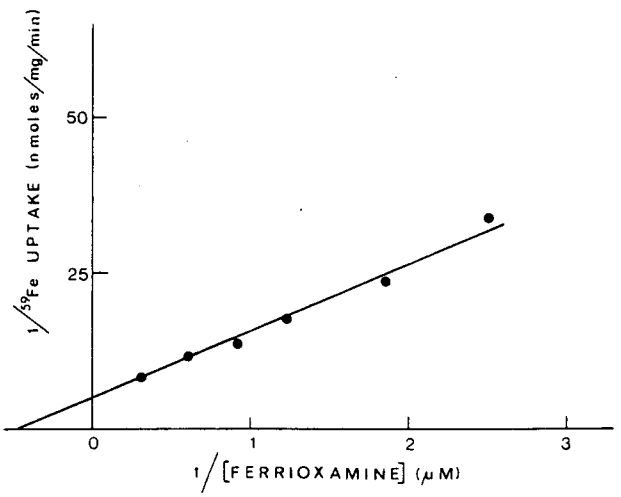

Fig. 5. Lineweaver-Burk plot of ^{59}Fe-desferrioxamine-B-mediated iron uptake by *G. candidum*. Transport conditions are described in Materials and methods

Table 2. Competition between $^{59}FeCl_3$ and Fe-desferrioxamine B or between ^{59}Fe-desferrioxamine B and $FeCl_3$

Substrate[a]	^{59}Fe uptake at	
	2 min (nmol · mg^{-1} · min^{-1})	5 min
$^{59}FeCl_3$	0.04	0.11
$^{59}FeCl_3$ + Fe-desferr. B	0.05	0.11
^{59}Fe-desferr. B	0.15	0.32
^{59}Fe-desferr. B + $FeCl_3$	0.17	0.32

[a] Each substrate was added at a concentration of 4 μM

Mössbauer study of iron assimilation

Cells of *G. candidum* grown under iron deficiency were incubated with either $^{57}FeCl_3$ (6 mM) or $^{57}FeCl_2$ (i.e. 6 mM $FeCl_3$ in the presence of 300 mM L-ascorbate) for 30, 60 or 120 min. Following the incubation period, the cells were treated as described in Materials and methods and used for Mössbauer measurements. A typical spectrum of the freeze-dried cells at room temperature after a 30-min incubation is shown in Fig. 6. A least-squares fit was attempted by assuming two quadrupole split components and an equal line width for both. Measurements done at this temperature range have not shown any magnetic interaction. This involves the existence of very fast relaxation times of high-spin Fe^{2+} and Fe^{3+} species. The solid line through the experimental points is the theoretical spectrum resulting from the computer fitting.

After uptake of $FeCl_2$, the reduced hyperfine constants for Fe^{2+} were $\delta = 1.37(1)$ mm/s and $\Delta Q = 3.02(4)$ mm/s, whereas for the Fe^{3+}, $\delta = 0.48(1)$ mm/s and $\Delta Q = 0.61(2)$ mm/s. After uptake of $FeCl_3$ the hyperfine constants for Fe^{2+} were, $\delta = 1.34(8)$ mm/s and $\Delta Q = 3.08(1)$ mm/s, whereas for Fe^{3+}, $\delta = 0.487(6)$ mm/s and $\Delta Q = 0.586(9)$ mm/s. δ and ΔQ stand for isomer shift with respect to iron and quadruple splitting, respectively. The half-line width (Γ) for both components was 0.25(1) mm/s, indicating a possible existence of site distribution. From the spectra we calculated the percentages of Fe^{2+}, namely 20% and 39% following the uptake of $FeCl_2$ and $FeCl_3$, respectively. These results suggest that iron is stored within the cells as both ferric and ferrous metabolites, regardless of the valence in which the iron enters the cell. The ferric iron species might represent the predominant fraction, under the experimental conditions used, and its relative amount is not affected by the valence of iron sup-

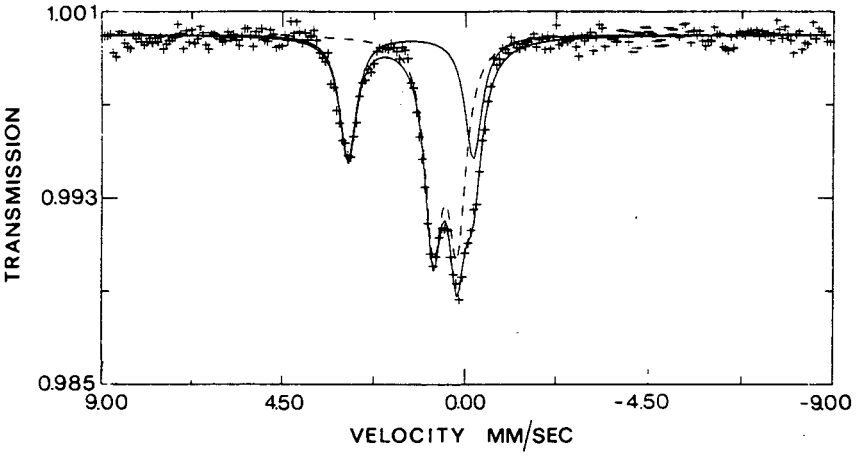

Fig. 6. Mössbauer spectrum at room temperature of freeze-dried cells of *G. candidum* after incorporation of $^{57}FeCl_3$ for 30 min. The spectrum is composed of two components corresponding to Fe^{3+} (- - -) and Fe^{2+}; (——) theoretical spectrum obtained by a least-squares fitting

plied to the cells. No significant changes in the pattern of stored iron was observed during longer incubation periods.

Discussion

Results of the present study have shown that *G. candidum* lacks siderophore production and its iron uptake is mediated by at least two distinct active transport systems. One of the systems is specific to ferric and ferrous ions, whereas the other displays specificity towards ferrioxamine B and, presumably, other hydroxamate siderophores. Although the two systems exhibit many similar properties, e.g. energy- and temperature-dependence, pH optima at 6, requirement for sulfhydryl groups, repression by iron (Fig. 3) and saturation kinetics, the possibility that they share the same recognition or transport system may be excluded on the basis of the following observations: (a) reciprocal competition experiments between $FeCl_3$ and ferrioxamine B gave negative results (Table 2); (b) the ferrioxamine B system is significantly more sensitive to temperature elevation than the Fe^{3+} system, as expressed by the distinctive Q_{10} values and energies of activation (Fig. 2); (c) the differential inhibitory effect of the electron acceptor PMS on the two systems (Table 1) may imply that the ferrioxamine-B-mediated iron uptake demands more cell-generated energy than ferric iron uptake. The latter conclusion may also be supported by the significantly higher sensitivity of the ferrioxamine system towards the uncoupler CCCP. It is also noteworthy that, in contrast to the excessive adsorption of iron on the cell-surface during Fe^{3+} uptake (Fig. 1), the iron adsorption by the ferrioxamine system was negligible.

Although the possibility that iron is being transported across the cell membrane in the ferric form by an energy-dependent saturable process has not been reported in fungi, a similar transport system was detected in *Yersiniae* (Perry and Brubaker 1979). Several cases have been reported in fungi (Manulis et al. 1987b; Rodriguez et al. 1984; Lesuisse et al. 1987) and bacteria (Evans et al. 1986; Cowart and Foster 1985), which indicate that iron is transported across the cell membrane only in the ferrous form. Reduction of ferric iron in the foregoing transport systems is achieved by either external reductants (Manulis et al. 1987b; Cowart and Foster 1985; Rodriguez et al. 1984; Lesuisse et al. 1987) and/or by a membrane-associated enzymatic process (Rodriguez et al. 1984; Lesuisse et al. 1987). Reduction of ferric iron prior to its transport does not seem to occur in *G. candidum*. The latter conclusion is supported by our failure to inhibit Fe^{3+} uptake with ferrous specific chelates (Fig. 4). Neither could we detect formation of Fe^{2+} when cells were incubated for a long period with ferric iron in the presence of the ferrous iron indicator BPDS as reported by Manulis et al. (1987b) for *Stemphylium botryosum* or Lesuisse et al. (1987) for yeast. It appears therefore, that the ferric iron might be directly transported also in the oxidized form.

Ferrous iron could be readily transported into cells of *G. candidum* with an identical Michaelis constant as the ferric iron, but with higher V_{max}. Fe^{2+} in the present study was obtained by reduction with L-ascorbate. Therefore, it might be difficult to assign the higher V_{max} merely to ferrous iron, since ascorbate also maintained the sulfhydryl groups of the cell surface in the reduced form. The latter appeared significant for the function of the present transport system (Table 1). Studies on iron assimilation through Mössbauer

spectroscopy indicate that iron is being stored as both ferric and ferrous metabolites. The ratio between the former and the latter iron forms appears not to be affected by the valence of the iron incorporated into the cells. The Mössbauer spectra for the Fe^{2+} and Fe^{3+} species illustrated in Fig. 6 are quite similar to spectra of yet unidentified metabolites reported by Matzanke et al. (1987) in *Neurospora crassa*. They assumed that the Fe^{2+} species represents a novel internal iron compound, whereas the Fe^{3+} species might correspond to an iron-storage compound, similar to bacterioferritin.

The absence of siderophore formation by citrus-pathogenic isolates of *G. candidum* is quite interesting, since this fungus maintains the capacity to transport iron via different hydroxamate siderophores. The ability of *G. candidum* to utilize iron efficiently from ferrioxamine B, an hydroxamate siderophore produced by *Streptomyces* spp. has also been observed in yeast (Lesuisse et al. 1987). However, the latter siderophore was not recognized by other fungi, such as *Stemphylium botryosum* (Manulis et al. 1987a) and *Verticillium dahliae* (Cordova and Barash, unpublished results). It should also be remembered that *G. candidum* comprises a complex of asexual fungi with different sexual stages (Butler and Peterson 1972). Thus, the possibility that isolates of *G. candidum* which maintain the production of siderophores will be found, cannot be entirely excluded.

