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Control of Fusarium wilt in carnation grown on rockwool by Pseudomonas sp. strain WCS417r and by Fe-EDDHA

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Abstract

In carnations grown on rockwool disease incidence of fusarium wilt caused by *Fusarium oxysporum* f.sp. *dianthi* (*Fod*) was reduced when Fe-EDDHA instead of Fe-DTPA was used as iron source in the nutrient solution. Addition of *Pseudomonas* sp. strain WSC417r intensified this reduction in the cultivar Pallas, moderately resistant to Fusarium, but not in the susceptible cultivar Lena. Treatment of plants with Fe-EDDHA instead of Fe-DTPA as iron source resulted in higher numbers and percentages on the roots, of *in vitro* antagonistic fluorescent pseudomonads. However, differences were only significant at 56 days after planting for cv. Lena and at 14 and 28 days after planting for cv. Palas. Both chelators, at different concentrations, had no effect on root colonization by either *Pseudomonas* sp. strain WCS417r or *Fod* strain WCS816. However, when coinoculated, reduced numbers of propagules of *Fusarium* were found at concentrations of Fe-EDDHA lower than 10^{-5} M.

Higher concentrations of the siderophore fusarine produced by *Fod* strain WCS816 were demonstrated when Fe-EDDHA instead of Fe-DTPA was used as iron source in culture media. At equal concentrations, no such differences were found in the amount of siderophore produced by WCS417r. Germ tube length of *Fod* was less with Fe-EDDHA than with Fe-DTPA. The reduction of germ tube length was stronger when the purified siderophore of WCS417r was added in excess to the culture media with Fe-EDDHA than those with Fe-DTPA. Therefore, the observed reduction of germ tube growth can not completely be explained by iron deprivation. It appeared that EDDHA exhibited a toxic effect for conidia of *Fod* strain WCS816 as well.

We conclude that the observed disease reduction by Fe-EDDHA is a consequence of a limitation of iron availability for *Fod*. This limitation is possibly intensified by the increase in number or percentage of antagonistic fluorescent pseudomonads that strongly compete for iron. The additional effect after bacterization with *Pseudomonas* strain WCS417r in Fe-EDDHA treated carnations of cv. Pallas is likely to be due, at least partly, to a direct competition for iron between the siderophores of *Fod* strain WCS816 and of *Pesudomonas* sp. strain WCS417r.

Additional keywords: biological control, carnation, iron, pseudomonads, rockwool, siderophores.

Introduction

Most aerobic and facultative anaerobic microorganisms respond to iron limiting conditions by producing siderophores that sequester iron (Neilands, 1981). The chelated

iron which is actively transported across the cell walls (Emery, 1974; Neilands, 1984) is utilized in enzyme systems (Neilands, 1974). Siderophores differ in their affinity for ferric iron. In general, siderophores produced by pseudomonads have a higher affinity (Log Kz >40) (Neilands et al., 1974) than the siderophores such as fusarine produced by Fusarium spp. (Log Kz 29) (Emery, 1965). Hence, many siderophore producing pseudomonads have been selected and implemented as biocontrol agents of soil-borne pathogens and were recently reviewed by Weller (1988).

Addition of synthetic iron chelators to soil suppressed Fusarium wilt pathogens (Scher and Baker, 1982). It was demonstrated that elongation of germ tubes of microconidia of *Fusarium oxysporum* f. sp. *lini* was inhibited *in vitro* by the addition of iron chelators ethylenediamine-tetraacetatic acid (EDTA), ethylenediaminedi-O-hydroxyphenyl-acetic acid (EDDHA) or diethylene-diaminetetraacetic acid (DTPA) and could be restored either partially or completely by the addition of $FeCl_3$ (Scher and Baker, 1982). Evidence was also presented by Scher and Baker (1984) that, when Fe-EDDHA was added to soil, the *Pseudomonas putida* biocontrol agent increased in numbers. No direct inhibition of the pathogen by Fe-EDDHA could be demonstrated. Simeoni et al. (1987) suggested a critical iron level for an iron mediated suppression of fusarium wilt. *In vitro*, suppression of chlamydospore germination in the presence of *Pseudomonas putida* A12, occured below critical levels of Fe^{3+} activities of 10^{-19} M – 10^{-22} M, whereas optimal suppression took place between Fe^{3+} activities of 10^{-22} M and 10^{-27} M (Simeoni et al., 1987).