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References

Butler EE (1960) Pathogenicity and taxonomy of *Geotrichum candidum*. Phytopathology 50:665-672

Butler EE, Petersen LJ (1972) *Endomyces geotrichum*, a perfect stage of *Geotrichum candidum*. Mycologia 49:820-830

Cowart RE, Foster BG (1985) Differential effects of iron on growth of *Listeria monocytogenes:* minimum requirements and mechanisms of acquisition. Infect Dis 151:721-730

Donald C, Passey BI, Swaby RJ (1952) A comparison of methods for removing trace metals from microbiological media. J Gen Microbiol 7:211-220

Eckert JW (1978) Postharvest disease of citrus fruits. Outlook Agric 9:225-259

Emmons CW, Binford CH, Utz JP (1970) Medical mycology. Lea & Febiger, Philadelphia

Evans SL, Arceneaux JEL, Byers BR, Martin ME, Aranha H (1986) Ferrous iron transport in *Streptococcus mutans*. J Bacteriol 168:1096-1099

Lesuisse F, Raguzzi F, Crichton RR (1987) Iron uptake by the yeast *Saccharomyces cerevisiae:* involvement of a reduction step. J Gen Microbiol 133:3229-3236

Manulis S, Kashman Y, Barash I (1987a) Identification of siderophores and siderophore-mediated uptake of iron in *Stemphylium botryosum*. Phytochemistry 26:1317-1320

Manulis S, Netzer D, Barash I (1987b) Acquisition of iron by *Stemphylium botryosum*. Can J Microbiol 33:652-657

Matzanke BF, Bill E, Muller GJ, Trautwein AX, Winkelmann G (1987) Metabolic utilization of ^{57}Fe-labelled coprogen in *Neurospora crassa*. Eur J Biochem 162:643-650

Mor H, Steinlauf R, Barash I (1984) Virus-like particles and double-stranded RNA in *Geotrichum candidum*, the causal agent of citrus sour rot. Phytopathology 74:921-924

Neilands JB (1981) Iron absorption and transport in microorganisms. Annu Rev Nutr 1:27-46

Neilands JB, Konopka K, Schwyn B, Coy M, Francis RT, Paw BH, Bagg A (1987) Comparative biochemistry of microbial iron assimilation. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, pp 3-33

Norrod P, Williams RP (1978) Growth of *Neisseria gonorrhoeae* in media deficient in iron without detection of siderophores. Curr Microbiol 1:281-284

Perry RD, Brubaker RR (1979) Accumulation of iron by *Yersiniae*. J Bacteriol 137:1290-1298

Reeves MW, Pine L, Neilands JB, Ballows A (1983) Absence of siderophore activity in *Legionella* species grown in iron-deficient media. J Bacteriol 154:324-329

Rodriguez RK, Klemm DJ, Barton LL (1984) Iron metabolism by an ectomycorrhizal fungus *Cenococcum graminiforme*. J Plant Nutr 7:459-468

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47-56

Winkelmann G, van der Helm D, Neilands JB (1987) Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim

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Characterization of siderophore-mediated iron transport in Geotrichum candidum, a non-siderphore producer

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Summary. Geotrichum candidum is capable of utilizing iron from hydroxamate siderophores of different structural classes. The relative rates of iron transport for ferrichrome, ferrichrysin, ferrioxamine B, fusigen, ferrichrome A, rhodotorulic acid, coprogen B, dimerum acid and ferrihodin were 100, 98, 74, 59, 49, 35, 24, 12 and 11 per cent respectively. Ferrichrome, ferrichrysine and ferrichrome A inhibited ^{59}Fe-desferrioxamine B-mediated iron transport by 71, 68 and 28 per cent respectively when added at equimolar concentrations to the radioactive complex. The inhibitory mechanism of ^{59}Fe-desferrioxamine B uptake by ferrichrome was noncompetitive (Ki 2.4 uM), suggesting that the two siderophores do not share a common transport system. Uptake of ^{59}Fe-ferrichrome, ^{59}Fe-rhodotorulic acid and ^{59}Fe-fusigen was unaffected by competition with the other two siderophores or with ferrioxamine B. Thus, G. candidum may possess independent transport systems for siderophores of different structural classes. The uptake rates of Fe-(^{14}C)desferrioxamine B and ^{67}Ga-desferrioxamine B were 30 and 60 per cent respectively, as compared to ^{59}Fe-desferrioxamine B. The specific ferrous chelates, dipyridyl and ferrozine at 6mM caused 65 and 35 per cent inhibition of ^{59}Fe-desferrioxamine uptake. From these results we conclude that, although about 70 per cent of the iron is apparently removed from the complex by reduction prior to being transported across the cellular membrane, a significant portion of the chelated ligand may enter the cell intact. The former and latter mechanisms seem not to be mutually exclusive.

Key words: Iron, siderophores, transport, Geotrichum candidum

Introduction

Geotrichum candidum Lk. ex Pers. is a versatile ubiquitous ascomycete fungus of considerable importance to man. It is responsible for a watery, soft decay in a wide range of fruits and vegetables (Butler 1960) and may also become pathogenic to man and animals (Emmons et al. 1970). The fungus is widespread in nature and a common inhabitant of soils (Eckert 1978). Although G. candidum has adapted to many different habitats, it is incapable of siderophore production (Mor et al. 1988).

It has previously been shown (Mor et al. 1988) that iron uptake by G. candidum is mediated by two distinct iron-regulated, energy- and temperature-dependent transport systems that require sulfhydryl groups. One system exhibits specificity for either ferric or ferrous iron, whereas the other exhibits specificity for ferrioxamine-B-mediated iron uptake and, presumably other hydroxamate siderophores. The two systems were distinguished as two separate entities by negative reciprocal competition and, on the basis of differential response, to temperature and phenazine methosulfate (Mor et al. 1988). The present study was undertaken to characterize the siderophore-mediated transport system in G. candidum.

Materials and Methods

Culturing. The fungal strain of G. candidum (I-9) used in this study and culturing conditions have previously been described (Mor et al. 1988). For uptake experiments the fungus was grown for 2 days at 26°C on a rotary shaker at 200 rpm in 1-l conical flasks containing 200 ml of an iron-deficient glucose-asparagine liquid medium (Mor et al. 1988).

Transport assays. The preparation of cell suspension and experimental conditions for siderophore-mediated iron uptake were as described (Mor et al. 1988). Samples were filtered through GF/C filter paper (2.5cm diameter) and rinsed three times with 5ml of 50mM EDTA. The filters with the mycelial pads were transferred to polyethelene tubes, dried for 1h at 70°C and counted in a Packard gamma counter C (model 5166).

The radioactive gallium complex of desferrioxamine B was prepared by adding $^{67}GaCl_3$ (2.5mCi/mg) to desferrioxamine B to a final concentration of 4 μM with 50 per cent excess ligand. The absence of free gallium in the chelate solution

was confirmed by chromatography on Whatman no. 1 paper (10cm) in 2 solvent systems consisting of methanol-water (85:15 v/v) and saline solution. The chelate complex had an Rf = 1 in both solvents, whereas the Rf of the Ga salt was close to zero.

Fe(^{14}C)desferrioxamine B was prepared by adding (^{14}C)desferrioxamine B (ca 0.35μ Ci/mg) to $FeCl_3$ solution to a final Fe concentration of 4μM with 50% excess ligand. Experimental conditions were as described earlier but with the following modifications: The samples were filtered through nitro- cellulose membrane filters (0.45μ, 2.5cm in diameter) rinsed with 50mM EDTA and dried for 1h at 70°C. The filters with the mycelial pads were counted in 5ml liquiscint scintilation fluid with a Tri Carb Packard Liquid Scintilation counter (model 1500).

Chemicals. ^{59}Fe (3-20mCi/mg) was purchased from Amersham International (Amersham, UK). (^{14}C) desferrioxamine B (ca 0.35μ Ci/mg) was a gift from Dr. H. H. Peter (Ciba Geigy Ltd, Basel). ^{67}Ga-citrate (neoscan) was obtained from Med-Physics Incorp., Richmond, California. The 1, 2, 4-triazine (ferrozine) and 2,2'-dipyridyl (dipyridyl) were purchased from Sigma Chemicals. Desferrioxamine B (desferal) was kindly provided by Ciba Laboratories. Siderophores were kindly provided by Prof. van der Helm and Prof. Winkelmann.

Results and discussion

Siderophore-mediated iron uptake. Results shown in Fig. 1 indicate that G. candidum can utilize iron from an array of different hydroxamate siderophores in a differential manner. The highest rate of uptake was recorded with ferrichrome and ferrichrysin (98-100%) followed by ferrioxamine B (76%), fusigen (61%), ferrichrome A (51%), rhodotorulic acid (35%) and coprogen B (24%). The rate of $^{59}FeCl_3$ uptake was considerably lower (26%) than ferrichrome (Fig. 1). The ability of G. candidum to transport iron differentially from siderophores of various structural classes may be analyzed in the light of two major hypotheses: (a) iron is first removed by a membrane-bound siderophore reductase and then taken up by the cells via the ferrous transport system described earlier (Mor et al. 1988). The broad range of the utilized siderophores could be attributed to a low specificity of this enzyme which, nevertheless, exhibits differential affinity towards the various siderophores; (b) siderophore-mediated iron

transport is performed via receptors as described for other fungi (Winkelmann and Huschka 1987).

When the variation of iron uptake within each siderophore class is analyzed according to structure-activity relationship, a rather unique interaction emerges which could favour the receptor hypothesis. Among the ferrichrome class of compounds (Fig. 1 B-F) the transport rate seems to be significantly affected by the N-acyl residues. Thus, ferrichrome and ferrichrysine, which showed the highest transport activity, contain three ornithyl N-acetyl residues. Ferrichrome A, which showed significantly lower activity, contains three trans-β-methylglutaconyls, whereas ferrirubin and ferrihodin, which exhibited the lowest activity, possess three anhydromevalonyl residues in the trans and cis configuration, respectively.

The foregoing results are in agreement with the structural requirements of the ferrichrome receptor in Neurospora crassa (Winkelmann and Huschka 1987) and Aspergillus ochraceus (Jalal et al. 1984). Using different ferrichrome-type siderophores including asperchromes, it was demonstrated that in both fungi the efficiency of siderophores seem to increase when various N-acyl residues in the ornithines are gradually replaced by acetyls. A similar phenomenon was also observed with two siderophores of the coprogen class. Thus, rhodotorulic acid, which was twice as active as dimerum acid (Fig. 1 I-J) contains two ornithyl N-acetyl residues, whereas the N-acyls of the latter are composed of trans-anhydromevalonyls.

Results in Fig. 1 G-H indicate that coprogen B is an effective iron transport molecule in G. candidum, whereas coprogen (acetylated coprogen B) is not. These results suggest that the positive charge of the free amino group supports recognition and transport. In contrast to G. candidum, coprogen was the effective and coprogen B the non-effective siderophore in N. crassa (Ernst and Winkelmann 1974). In the latter study, substitution of the N-acetyl group in coprogen by N-propyl or N-butyryl residues, revealed a signficant increase in uptake, suggesting that this part of the molecule has to be non-polar for N. crassa. The necessity of a positive charge for an effective uptake was also observed with fusigen in G. candidum (Fig. 1 K-L). Acetylation of the free ornithyl amino groups of fusigen and formation of triacetyl fusigen almost completely retarded iron uptake. The effect of a positive charge on uptake rate was also demonstrated with ferrioxamines in Streptomyces pilosus, a natural producer of these siderophores (Muller and Raymond 1984). The lack of a

positive charge in ferrioxamine D_1 reduced the uptake rate by 40 per cent as compared to ferrioxamine B, which has identical architecture but the amino group remained unacetylated.