In artificial substrates such as nutrient film cultures or rockwool, nowadays widely used in horticulture, the iron availability for plant growth is controlled by iron chelators which are used as sole iron source. Manipulation of iron availability by adding different chelators to the substrate provides the opportunity to study the effects of variation in iron availability fusarium wilt caused by *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) in a more defined and controlled system. The affinity of EDDHA for Fe^{3+} is higher that that of DTPA (Log Kz 33.9 and 27.4, respectively). Although iron is firmly bound by both chelators it has been demonstrated that divalent ions such as Cu^{2+} and Zn^{2+} can be bound as well (Lindsay, 1979).

This investigation presents data on the possibility to control fusarium wilt of carnation in rockwool cultures by using Fe-EDDHA as the source of iron and by adding the biocontrol agent *Pseudomonas* sp. WCS417r to the substrate. To investigate the mechanisms underlying the observed disease reduction by Fe-EDDHA and/or *Pseudomonas* sp. strain WCS417r the following hypotheses were tested: 1) Populations of rhizosphere pseudomonads and of *Pseudomonas* sp. strain WCS417r, producing high affinity siderophores that are competing for Fe(3+) with *Fod*, are favored by Fe-EDDHA. 2) Lowering iron availability by Fe-EDDHA and/or *Pseudomonas* sp. strain WCS417r, reduces elongation of germ tube length and population densities of *Fod*. Preliminary results have been published earlier (Van Peer 1988; Van Peer et al., 1989).

Materials and methods

Microorganisms. A rifampicin resistant mutant (WCS417r) of *Pseudomonas* sp. strain WCS417, which had been selected from wheat rhizosphere, was used to bacterize the plants. This strain had demonstrated to suppress *Gaeumannomyces graminis* var. *tritici* in wheat (Lamers et al., 1988). It also promoted the growth of several crops grown in hydroponic culture (Van Peer and Schippers, 1989) including carnation (R. van Peer,

unpublished results). A strain (WCS816) of *Fusarium oxysporum* Schlecht. f.sp. *dianthi* (Prill. & Delacr.) Snijder & Hansen, virulent on carnation, was used to inoculate the plants. This strain was obtained from the Research Station for Floriculture, Aalsmeer, the Netherlands.

Bacterization and inoculation with Fusarium. A bacterial suspension of *Pseudomonas* sp. WCS417r was prepared by diluting cultures, grown for 24 h at 27 °C on King's medium B (KB) agar (King et al., 1954), in sterile tap water to approximately 10^7 cells ml^{-1}. One week after planting, the plants were bacterized using 25 ml of the bacterial suspension for each plant. Microconidial suspensions for inoculation of plants were obtained from a culture of *Fusarium oxysporum* f.sp. *dianthi* (*Fod*) strain WCS816 grownin liquid Czapek Dox medium on a reciprocal shaker for 5 days at 23 °C. Spores and hyphae were centrifuged for 20 min at 6000 *g*, rinsed three times with sterile distilled water (SDW) and filtered through sterile glasswool. One week after bacterization, plants were inoculated by addition of 10 ml of a microconidial suspension (10^6 conidia ml^{-1}) to the roots of each plant.

After inoculation, the plants were completely randomized. Disease symptoms and number of diseased plants were recorded at least weekly for 48 plants per treatment. A plant was considered to be deseased when the first local symptoms on leaves were present (index 2 on the scale used by Baayen and Niemann, 1989).

Plant material. Carnation cuttings of the cultivar Lena, susceptible to *Fod* and of the moderately resistant cultivar Pallas, were rooted on rockwool granulate. The carnation cuttings were obtained from van Staaveren B.V. and/or Hilwerda B.V.. These cuttings were placed on rockwool (GRODAN, the Netherlands) and grown in the glasshouse at 22 °C for one week prior to bacterization. A nutrient solution (De Voogt 1981), modified for iron (15 μM Fe-EDDHA or 15 μM Fe-DTPA) was given by hand. Depending on growth season and evaporation 25-50 ml was given three times a week. The pH and Ec of the nutrient solution were measured weekly and were kept at 6.5 and 2.5, respectively. The nutrient solution was analyzed every four weeks and adjusted if necessary.