Specificity of siderophore transport systems. The question as to whether different transport systems might be involved in siderophore-mediated iron uptake of G. candidum was investigated by competition studies. Results shown in Table 1 reveal that ^{59}Fe-desferrioxamine B uptake is highly inhibited in the presence of equimolar concentration of ferrichrome or ferrichrysin, whereas fusigen, rhodotorulic acid or $FeCl_3$ showed only negligible inhibition. Interestingly, the inhibition rate by the ferrichrome-type compounds was signficantly higher (68-71%) than the control with ferrioxamine (40%). Nevertheless, the apparent Km for ferrichrome and ferrioxamine B, as calculated from Lineweaver-Burk plots (Fig. 2) was identical (2 μ M) suggesting that the affinity of each compound to its own membrane carrier is similar.

The nature of inhibition of ^{59}Fe-desferrioxamine B by ferrichrome was examined by using the Dixon plot (Segel 1968). When the reciprocal velocities of two ^{59}Fe-desferrioxamine concentrations were tested with increasing ferrichrome concentrations, the 2 lines coincided in the X axis (Fig. 3), indicating a non-competitive inhibition mechanism. The apparent inhibition constant (Ki) obtained for ferrichrome was 2.4 μ M. These results may imply that the two structurally different siderophores do not share a common receptor. The effective inhibitory action of ferrichrome could result from its binding to the ferrioxamine B receptor and interfering with iron transport by causing either conformational changes of the receptor protein or other mechanism. Alternatively, it is possible that the receptors of ferrioxamine B and ferrichrome are closely situated within the membrane and may, under certain conditions, interact with each other. Thus, the binding of the ferrichrome to its own receptor could lead to changes in the ferrioxamine B receptor protein which adversely affect the binding of its specific siderophore. It is noteworthy that ^{59}Fe-ferrichrome-mediated iron uptake was also inhibited by the presence of ferrioxamine B (30% inhibition), suggesting that the interaction between the two siderophores is reciprocal. This finding might favour the latter hypothesis.

The possibility that other siderophores recognized by G. candidum (Fig. 1) may share common transport systems was further investigated. Results shown in Fig. 4 indicate that neither ferrichrome nor rhodotorulic acid, nor fusigen

could inhibit siderophores of the different structural classes. Similar results were obtained when iron uptake via these siderophores was performed in the presence of ferrioxamine B (not shown). The foregoing data suggest that G. candidum possesses independent transport systems for the different classes of siderophores.

Muller et al (1984) have shown that the kinetically inert chromic complex of desferrioxamine B was a strong inhibitor of iron uptake mediated by ferrichrome and ferric-enantio rhodotorulic acid in S. pilosus. These inhibition experiments may indicate that, in contrast to G. candidum, iron from these exogenous siderophores is transported by the same uptake system as ferrioxamine B. Since the ligands have no structural similarity to ferrioxamine B, except the presence of three hydroxamate groups, it was concluded that only the hydroxamate iron centre and its direct surroundings are important for recognition and uptake (Muller et al. 1984). However, due to lack of information on the mechanism of inhibition, the possibility that these siderophores compete for the same transport system cannot be conclusive. A shared transport system for siderophores of different structural types (i.e. ferrichrome and coprogen) was demonstrated in N. crassa.

Mechanism of ferrioxamine B-mediated iron transport. Further experiments were designed to distinguish between two basic mechanisms for iron uptake via siderophores : (a) the iron chelate is transported through the cytoplasmic membrane as an intact complex ; (b) removal of the iron from the siderophore is a prerequisite for transfer of the metal across the cellular membrane, whereas the ligand remains extracellular. To distinguish between the former and latter possibilities, the transport of ^{59}Fe-desferrioxamine B was compared with Fe-(^{14}C)desferrioxamine B and ^{67}Ga-desferrioxamine B. The uptake of the ^{14}C-labelled siderophore allows the close pursuit of the fate of the ligand during transport. Since Ga^{3+} cannot be reduced by reductases, it can be used as a probe for the uptake of the intact complex and/or the ability of the cells' surface to remove the metal by an exchange mechanism rather than reduction.

Results shown in Fig. 5 indicate that the uptake rates of Fe-(^{14}C) desferrioxamine B and ^{67}Ga-desferrioxamine B were 30 and 60 per cent, respectively, as compared to the ^{59}Fe-desferrioxamine B. The reduced uptake of the ^{14}C-labelled ligand suggests that most of the iron ($\sim$ 70 per cent) is removed from the complex prior to being transported into the cell. Nevertheless, a significant portion of the ligand ($\sim$ 30 per cent) can be transported across

the cellular membrane. The observation that ^{67}Ga uptake was 30 per cent higher than the ^{14}C-labelled ligand (Fig. 5) suggests that this metal can drag a higher proportion of the ligand into the cell than can iron. The latter conclusion is based on the assumption that reduction, rather than exchange, is the only extracellular mechanism for decomplexation of iron from ferrioxamine B in G. candidum. In S. pilosus ^{67}Ga-desferrioxamine B, as well as chromic-desferrioxamine B were transported at rates similar to ^{59}Fe-desferrioxamine B (Muller and Raymond 1984). This implies that decomplexation of the metal does not take place as in S. pilosus.

Evidence for reduction of Fe^{3+}-desferrioxamine B by the fungal cells is given in Table 2. Dipyridyl and ferrozine, two effective and specific chelators of Fe^{2+} inhibited iron uptake of ^{59}Fe-desferrioxamine B by 65 and 35 per cent, respectively at 6mM concentration. Dipyridyl, at identical concentration completely inhibited ferrichrome A uptake by Ustilago sphaerogena (Emery 1987), whereas ferrozine at 1mM caused >80 per cent inhibition of ferrioxamine B transport in Saccharomyces cerevisiae (Lesuisse et al. 1987). The former and latter siderophores have been proven to mediate iron transport by the so-called "taxi mechanism", where the ligand remains extracellular. The observation that only partial inhibition of ^{59}Fe-desferrioxamine B was obtained in the presence of dipyridyl or ferrozine (Table 2) further indicates that the 2 mechanisms exist in G. candidum for ferrioxamine B-mediated iron uptake and that they are not mutually exclusive. It is of interest to note that ^{59}Fe-desferrioxamine B is taken up intact by S. pilosus (Muller et al. 1984) completely decomplexed by reduction prior to transport by S. cerevisiae (Lesuisse et al. 1987) and both occur in G. candidum.

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References

Butler EE (1960). Pathogenicity and taxonomy of Geotrichum candidum. Phytopathology 50:665-672

Eckert JW (1978). Postharvest disease of citrus fruits. Outlook Agric 9:225-259

Emery T (1987). Reductive mechanisms of iron assimilation. in: Iron transport in microbes, plants and animals. Winkelmann G, van der Helm D, Neilands JB (eds) Weinheim, VCH Verlagsgesselschaft pp 317-336

Emmons CW, Binford CH, Utz JP (1970). Medical mycology. Lea & Febiger, Philadelphia

Ernst J, Winkelmann G (1974). Metabolic products of Microorganisms. Uptake of iorn by Neurospora crassa Arch Microbiol 100:271-282

Huscka H, Naegeli HU, Leuenberger-Ryf H, Keller-Schierlein W, Winkelmann G (1985). Evidence for a common siderophore transport system but different siderophore receptors in Neurospora crassa. J Bacteriol 162:715-721

Jalal MAF, Mocharla R, Barnes C, Hossain MB, Powell DR, Eng-Wilmot DL, Grayson SL, Benson BA, van der Helm D (1984). Extracellular siderophores from Aspergillus ochraceous. J Bacteriol 158:683-688

Lesuisse F, Raguzzi F, Crichton RR (1987). Iron uptake by the yeast Saccharomyces cerevsiae : involvement of a reduction step. J Gen Microbiol 133:3229-3236

Mor H, Paternak M, Barash I (1988). Uptake of iron by Geotrichum candidum, a non-siderophore producer. Bio Metals 1:99-105

Muller G, Raymond KN (1984). Specificity and mechanism of ferrioxamine B-mediated iron transport in Streptomyces pilosus. J Bacteriol 160:304-312

Muller G, Matzanke BF, Raymond KN (1984). Iron transport in Streptomyces pilosus mediated by ferrichrome siderophores, rhodotorulic acid and emantio-rhodotorulic acid. J Bacteriol 160:313-318

Table 1. Inhibition of ^{59}Fe-desferrioxamine-B-mediated iron uptake in the presence of various siderophores

Siderophore [a]	Inhibition (per cent)
Ferrioxamine B	40
Ferrichrome	68
Ferrichrysin	71
Ferrichrome A	28
Fusigen	8
Rhodotorulic acid	7
$FeCl_3$	6

[a] The concentration of ^{59}Fe-desferrioxamine B and each of the added siderophores was 10 μ M. Uptake conditions are as described in "Materials and Methods".

Table 2. Effect of ferrous-specific chelates on ^{59}Fe-desferrioxamine-B-mediated iron uptake

Chelate added [a]	^{59}Fe uptake (per cent)
-	100
2,2'dipyridyl	65
Ferrozine	35

[a] Chelate concentration was 6mM

Legends to Figures

Fig. 1. Comparative uptake of ^{59}Fe-siderophores by G. candidum. Uptake conditions are as described in "Materials and Methods". Conditions for ^{59}Fe-Cl_3 uptake were as described elsewhere (Mor et al. 1988). A-$FeCl_3$ B-Ferrichrysin C- Ferrichrome D-Ferrichrome A E-Ferrirubin F-Ferrirhodin G-Coprogen H-Coprogen B I-Rhodotorulic acid J-Dimerum acid K-Fusigen L-Triacetyl Fusarinine M-Ferrioxamine B N-Aerobactin.

Fig. 2. Lineweaver-Burk plot of ^{59}Fe-desferrichrome and ^{59}Fe-desferrioxamine-B-mediated iron uptake. Transport conditions are as in Fig. 1.

Fig. 3. The Dixon plot of ^{59}Fe-desferrioxamine-B-mediated iron uptake with ferrichrome as the inhibitor. Uptake was measured at two different concentrations of ^{59}Fe-desferrioxamine B (i.e. 1 and 4 μM). Each line was obtained by plotting 1/v of ^{59}Fe-desferrioxamine B versus concentration of ferrichrome. Transport conditions are as in Fig. 1.

Fig. 4. Iron uptake mediated by ^{59}Fe-ferrichrome, ^{59}Fe-rhodotorulic acid and ^{59}Fe-fusigen in the presence of siderophores from other structural classes. The radioactive and unlabelled siderophores were given in equimolar concenttrations (6 μ M). Transport conditions are as in Fig.1.

Fig. 5. Uptake of ^{59}Fe-desferrioxamine B, Fe-(^{14}C)desferrioxamine B and ^{67}Ga-desferrioxamine B. The metal complexes were added at 4uM concentration. Conditions for transport and preparation of the labelled siderophores are described in "Materials and Methods".

Fig 1

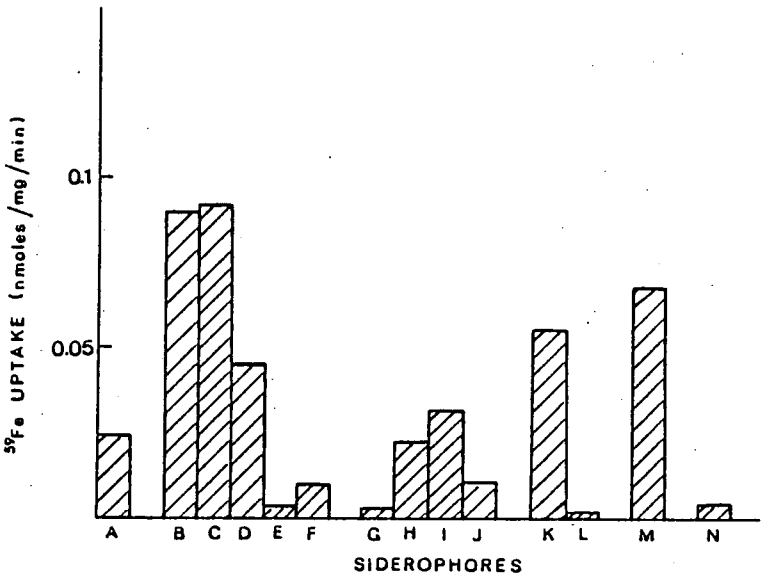


Fig 2

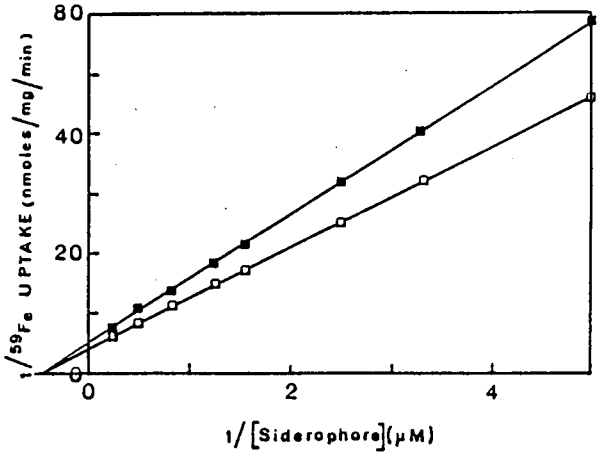


Fig 3

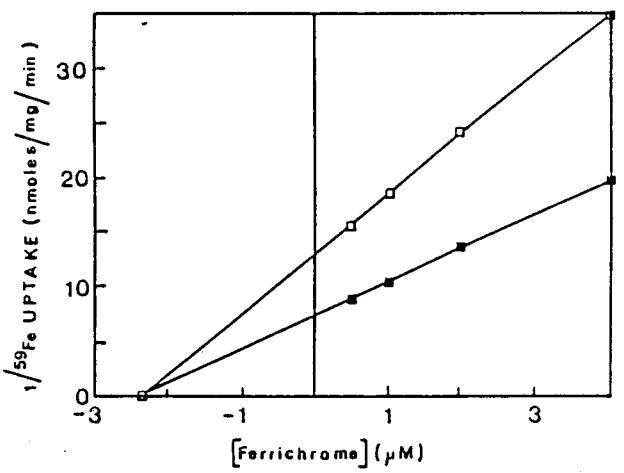


Fig 4

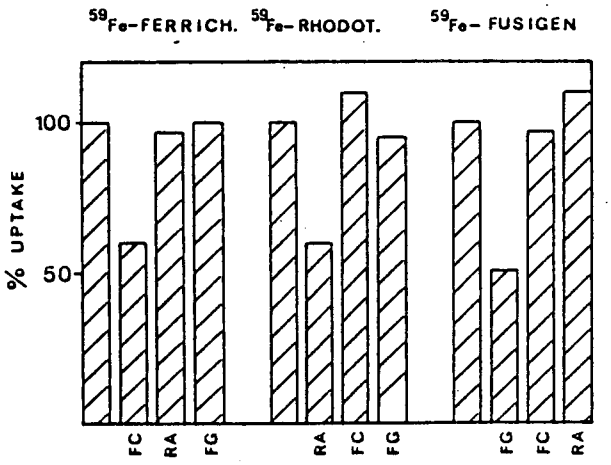
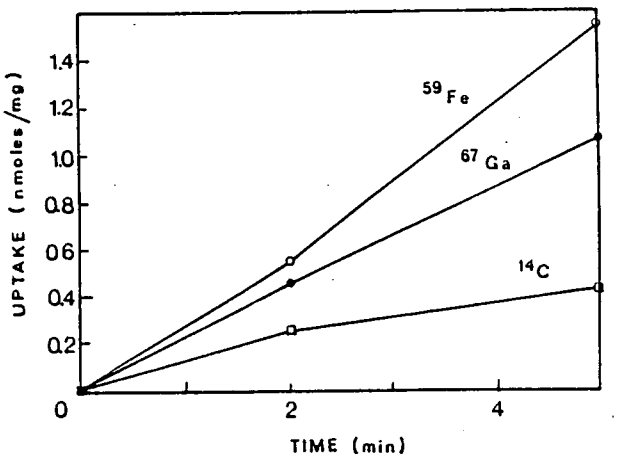

59Fe-FERRICH.
59Fe-RHODOT.
59Fe- FUSIGEN
% UPTAKE
100
50
FC
RA
FG
RA
FC
FG
FG
FC
RA

Fig 5


UPTAKE (nmoles/mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
2
4
TIME (min)
59Fe
67Ga
14C

Characterization of hydroxamate siderophores and siderophore-mediated iron uptake in Gaeumannomyces graminis var. tritici

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(ABSTRACT)

Under iron-deficient conditions, Gaeumannomyces graminis var. tritici produces dimerum acid and coprogen B as well as traces of two other hydroxamate siderophores. An inverse correlation between the production of coprogen B and dimerum acid with a rise in pH was established. Although secretion of siderophores was completely repressed in the presence of 3 uM iron, a low iron concentration (0.06 uM) was necessary for optimal production.

Siderophore-mediated iron uptake for dimerum acid and coprogen B exhibited active transport and optimum at pH 4.5-5 and 30°C. Michaelis kinetics with apparent Kms of 6.8 and 11 uM and Vmax of 0.26 and 0.54 nmoles.mg^{-1}.min^{-1} was obtained for dimerum acid and coprogen B respectively. Ggt could utilize iron effectively from foreign siderophores produced by other fungi. However, utilization of iron from pseudobactin, ferrioxamine B, EDTA and EDDHA was ineffective. Competition studies between dimerum acid and various siderophores suggest a common transport system of the latter siderophore with coprogen B, rhodotorulic acid and ferrichrome but not with fusigen and triacetylfusigen. Wheat seedlings were capable of utilzing iron efficiently from dimerum acid and coprogen B. The possible influence of the pathogens siderophores on its survival and virulence is discussed.

Abreviations used in text: Ggt, Gaeumannomyces graminis var. tritici

INTRODUCTION

The acquisition of iron in fungi and other microorganisms under iron-deficient conditions is generally mediated by siderophores [9]. Siderophores are low-molecular weight ferric chelating agents which supply the metal to the cell by binding to specific receptors in the cell membrane. An array of hydroxamate, phenolate and miscellaneous siderophores have been isolated from a relatively small number of fungi and bacteria [9,17] including some plant pathogens [2,14,17].

Take-all disease of wheat (Triticum aestivum L.) caused by Gaeumannomyces graminis (Sacc) von Arx and Olivier var. tritici Walker (Ggt) is a destructive disease in many wheat growing areas throughout the world. Microbial competition for iron is known to be an important mechanism of antagonism in the rhizosphere [17,21]. Fluorescent pseudomonads and their siderophores appear to be involved in the natural microbial suppression of Ggt [3,11,23,25]. The suppressive ability of some but not all strains of Pseudomonas fluorescence and P. putida was reduced or completely eliminated by the addition of FeEDTA to the soil [3,23] suggesting that these strains suppressed take all by siderophore production. Iron may also exert a direct effect on the sensitivity of wheat plants to Ggt. Thus application of Fe^{+3} or Mn^{+2} reduced take-all severity when supplied through the roots [19].

Since competition for iron is at least partially responsible for suppression of Ggt in the rhizosphere by pseudomonads, the relative ability of Ggt strains to sequester iron from the vicinity may affect their survival and pathogenicity. Furthermore, the capacity of the pathogen to reduce the iron status of the host via siderophores could render the wheat more susceptible to take all as reported with other diseases [2,7]. The present study was therefore

undertaken to charcterize the siderophore production and the siderophore-mediated iron uptake system in Ggt.

MATERIALS AND METHODS

Isolates and Cultures

All isolates of Ggt were obtained from diseased wheat plants collected in Israel. The isolates were maintained on half strength potato dextrose agar (PDA) or glucose asparagine agar (GAA) [13]. For siderophore production the fungus was cultured in 1-l Roux flasks containing 100 ml of a sucrose-glutamate broth composed of 20 g of sucrose, 9 g of L-glutamate, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of KCl, 5 mg of pyridoxin and 5 mg thiamine HCl in 1 liter of double distilled water (pH 7). Altenatively, the fungus was cultured in a glucose-nitrate broth containing 25 g of glucose, 1.5 g of NH_4NO_3, 1 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.5 g of KCl, 0.25 g of $MgSO_4$, 2 mg of thiamine HCl, 3.3 mg of $CuSO_4.5H_2O$, 4.3 mg of $MnCl_2.4H_2O$ and 0.7 mg of $ZnSO_4.7H_2O$ in liter double distilled water (p 5.5). The Roux flasks were inoculated with discs obtained from GAA cultures of Ggt and incubated at 26 ^{o}C for 21 days under fluorescence illumination.

Extraction and purification of siderophores

At the end of the growth period the mycelia were removed by filtration through Whatman no. 1 paper and culture filtrates (1500 ml) were collected. The filtrate was evaporated under vacuum at 35 ^{o}C to 1/10 of the original volume and $FeCl_3$ was added to convert siderophores to iron chelates. The reddish solution was saturated with ammonium sulfate and left overnight at 4 ^{o}C. The filtrate was then centriguged at 10.000 g for 10 min and the clear supernatiant was extracted with benzoyl alcohol according to the method of Nielands [16] and transferred to aqueous solution as described by Manulis et al. [14]. The siderophores were lyophilized and stored until used.