Effect of Fe-EDDHA on the development of pseudomonas populations, competing for iron with Fod strain WCS816. At five dates after planting of rooted carnations in rockwool, pieces were randomly taken from the roots of 12 plants (0.3 g per plant) only receiving Fe-DTPA or Fe-EDDHA. Root pieces were then dilution plated on King's medium B (KB). The plates were incubated for 48 h at 27 °C. All colonies from agar plates of the appropriate dilution (20-50 colonies per plate) were isolated and pure cultured. To determine the development of *Pseudomonas* populations competing for ferric iron with *Fod* on the roots, all the purified *Pseudomonas* isolates obtained from the carnation root pieces were spotted on KB agar plates supplemented with 10 μM EDDHAand 8 μM $FeCl_3$) and sustaining fluorescent siderophore production (10 colonies per agar plate). After incubation for 12 h, plates were sprayed with a microconidial suspension of *Fod* (10^6 microconidia ml^{-1}) and incubated for 24 h at 27 °C. The number and percentage of Pseudomonas isolates, surrounded by zones of inhibition of *Fod*, were then recorded. On KB agar supplemented with 50 μM EDDHA and 40 μM FeCl (sustaining no fluorescent siderophore production) the same strains were tested

against *Fod*. When the degree of inhibition was much less or absent than with 10 μM EDDHA and 8 μM FeCl, it was concluded that the Pseudomonas strains compete for iron with *Fod*. At the repeat of the experiment, the sampling dates were limited to four: 7, 14, 30 and 42 days after planting using 10 plants at each sampling date.

Effect of Fe-EDDHA and Fe-DTPA on population densities of Pseudomonas sp. strain WCS417r and Fod strain WCS816. Rooted cuttings of carnation cv. Pallas were transferred to continuously aerated hydroponic cultures (Van Peer and Schippers, 1989). The nutrient solution was inoculated with a microconidial suspension of *Fod* strain WCS816 (final concentration 10^4 conidia ml^{-1}), with a bacterial suspension of *Pseudomonas* sp. strain WCS417r (final concentration 10^6 cells ml^{-1}) or with both microorganisms simultaneously. The effect of various concentrations of the iron chelators Fe-EDDHA or Fe-DTPA on the colonization of roots by the bacterium and the fungus was determined 7 days after the addition of the microorganisms to the nutrient solution.

Colonization of roots by Pseudomonas sp. strain WCS417r and Fod strain WCS816. Microbial colonization of the roots was determined by shaking 0.3 g of root pieces for 1 min in glass test tubes containing 5 ml $MgSO_4$ (0.1 M) and 2.5 g glass beads (3 mm diam.). Serial dilutions were plated on KB medium (Geels et al. 1986), supplemented with 200 mg rifampicin (Serva) per liter to recover *Pseudomonas* sp. strain WCS417r and on a slightly modified Komada medium (Komada, 1975) as described by Gams and Van Laar (1982) to determine the number of viable propagules of *Fod* strain WCS816. The plates were incubated at 27 °C and 23 °C and counted after 2 and 5 days, respectively.

In vitro growth and siderophore production of Pseudomonas sp. WCS417r and *Fod strain WCS816. Pseudomonas* sp. strain WCS417r and *Fod* strain WC816 were cultured in a liquid medium described by Scher and Baker (1982). Traces of iron in this medium were removed by complexation with 8-hydroxyquinoline (8-OHQ) (Waring and Werkman, 1942) followed by chloroform extraction of the iron complex. After autoclaving of the medium, different concentrations of sterile filtrated Fe-EDDHA or Fe-DTPA were added to the medium. Cultures of *Pseudomonas* sp. strain WCS417r and *Fod* strain WCS816 were grown for 2 and 5 days, respectively, in 50 ml of the medium in Erlenmeyer flasks (250 ml) on a reciprocal shaker at 27 °C. Growth of WCS417r was estimated by reading the absorbance at 660 nm, whereas growth of *Fod* strain WCS816 was estimated by determination of biomass dry weight. Siderophore production by *Pseudomonas* sp. strain WCS417r was estimated by reading the absorbance of the supernatant (adjusted at pH 7.0) of centrifuged samples at 400 nm . Siderophore production by the fungus was only assessed after the addition of iron, inducing the formation of a fusarine-Fe complex (Emery, 1965). The presence of this complex was demonstrated by the absorbance of the filtrate (adjusted to pH 3.0) at 440 nm. Optimal absorbance was found after addition of $FeCl_3$ to the level of 10^{-2} M (R. van Peer, unpublished results).