Prior to purification, the siderophores were dissolved in 50 ml methanol and the precipitate was removed by centrifugation at 10,000 g for 10 min. The supernatant was taken to dryness in vacuo. The siderophoes were dissolved in 5 ml distilled water and purified by gel filtration through a Bio-Rad column of Bio-Gel P-2 (190 x 2 cm) following by chromatography on Whatman cation exchange column CM-52 (16 x 2.6 cm) and a Pharmacia Sephadex LH-20 column (25 x 2.5cm) according to Manulis et al. [14].

Analytical procedures

The amount of total siderophores was measured according to Subramanian et al. [20]. Purity of the compounds was established by high voltage electrophoresis [6] and silica gel thin layer chromatography (TLC) with various solvents [14]. The hydroxamate siderophores were detected by spraying with Folin-Ciocaltaeus reagent [20].

For structural determination the siderophores were deferrated with 8-hydroxyquinoline [22]. ^{1}HNMR and ^{13}CNMR spectra were recorded on a Bruker WM-360 spectrometer. All chemical shifts were reported with respect to TMS(0).

^{55}Fe uptake assay conditions

Twelve-day-old mycelium was removed from the sucrose-glutamate growth medium by suction filtration with an Ederol filter paper (no. 15) and washed several times with deionized water. The mycelium was then suspended (1.4 g fr. wt. per 100 ml) in 0.1 M acetate buffer, pH 4.5 for 45 sec in a Waring Blendor (low speed). The homogenous suspension of the fungal cells obtained by the latter procedure contained negligible amounts of broken cells which did not interfer with the uptake assay.

The standard procedure for iron uptake was carried out in 100 ml flasks containing 10 ml of cell suspension as previously described [14]. The reaction was started by a rapid addition of ^{55}Fe-chelated siderophore solution. Samples

of 3 ml were removed at various time intervals and immediately filtered through GF/C Whatman filter paper (2.5 cm diameter) and rinsed x3 times with 5 ml 50 mM EDTA (ethylene diaminetetra-acetic acid). The filters with the mycelial pads were dried for 2 hours at 68 ^{o}C and counted with Permablend scintilation liquid in a Packard 1500 Tri-carb scintilation counter. Counts were corrected for the reading obtained at zero time. Results are an average of at least 3 independent experiments.

The ^{55}Fe-labelled siderophores were prepared by addition of $^{55}FeCl_3$ in 0.1 M HCl (1.72 mCi mg^{-1} $FeCl_3$) to an approximately 50% excess of ligand in water to give a stock solution of 1.3 mM in complex. The molar ratio between the ferric iron and the ligand was 2:3 for dimerum acid and 1:1 for coprogen B.

Utiliztation of iron by wheat seedlings were carried out essentially as described by Reid et al. [18]. Surface sterilized seeds of T. aestivum cv. 'Barkai' were germinated in vermiculite and then transferred to aerated hydroponic culture in 500 ml iron-free, 0.5 x Hoagland's nutrient solution at pH 7 buffered with $CaCO_3$. Plants were grown under a 14 h photoperiod at 26 ^{o}C. After 12 days, when the plants displayed visible signs of iron chlorosis, they were transferred into containers with 20 ml of fresh nutrient solution containing 5.3 x 10^{-3} uM ^{55}Fe (1 mCi/0.58 mg $FeCl_3$; 5 uCi/container) as $^{55}FeCl_3$, ^{55}Fe-EDDHA, ^{55}Fe-desferrioxamine B, ^{55}Fe-coprogen B and ^{55}Fe-dimerum acid. Each treatmeant contained 18 plants. Over a 6 day period plants were harvested every 2 days according to Reid et al. [18]. The ^{55}Fe content was determined with Packard 1500 TRI-CARB using Liquiscint scintilation liquid.

Chemicals

Rhodotorulic acid, fusigen, triacetyl fusarinine, coprogen B, dimerum acid, ferrichrome and aerobactin were kindly provided by Professors D. van der Helm, University of Oklahoma, U.S.A. and G. Winkelmann, University of Tubingen, West

Germany. Pseudobactin and desferrioxamine B were kindly provided by Drs. J.S. Buyer, Beltsville, U.S.A. and B Hemming, Monsanto Co. St. Louis, U.S.A. respectively. Isotopic ferric chloride was purchased from the Radiochemical Centre, Amersham.

RESULTS

Isolation and identification of siderophores

Siderophoes were extracted from filtrates of 21-day-old cultures (Ggt isolate N) grown on sucrose-glutamate medium as described in Materials and Methods. Following the methanol precipitation step they were chromatographed on Bio-Gel P-2 column (Fig. 1). The elution profile of the siderophores shows three major components designated as A, B and C according to their migration towards the cathode on paper electrophoresis (Fig. 1). Siderophore C was an highly cationic compound whereas B and A were only slightly cationic in pyridine acetate buffer, pH 5.2. Further purification was achieved by chromatography on a CM-52 column which exhibited differential adsorption towards the 3 siderophores. Thus siderophore A was eluted with water whereas siderophores B and C remained adsorbed on the column and separated by a linear gradient elution with KCl (0-0.5 M). Each siderophore was finally brought to homogenity by employing Sephadex LH-20 column chromagraphy. It should however be emphasized that 2 minor peaks which were eluted from the bio gel P-2 column and may represent additional siderophores were not further investigated.

Initial identificaton of siderophore B as dimerum acid and C as coprogen B (Fig. 2) was carried out by co-chromatography with authentic samples in silica gel TLC using 4 different solvents as previously described [14]. Identical migration rates of siderophore B with dimerum acid and siderophore C with coprogen B were also demonstrated with high voltage electrophoresis.

The ^{1}HNMR obtained for siderophore B (360 MHz, D_2O): 4.03 brs (2H), 1.67 brs (4H), 1.59 brm (4H), 3.55 brs(4H), 6.05 brs (1H), 5.75 brs (1H), 1.82 brs (6H), 2.24 brs (4H), 3.61 brs (4H). The ^{13}CNMR for siderphore B (360 MHz, D_2O):172.5, 172.0, 169.4, 153.9, 150.5, 120.1 (br), 119.1, 66.1, 61.8 t, 56.7 t, 53.6, 49.9, 44.7, 43.5, 33.2, 24.6, 24.2, 20.4, 17.1 ppm. The ^{1}HNMR obtained for siderophore C (360 MH_z, D_2O) 6.08 brs (3H), 4.31 brm (2H), 4.08 brs (2H), 3.65 brs (6H), 3.58 brs (6H), 2.45 brs (2H), 2.29 brs (4H), 2.21 brs (2H), 1.86 brs (9H:), 1.171 brs (6H), 1.60 brm (6H:). ^{13}CNMR for siderophore C 360 MHz, d_6-DMSO):172.2s, 169.8s, 168.1s, 117.4d, 116.5d, 64.8, 62.5t, 59.4t, 54.0d, 47.1d, 43.8t, 41.2t, 30.6t, 28.2t, 23.3t, 22.4t, 22.3, 20.49, 1;8.4q, 18.2q ppm. The spectral data obtained for each compound were similar if not identical with values reported for dimerum acid [5,8] and coprogen B ([2,26] respectively.

Siderophore A showed identical ^{1}HNMR and ^{13}CNMR to dimerum acid (DA). Its ^{13}CNMR (d_6-DMSO):167.9s, 166.6d, 150.6s, 116.5d, 58.2t, 53.8d, 46.7t, 40.7t, 30.4t, 22.2t, 18.3 ppm was also identical with the ^{13}CNMR reported for dimerum acid by Jalal et al. [10]. A FAB-mass spectra: (MS) of siderophore A exhibited a base peak (100%) at m/z 538.2 corresponding to a fragment that could be $FeDA^{+}$ (M.W 538.17): FAB-MS of $Fe2(DA)_3$, the common form of ferric-dimerum acid (i.e. siderophore B at the present work) is expected to give m/z=1582.6 ($FeDA_3Na^{+}$, with 1 C^{13} isotope); m/z=1614.8$(Fe_3DA_3+2e^{-})^{+}$. Based on the NMR spectra and FAB-MS it may be assumed that siderophore A is $FeDA^{+}$. Further support to the identical ligands in siderphores A and B is the fact that the former was spontaneously converted to the latter siderophore namely dimerum acid during long storage.

Production of siderophores

Siderophores production was detected during the logarithmic growth phase and reached a maxima after approximately 18 days at the stationary phase (Fig. 3).

L-glutamatae was found as the best nitrogen source for biosynthesis of siderophores and caused 7 fold increase in their secretion as compared to asparagine or nitrate. All 15 isolates of Ggt, which were obtained from various locations in Israel, produced coprogen B and dimerum acid in significant amounts.

The secretion of siderophores by Ggt was dependent on iron deficiency and was completely repressed in the presence of 0.5 ppm (3 μM) iron (Fig. 4). Nevertheless traces of iron (0.01 ppm) were necessary for maximal siderophores production. The ratio between coprogen B and dimerum acid was affected by the pH of the media (Fig. 5). Elevation in the pH caused a decrease in coprogen B with a concomitant increase in dimerum acid. Therefore dimerum acid was the predominant siderophore in sucrose-glutamate medium (pH 7) whereas coprogen B remained the major siderophore in glucose-nitrate medium (pH 5.5).

Characterization of siderophore-mediated iron uptake by Ggt.

Uptake of ^{55}Fe-labelled coprogen B and dimerum acid was found to be linear at least within 1 h. The two siderophores exhibited optimal transport at pH 4.5-5.0 and 30 ^{o}C. The siderophore-mediated iron transport in Ggt was energy dependent as implied from inhibition by 1 mM sodium azide (40%) and m-chlorophenylhydrazone (60%). Kinetic studies of initial uptake versus siderophore concentration exhibited Michaelis kinetics with apparent Kms of 6.8 and 11 uM and Vmax of 0.26 and 0.54 nmoles mg^{-1} min^{-1} for dimerum acid and coprogen B, respectively (Fig. 6).

Results shown in Table 1 indicate that in addition to its own siderophores, Ggt is capable of utilizing iron effectively from siderophores of exogenous origin such as rhodotrulic acid, ferrichrome, fusigen and triacetyl fusigen. The foreign siderophores could mediate iron uptake as well or better than the natural siderophores. However, ferrioxamine B and pseudobactin, both

siderophores of bacterial origin, and the synthetic chelates EDTA and EDDHA, were ineffective (Table 1).

The specificity of the dimerum acid transport system was examined by competition studies. It can be observed (Table 2) that ^{55}Fe-dimerum acid uptake is significantly inhibited not only by equimolar concentration of rhodotorulic acid (40%) and coprogen B (30%), which are structurally related to dimerum acid, but also by ferrichrome (27%), which represent a different structural class of siderophores [9]. Only a weak inhibition (10-17%) could be detected with representative of the fusarinine class namely triacetyl fusarinine and fusigen.

It is possible that the siderophores secreted by Ggt be involved in iron deprivation of the host plant. This could particularly be true if they remain specific to the pathogen and not to the host. Therefore the ability of wheat seedlings to utilize iron from the natural siderophores of Ggt was investigated. Results illustrated in Fig. 7 indicate that wheat seedlings can mobilize iron from ^{55}Fe-dimerum acid and ^{55}Fe-coprogen B at a rate equivalent to $^{55}FeCl_3$, whereas low iron uptake could be detected from ferrioxamine B or FeEDDHA.

DISCUSSION

Coprogen B and dimerum acid are being produced by a variety of phytopathogenic fungi, including Verticillium dahliae [2,8], Stemphylium botryosum [14], Fusarium sp. [5] and now Ggt. Siderophores production by Ggt was highly enhanced by the presence of L-glutamate, a well known precursor of L-ornithine, which serves as an essential component of the hydroxamate siderophores [9]. The repression of siderophores by 3 uM iron (Fig. 4) is similar to iron concentration reported for other fungi [2,14]. However, a low iron concentration (0.06 uM) was mandatory for optimal secretion of siderophores as reported elsewhere [1].

The inverse correlation between the production of coprogen B and dimerum acid with a rise in pH (Fig. 5) strongly resembles the kinetics of siderophore secretion in V. dahliae [2]. This could imply that dimerum acid is formed as a breakdown product of coprogen B. The latter phenomenon may occur at high pH by enzymatic cleavage of the ester bond which links a fusarinine unit to dimerum acid to form coprogen B (Fig. 2). It should also be pointed out that although coprogen B and dimerum acid were the major siderophores produced by Ggt, traces of two additional hydroxamate siderophores could be detected in culture filtrates.

The high-affinity, siderophore-mediated iron assimilation characterized in Ggt has been detected in most [9,24] but not all [15] fungi which were so far investigated. Like other fungi [14,24], Ggt displays variable capacity to utilize siderophores of exogenous origin (Table 1). The fact that coprogen B, rhodotorulic acid and ferrichrome exerted a significant inhibition of ^{55}Fe-dimerum acid uptake (Table 2) could imply that these compounds share a common transport system with dimerum acid. Coprogen B and rhodotorulic acid are structurally related to dimerum acid and constitute the coprogen class of siderophoes, whereas ferrichrome represents an entirely different class of hydroxamate siderophores [9]. Similar to Ggt, the former and latter classes of siderophores were found to share a common transport system in Neurospora crassa [24]. Although representatives of the fusarinines class (e.g. triacetylfusarinine and fusigen) can effectively mediate iron transport in Ggt (Table 1), they exhibited only a low and insiginificant inhibition of ^{55}Fe-dimerum acid (Table 2) and may possess a separate transport system.

Take-all decline results from microbial suppression of the pathogen in the soil [3]. Kloepper et al. [11] demonstrated that conducive soil can be converted into suppressive soil by adding Pseudomonas strain B-10 or its siderophore

pseudobactin. Conversely, the suppressiveness of the soil was eliminated by adding exogenous iron in the form of FeEDDHA to the soil and thus shutting down siderophore production. This implies that deprivation of the pathogen of iron may be a significant mechanism for biocontrol of Ggt by some, but not all strains of pseudomonads [3,23]. In the present study we have demonstrated that Ggt does have the potential for an efficient iron uptake via hydroxamate siderophores under iron deficiency. Factors which might determine which species predominate in an iron-limiting environment include [12]: a) the relative equilibrium binding constants for ferric iron, b) the relative kinetics of ferric iron-complex formation, c) the amount of siderophore production and d) the efficiency of iron assimilation. Variations in these factors among pseudomonad strains on one hand and among Ggt strains on the other hand could determine the contribution of bacterial siderophores to Ggt biocontrol in the rhizosphere. It is noteworthy that Ggt cannot significantly utilize iron from pseudobactin (Table 1), a major siderophore released by fluorescent pseudomonads [12]. The latter observation could explain why pseudobactin withdraw iron efficiently from Ggt, inspite of its own siderophores systems.

It has been demonstrated with oat that iron uptake rates from hydroxamate siderophores are sufficient to supply iron to plants in physiologically relevant quantities [4,18]. Results presented (Fig. 7) suggest that a similar phenomenon may occur with wheat. The ability of wheat seedlings to utilize iron from natural siderophores of Ggt eliminates the possiblity that they may sequester iron from the plant prior or during infection. Although iron deficient wheat is probably more susceptible to take-all [19], it is not at all clear whether the iron status of the plant is affected by the pathogen.

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REFERENCES

1. ATKIN, C.L., NEILANDS, J.B. & PHAFF, H.J. (1970). Rhodotorulic acid from species of Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus and Sporobolomyces and new alanine containing ferrichrome from Cryptococcus melibiosum. Journal of Bacteriology 103, 722-733.

2. BARASAH, I., ZION, R., KRIKUN, J. & NACHMIAS, A. (1988). Effect of iron status on Verticillium wilt disease and on in vitro production of siderophores by Verticillium dahliae. Journal of Plant Nutriton 11, 893-905.

3. COOK, R.J. & WELLER, D.M. (1987). Management of take-all in consectutive crops of wheat or barley. in Innovative approaches to plant disease control, Ed. by I. Chet, pp. 41-76. John Wiley, New York.

4. CROWLEY, D.E., REID, CP.P., SZANISZLO, P.J. (1988). Utlization of microbial siderophores in iron acquisition by oat. Plant Physiology 87, 680-685.

5. DIEKMANN, H. (1970). Stoffwechselprodukte von Mikroorganismen. Vorkommen und strukturen von voprogen B und dimereumsaure. Archives Mikrobiology 73, 65-76.

6. EMRY, T. (1965). Isolation, characterization and properties of fusarinine A. -hydroxamic acid derivative of ornitin. Biochemistry 4, 1410-1417.

7. GUERRA, D. & ANDERSON, A.J. (1985). The effect of iron and boron amendments on infection of bean by Fusarium solani. Phytopathology 75, 989-991.

8. HARRINGTON, G.L. & NIELANDS, J.B. (1982). Isolation and characterization of dimerum acid from Verticillium dahliae. Journal of Plant Nutriton 5, 675,-682.

9. HIDER, R.C. (1984). Siderophore mediated Absorption of iron. Structure and Bonding 58, 25-87.

10. JALAL, M.A.F., LOVE, S.K. & VAN DER HELM, D. (1986). Siderophore mediated iron (iii) uptake in Gliocladium virens. I. Properties of cis-fusarinine, transfusarinine, dimerum acid, and their ferric complexes. Journal of Inorganic Biochemistry 28, 417-430.

11. KLOEPPER, J.W., LEONG, J., TEINTZE, M. & SCHROTH, M.N. (1980). Pseudomonas siderophores: A mechanism explaining disease suppressive soils. Current Microbiology 4, 317-320.

12. LEONG, J. (1986). Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Annual Review of Phytopathology 24, 187-209.

13. LILY, V.G. & BURNETT, H.L. (1951). Physiology of fungi. McGraw Hill book Co., New York, 427 pp.

14. MANULIS, S., KASHMAN, Y. & BARASH, I. (1987). Identification of siderophores and siderophore-mediated uptake of iron in Stemphylium botryosum. Phytochemistry 26, 1317-1320.

15. MOR, H. & BARASH, I. (1988). Uptake of iron by Geotrichum candidum, a non-siderophore producer. Biology of Metals 1, 99-105.

16. NEILANDS, J.B. (1952). A crystaline organo-iron pigment from a fungus. Ustilago sphaerogena. Journal Amearican Chemical Society 74, 4846-4847.

17. NEILANDS, J.B. & LEONG, S.A. (1986). Siderophores in relation to plant growth and disease. Annual Review of Plant Physiology 37, 187-208.

18. REID, C.P.P., CROWLEY, D.E., KIM, H.J. POWELL, PE.E. & SZANISZLO, P.J. (1984). Utilization of iron by oat when supplied as ferrtaed synthetic chelate or as ferated hydroxamate siderophore. Journal of Plant Nutrition 7, 437-447.

19. REIS, E.M., COOK, R.J. & McNEAL, B.L. (1982). Effect of mineral nutriton on take-all of wheat. Phytopathology 72, 224-229.

20. SUBRAMANIAN, K.N., PADMANBAN, G. & SARMA, P.S. (1965). Folin-Ciocalteus reagent for the estimation of siderophores. Analytical Biochemistry 12, 106-112.

21. SWINBURNE, T.R. (1986). Irone, siderophores and plant diseases. Plenum Press New York.

22. WEIBE, C. & WINKELMANN, G. (1975). Kientic studies on the specificty of chelate iron uptake in Aspergillus. Journal of Bacteriology 123, 837-842.

23. WELLER, D.M., HOWIE, W.J. & COOK, R.J. (1985). Relationship of in vitro inhibition of Gaeumannomyces graminis var. tritici and in vivo suppression of take-all by fluorescent pseudomonads. Phytopathology 75, 1301.

24. WINKLEMANN, G. & HASCHKA, H.G. (1987). Molecualr recognition and transport of siderophores in fungi. In: Iron transport in microbes plants and animals, Ed. by G. Winkelmann, D. van der Helm & J.B. Neilands pp. 317-336 VCH Vearlagasgesellschaft, Weinheim.

25. WONG, P.T.W. & BAKER, R. (1984). Suppression of wheat take-all and Ophiobolus patch by fluorescent pseudomonas from Fusarium-suppressive soil. Soil Biology & Biochemistry 16, 397-403.

26. ZAHNER, H., KELLER, SCHIERLEIN, W., HUTTER, R., HESS-LEISINGER, K. & DEER, A. (1963). Stoffwechsel-produkte von microorganismen. archives mikrobiology 45, 119-135.

Table 1

Relative uptake of $^{55}Fe^{+3}$ via various siderophores in Gaeumannomyces graminis var. tritici

Siderophore[a]	Relative uptake (%)
Coprogen B	100
Dimerum acid	129
Rhodotorulic acid	124
Ferrichrome	98
Fusigen	200
Triacetyl fusigen	130
Ferrioxamine B	11
Pseudobactin	16
EDTA	0
EDDHA	0

[a]The final iron concentration was 3.4 uM. Uptake conditions as described in "Materials and Methods".

Table 2

Competition of various siderophores with ^{55}Fe-dimerum acid

Siderophore[a]	Inhibition %
Dimerum acid	42
Coprogen B	30
Rhodotorulic acid	40
Ferrichrome	27
Triacetyl fusigen	17
Fusigen	10

[a]Final iron concentration was 0.5 uM. Siderophores were added at equimolar aconcentration. Uptake conditions as in Table 1.

LEGENDS TO FIGURES

Fig. 1. Chromatography of siderophores on Bio-Gel p-2 column and their electrophoretic separation. Siderophores (20 mg) applied to the column were obtained by benzoyl alcohol extraction. The column was eluted with double distilled water and 5 ml fractions were collected. The siderophores were detected by absorbeance at 440 nm. Paper electrophoresis was performed in acetic acid pyridine water (14:10:930 v/v) pH 5.2. Further details are described in "Materials and Methods".