Purification of the siderophore of Pseudomonas sp. strain WCS417r. A stationairy phase culture of *Pseudomonas* sp. strain WCS417r was diluted 100 fold in succinic acid

media (Meyer and Abdallah, 1978) and cultured for five days at 25 °C. Thereafter ferri-siderophores were isolated following the method described by Van der Hofstad et al. (1986). To prepare the iron free siderophore, the lyophilized siderophore of *Pseudomonas* sp. strain WCS417r was resuspended in 25 ml sterile distilled water (SDW) and the pH was adjusted to 4.0 with 10% (v/v) acetic acid. Iron was extracted with 8-hydroxyquinoline in chloroform (8-OHQ-Ch) (5% w/v) by vigorously stirring for 30 min at 4 °C. This procedure was repeated twice using 10% (w/v) 8-OHQ-Ch followed by another repeat of the procedure using 15% (w/v) 8-OHQ-Ch. The aqueous phase was washed three times with chloroform to remove 8-OHQ followed by lyophilization. Ferri siderophores were prepared by addition of small quantities of 0.1 M $FeCl_3$ until no fluorescense of the solution (pH 7.0) was detected with a fluorescense spectrophotometer (Optica Model 115). Assuming a 1 : 1 ratio of iron bound to the siderophore of *Pseudomonas* sp. strain WCS417r, the ferri-siderophore concentration was measured by fluorimetric titration of the desferri-siderophore solution with the $FeCl_3$ solution.

Interaction of the siderophore of Pseudomonas sp. strain WCS417r with Fe-EDDHA and Fe-DTPA and its effects on germ tube length of Fod strain WCS816. Microconidia were obtained from 7 days old cultures of *Fod* strain WCS816 on KB agarplates. Microconidia and hyphae were separated by filtration through sterile glasswool. EDDHA or DTPA were dissolved in 0.1 N NaOH and these stock solutions were adjusted to pH 6.0 with 1 N HCl or 1 N NaOH. Serum flasks (25 ml) containing 2.5 ml of 0.4 strength nutrient solution (De Voogt, 1981) were used in these experiments. The pH of the nutrient solution was adjusted to 7.0 with 1 N NaOH and buffered with the addition of 0.4 g $CaCO_3$ per 200 ml. The solution also contained 0.05% glucose as a readily available carbon source.

In the first set of experiments equal concentrations (20 μM) of filter sterilized chelators and siderophores were used and concentrations of ferric iron (added as $FeCl_3$) were varied. In the second set of experiments, concentrations of the siderophore were varied while concentrations of chelators (20 μM) which were saturated for 80% with ferric iron (16 μM $FeCl_3$) remained constant. All serum flasks contained approximately 10^4 microconidia ml^{-1} solution. Each treatment was replicated four times. Germ tube length of 100 microconidia of *Fod* per replicate was recorded after 13 h of incubation at 27 °C.

All experiments described above were repeated at least twice, unless indicated otherwise.

Statistics. Results were analyzed by analysis of variance, if necessary after log transformation followed by Student's t-test to calculate minimum significant difference (Sokal and Rohlf, 1981).

Results

Disease development. As expected, plants of susceptible cv. Lena were more diseased than those of the moderately resistant cv. Pallas. In both cultivars, the percentage of diseased plants was significantly lower when plants had been treated with Fe-EDDHA instead of with Fe-DTPA as iron source (Figs 1A and 1B). Bacterization of carnation