Fig. 2. Chemical structrues of coprogen B and dimerum acid.

Fig. 3. Production of siderophores as a function of growth. Growth was taken place in iron-deficient sucrose-glutamate medium. Siderophores (▲), Growth (•) and pH (▪).

Fig. 4. Production of siderophores as a function of iron concentration. Growth was performed in sucrose-glutamate medium. Iron concentration was determined after 21 days.

Fig. 5. Production of individual siderophores as a function of pH. The fungus was grown in glucose nitrate broth with 0.1M acetate buffer in pHs 4-5 and 0.1 phosphate buffer in pHs 6-7. Cop-coprogen B DA=dimerum acid.

Fig. 6. Lineweaver-Burk plots of (-X-) ^{55}Fe-coprogen B - and (□) ^{55}Fe-dimerum acid-mediated iron uptake. Transport conditions are described in "Materials and Methods".

Fig. 7. Iron uptake by wheat seedlings mediated by EDDHA and siderophores. Twelve-day-old seedlings were incubated in nutrient solution at pH 7 with 5.5×10^{-3}M ^{55}Fe (1 mCi/0.58 mg $FeCl_3$. Experimental details are described in "Materials and Methods". DA=dimerum acid, Cop.b=coprogen B, DES=ferrioxamine B.

Fig. 1

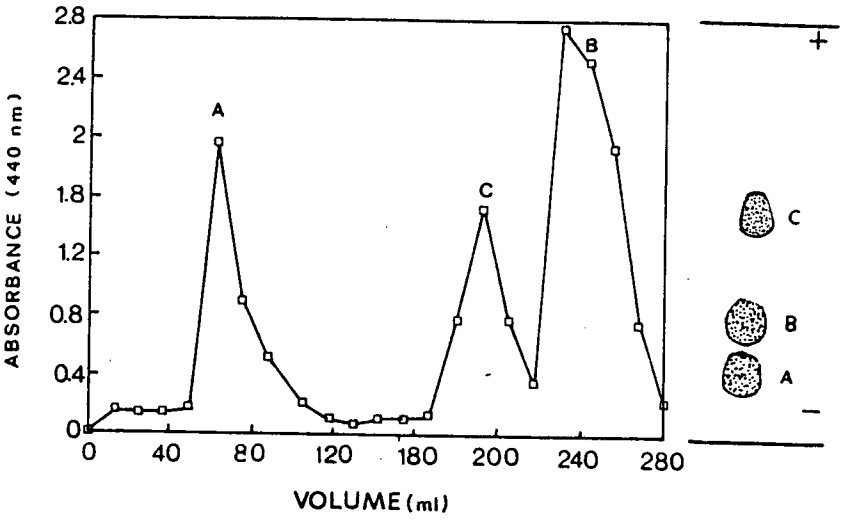
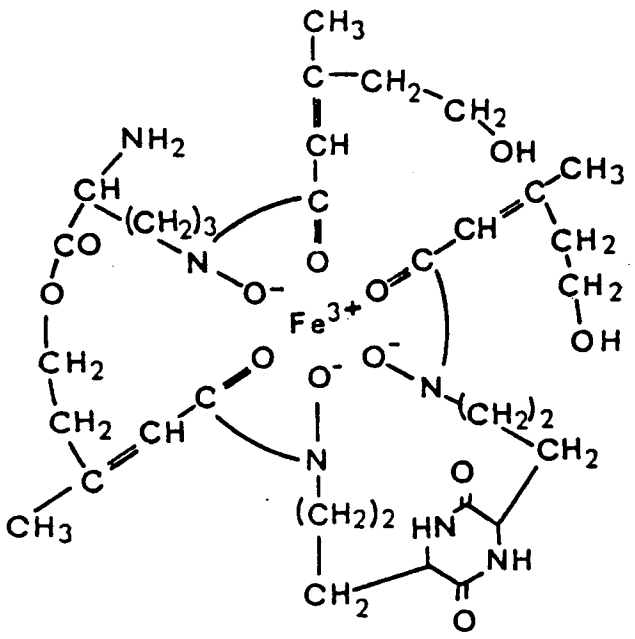
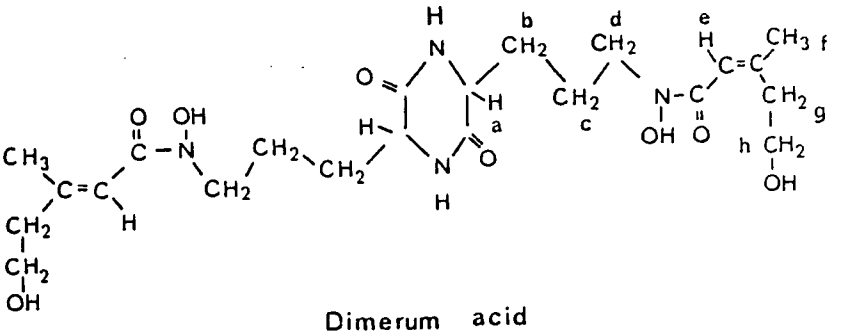