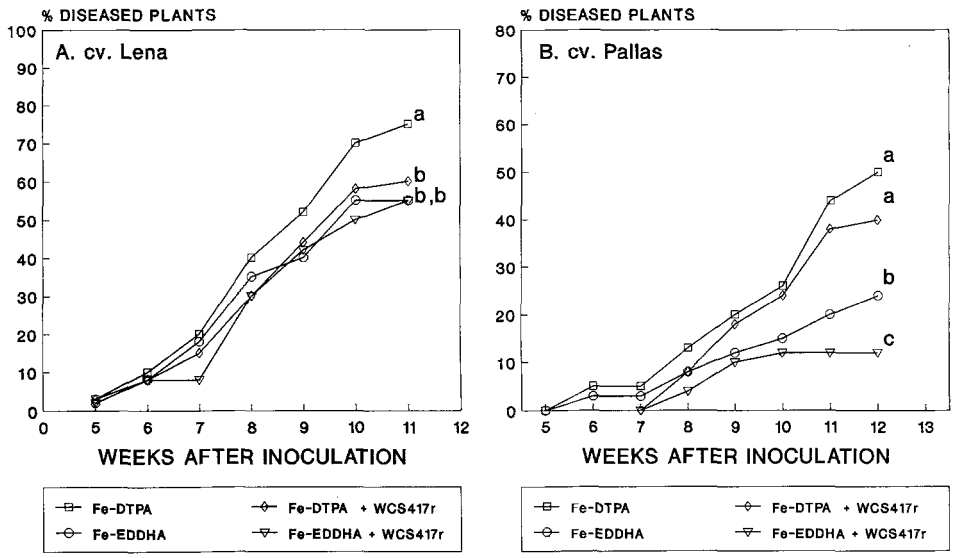

Fig. 1. Development of Fusarium wilt in susceptible carnation cultivar Lena (A) and a moderately resistant cultivar Pallas (B), inoculated with *Fusarium oxysporum* f.sp. *dianthi* strain WCS816 and grown on rockwool. Carnations received either Fe-DTPA or Fe-EDDHA as iron source with or without the addition of *Pseudomonas* sp. WCS417r. Significant differences ($P \leq 0.05$) are indicated by different letters. Each value is the mean of 48 plants.

cv. Lena with *Pseudomonas* sp. strain WCS417r, receiving Fe-DTPA as iron source, resulted in a significant reduction of the percentage of diseased plants. No such effect of bacterization was found with cv. Lena receiving Fe-EDDHA (Fig. 1A). In contrast, bacterization of cv. Pallas receiving Fe-EDDHA, did result in a further reduction of the disease compared to the non-bacterized treatment (Fig. 1B). *Pseudomonas* sp. strain WCS417r colonized the roots of carnations of both cultivars up to 5×10^4 cfu g^{-1} root fresh weight, 13 weeks after bacterization. No differences in colonization densities of *Pseudomonas* sp. strain WCS417r were found between the treatments.

Pseudomonads competing for Fe(3+) with Fod strain WCS816. All but 26 of the 5216 pseudomonads isolated from the roots and spotted on the KB agar plates supplemented with 10 μM EDDHA and 8 μM $FeCl_3$ produced siderophores, as was demonstrated by their fluorescense under U.V. light. In addition, 212 isolates did not fluoresce on KB agar with 50 μM EDDHA and 40 μM $FeCl_3$ but they inhibited growth of *Fod* at both iron levels. As no conclusion can be made on the involvement of siderophores besides other components produced by these pseudomonads they were excluded from the results as well.

Only at 56 days after planting, significantly higher numbers of fluorescent pseudomonads that inhibited *Fod* strain WCS816 *in vitro*, were obtained from non-bacterized, non- Fusarium inoculated roots of cv. Lena receiving Fe-EDDHA compared to those obtained from roots of cv. Lena receiving Fe-DTPA (Table 1, Fig. 2A). Generally, pseudomonads isolated from roots of cultivar Pallas treated with Fe-EDDHA yielded

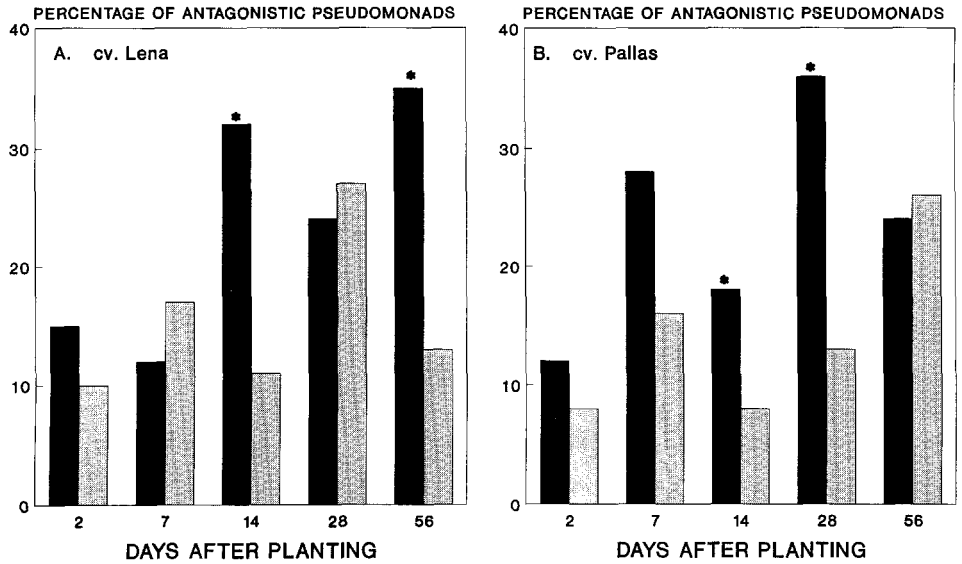

Fig. 2. Percentage of fluorescent pseudomonads antagonistic *in vitro* to *Fusarium oxysporum* f.sp. *dianthi* on KB media supplemented with 10 μM EDDHA and 8 μM $FeCl_3$. Fluorescent pseudomonands were isolated at different times after planting on rockwool from roots of carnations cv. Lena (A) and Pallas (B) treated with either Fe-EDDHA (■) or Fe-DTPA (□). Bars topped with an asterix (*) are significantly different at that sampling time.