Fig. 2



COPROGEN B



Dimerum acid

Fig. 3

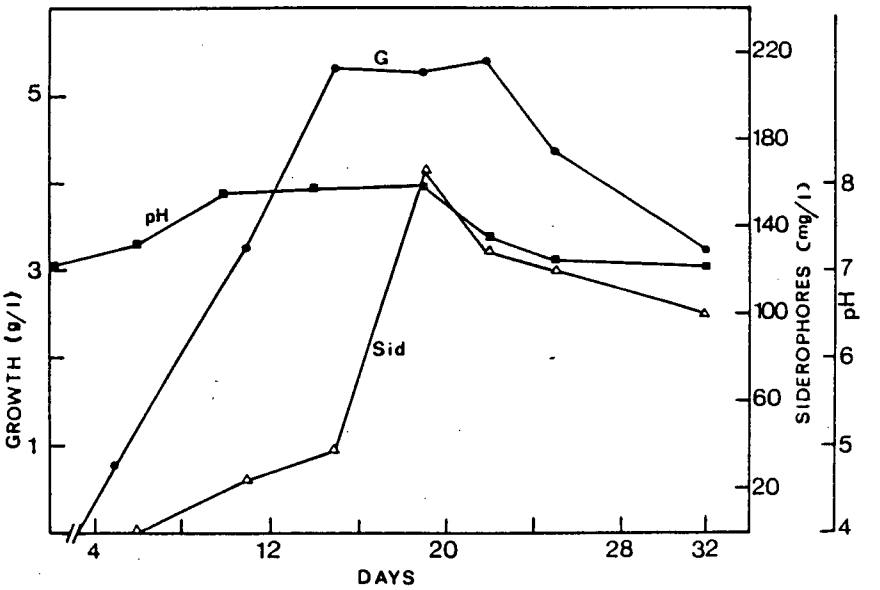


Fig. 4

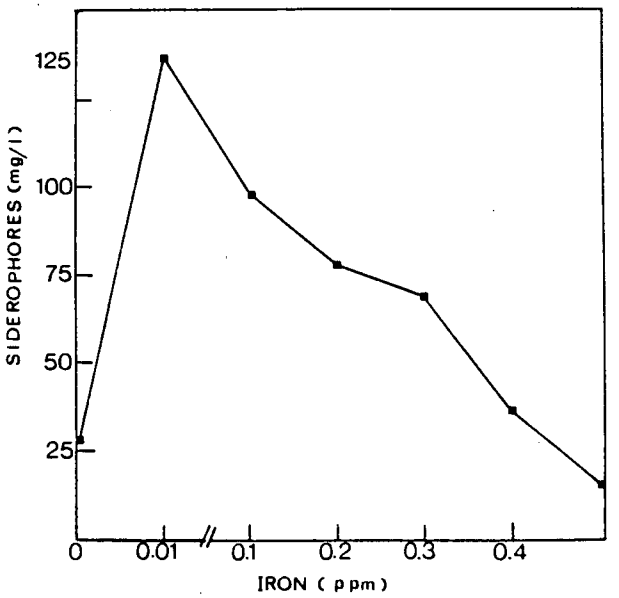


Fig 5

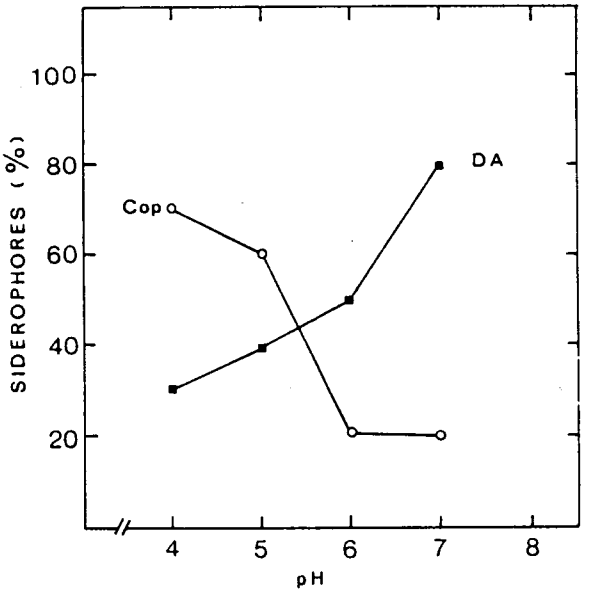


Fig. 6

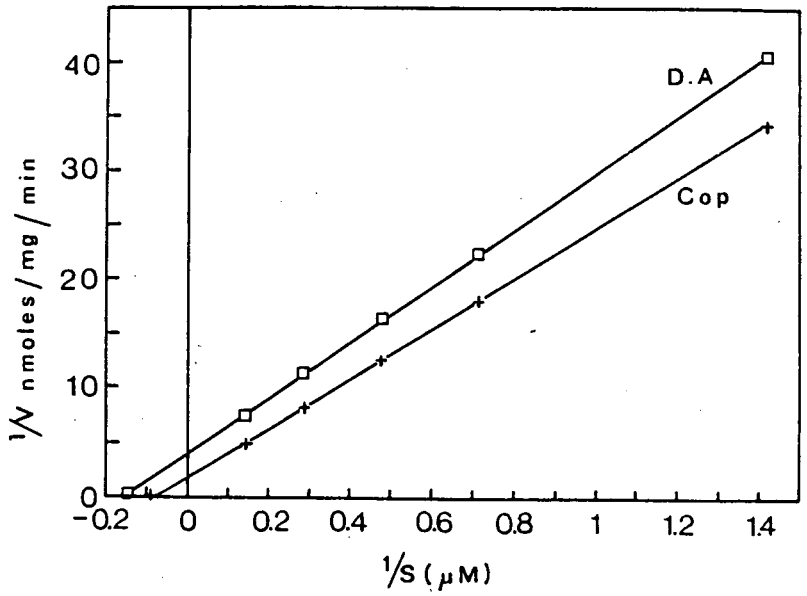
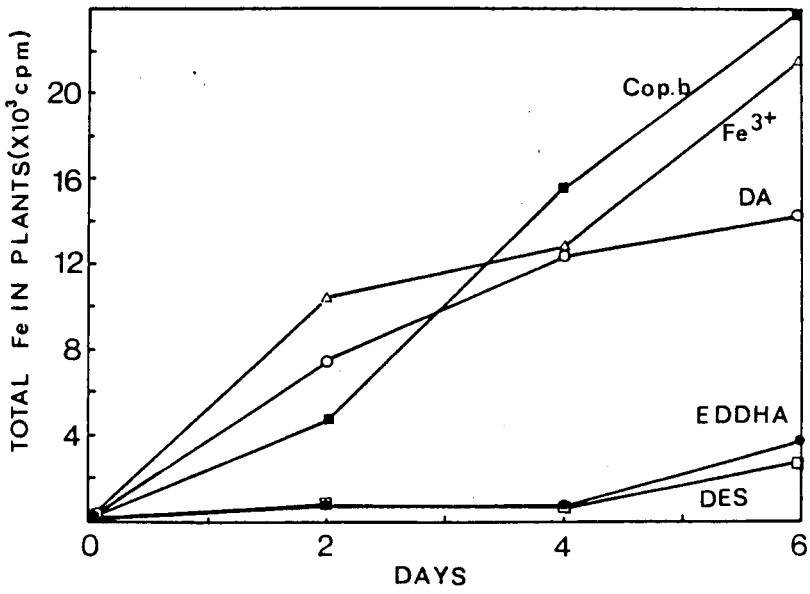

40
30
20
10
0
1/V nmoles / mg / min
D.A
Cop
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1/S (μM)

Fig. 7


TOTAL Fe IN PLANTS(X10³cpm)
20
16
12
8
4
Cop.b
Fe3+
DA
EDDHA
DES
0
2
4
6
DAYS

D. DESCRIPTION OF COOPERATION

Cooperation between the labs of Dr. Barash at TAU and Drs. Olsen and Stobel at MSU was achieved by visits of Dr. Barash (1987, 1989) to MSU and frequent bitnet communication. Dr. Strobel visited Israel in 1988 and Mr. Macur, a graduate student of Dr. Olsen, participated in an international meeting on Iron and plants which was held last June in Jerusalem. Parts of the work supported by the project were presented during this meeting as well as other scientific meetings in previous years.

E. EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL AIMS

As pointed out previously, the main goal of the present project was to define the role of iron in virulence of plant pathogens, particularly in Verticillium dahliae. In order to gain an insight into the interaction of iron with virulence, we sought answers to the following questions a) What is the relationship between the iron status of the host plant and verticillium wilt severity? b) How does iron limitation affect development of Verticillium dahliae within the plant and plant defense mechanisms? c) What are the mechanisms for iron sequestration by plant pathogens during saprophytic life and pathogenesis? Are siderophores involved or other mechanisms? Results obtained provided unequivocal answers to some of these questions whereas only partial answers were obtained for others. Each of this questions will be addressed in the following discussion.

1. Relationship between iron status of host plant and disease severity

In case of verticillium wilt caused by Verticillium dahliae, plants grown under iron deficient conditions were significanlty more sensitive to symptoms development and yield reduction than plants grown under iron sufficiency. In the present project, this phenomenon has been demonstrated in peanut, tomato, eggplant, potato and cannola under field as well as greenhouse conditions.

Supplemental of iron either applied via the soil (as EDDHA) or the folliage generally alleviated disease symptoms and increased yields compared to control in infested plots. These supplements had no effect on yield of plants growing in non-infested plots. In peanuts, soil application of iron was significantly more effective than foliar application. Moreover, the highly significant effect of FeEDDHA soil

additions on yield increase of peanuts grown in Verticillium infested soil justifies it as a practial control measure against the disease. As a matter of fact, FeEDDHA application has been recently successfully used in Israel to recover stunting and chlorotic peanuts in fields infested with Verticillium. Such results encouraged the recommendation of iron application as a practical mean of control. It should however be stressed that peanuts are known to be particularly sensitive to iron deficiency. Although, as demonstrated, supplemental of iron reduced Verticillium wilt in other crops as well, it may not be significant enough to recommend such a treatment as a contol mean against the disease. Iron application as a control measure against verticillium wilt should be considered for each crop separately. It may be justified in other plants which exhibit high sensitivity to iron deficiency (e.g. avocado, mango, olives, etc.).
During the course of this project, the effect of Verticillium dahliae infection on the nutrient balance of several host species was investigated. The results of our studies (Krikun et al) indicate that the primary effect of infection with regard to macroelement composition is a decrease in K. Depending on the crop and CV. there is also a decrease in Fe. In canola, in all 4 CVs examined, infected plants had one-half the Mn content of control plants. That there seem to be intereactions between Fe. Mn and K is known from the literature and from some of the results presented here.

2. Effect of iron on advancement of V. Dahliae within the host and host defense mechanisms.

Further experiments were aimed at elucidating the effect of iron on the development of the fungus in the plant. No significant difference could be detected in peanut (Bitan & Barash, Krikun et al.) or tomato (Macur & Olsen) between iron-deficient and iron-sufficient plants. In peanuts colony forming units were examined under greenhouse and field conditions with similar results in treated and control plants. In tomatoes, the distance of longitudinal invasion was approximately the same in both, iron replete and iron limited conditions.
The fact that the increased sensitivity of iron-deficient plants to V. dahliae is not due to enhanced colonization by the pathogen was also

supported by following phytoalexins production in peanuts (Bitan & Barash). It is noteworthy that the content of phytoalexins was significantly induced not only by infection but also due to iron deficiency. Thus, if the phytoalexins examined in peanut exert any effect on the progress of the mycelium within the host, than iron-stressed plants should become more resistant which was not the case in our studies. We therefore hypothesize that iron may exert an effect on expression of virulence mechanisms such as toxins, pectic enzymes or hormones production by the pathogen or alternatively, render the plant more tolerant towards these virulence determinants

3. Mechanisms for iron acquisition by plant pathogens

Many efforts have been invested in the present project in characterizing mechanisms for iron uptake by phytopathogenic fungi. These studies were dual-purposed: a) to elucidate the survival capacity of these pathogens as far as competition for iron is concerned and b) to find out whether V. dahliae can deprive the host of iron. In recent years, the role of iron competition in biological control exerted by pseudomonads has been elucidated. Although the latter is certainly not the only mechanisms involved in biocontrol, in several cases it has been proven to be a significant factor. Obviously fungal pathogens, especially soil-born fungi, should possess mechanisms for iron sequestration in order to survive. In view of the very scanty information on such mechanisms in phytopathogenic fungi we have extended this portion of the project beyond what was originally planned. Thus, in addtion to V. dahliae, other pathogens were extensively investigated.

Production of siderophores and siderophore-mediated iron uptake were investigated in V. dahliae, Stemphylium botryosum f. sp. lycopersici (causal agent of leaf spot and foliage blight in tomato), Gaeumannomyces graminus var tritici (causal agent of take-all disease in wheat) and the non-siderophore producer Geotrichum candidum (causal agent of sour rot in citrus). Quite interestingly, all the first three pathogens, which belong to the same or close groups of the Ascomycetes (all produce perithecia during sexual stage) secrete coprogen B and dimerum acid as their major siderophores. The

investigated transport systems generally exhibit characteristic properties for siderophore-mediated iron transport detected in other fungi. Properties unique to each system has been discussed in the papers presented in the "Body of the report". However, some general comments might be of interest. All of the investigated fungi were capable of utilizing iron from foreign hydroxamate siderophores produced by other fungi of special interest is Geotrichum candidum, which cannot produce siderophores but nevertheless exhibit high capacity to utlizie iron from an array of various hydroxamate siderophores. These results suggest that fungi are generally competitive for hydroxamate siderophores regardless of their origin. Hydroxamate siderophores have been detected in the rhizosphore (Reid et al. 1986 in Iron, siderophores and plant diseases).

Another point which should be emphasized is that the siderophore-mediated transport systems are designed for iron-stress conditions (i.e. high iron deficiency). Such conditions may not be available during pathogenesis and sequestration of iron may be achieved by mechanisms other than siderophores. Examples for such mechanisms have been demonstrated in Stemphylum botroysum (Menulis et al. 1987) and Geotrichum candidum (Mor et al. 1988). In the former case, the pathogen released ferric chelates (i.e. stemphytoxins I & II) to facilitate iron uptake. These chelates have been also shown to be phytotoxic.

The question of whether V. dahliae can deprive the host of iron has been examined. The work of Macur & Olsen with tomato indicate that Verticillium dahliae did not enhance iron stress severity as quantified by root peroxidase and chlorophyll content of young leaves. This conclusion may also be supported by the facts that peanuts could utilize iron from hydroxamate siderophores of V. dahliae and siderophores could not be detected in plants. Thus, the siderophore-mediated iron uptake systems may not be involved in iron sequestration during pathogenesis. However, although V. dahlie may not cause iron deficiency to the host plant during infection, it may do so indirectly. Thus a dramatic increase in peanut yield following FeEDDHA application to infested but not to non-infested soil was observed (Barash et al. 1988). This may indicate that the fungus can

efficiently sequester iron from the rhizosphere and compete indirectly for iron with the host plant.

F. LIST OF PUBLICATION RESUTLING FROM THE RESEARCH PROJECT

1. Manulis, S., Kashman, Y. & Barash, I. 1987. Identification of siderophores and siderophore-mediated uptake of iron in Stemphylium botryosum. Phytochemistry 26: 1317-1320.

2. Manulis, S., Netzer, D. & Barash, I. 1987. Acquisition of iron by Stemphylium botryosum under iron-replete conditions. Canadian Journal Microbiology 33: 652-657.

3. Barash, I., Zion, R., Krikun, J. & Nachmias, A. 1988. Effect of iron status on verticillium wilt disease and on in vitro production of siderophores by Verticillium dahliae. Journal of Plant Nutrition 11: 893-905.

4. Mor, H., Pasternak, M. & Barash, I. 1988. Uptake of iron by Geotrichum candidum, a non-siderophore producer. Biology of Metals 1: 99-105.

5. Mor, H. & Barash, I. 1989-90. Characterization of siderophore-mediated iron transport in Geotrichum candidum, a non-siderophore producer Biology of Metals (in press).

6. Dori, S., Solel, Z., Kashman, Y. & Barash, I. 1989. Characterization of hydroxamate siderophores and siderophore-mediated iron uptake in Gaeumannomyces graminis var. tritici. Physiological and Molecular Plant Pathology (submitted).

7. Macur, R.E. & Olsen, R.A. 1989. The relationship between iron nutrition and verticillium wilt resistance in tomato. (in preparation).