Table 1. Average number (Log cfu g^{-1} root fresh weight) of fluorescent pseudomonads antagonistic to *Fusarium oxysporum* f.sp. *dianthi* on KB medium supplemented with 10 μM EDDHA and 8 μM $FeCl_3$. Fluorescent pseudomonads were isolated from the roots at different times after planting of carnation cultivars Lena and Pallas receiving either Fe-EDDHA or Fe-DTPA as iron source.

Cultivar	Iron source	Days after planting				
		2	7[a]	14[b]	28	56
Lena	Fe-EDDHA	4.9	3.7	4.0	4.4	4.6*
Lena	Fe-DTPA	4.3	3.8	4.4	4.2	3.7
Pallas	Fe-EDDHA	4.7	4.3	5.2*	4.9*	4.7
Pallas	Fe-DTPA	4.3	3.8	4.0	4.1	4.8

Each value is the mean of 12 plants. Within each cultivar, significant differences between antagonistic pseudomonads on roots of plants receiving either Fe-EDDHA or Fe-DTPA are indicated by an asterix (*).

[a] Time of bacterization with *Pseudomonas* sp. strain WCS417r.

[b] Time of inoculation with *Fusarium oxysporum* f.sp. *dianthi* strain WCS816.

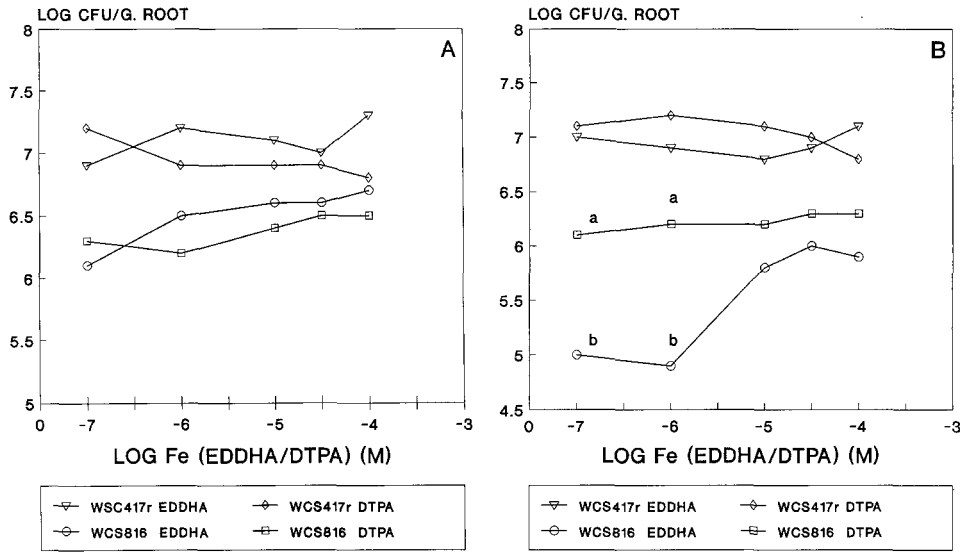

Fig. 3. Colonization of roots by *Pseudomonas* sp. WCS417r and/or *Fusarium oxysporum* f.sp. *dianthi* (*Fod*) strain WCS816 on roots of carnation cv. Pallas after 7 days of growing in hydroponic culture supplemented with different concentrations of Fe-EDDHA or Fe-DTPA. The micro-organisms were inoculated separately (A) or co-inoculated (B). Significant differences ($P \leq 0.05$) between the chelators are indicated by different letters. Each value is the mean of 10 plants.

a higher percentage of *Fod* strain WCS816 inhibiting pseudomonas isolates compared to those isolated from roots treated with Fe-DTPA (Fig. 2B). However, differences were only significant 14 and 28 days after planting. The repeat of the experiment demonstrated similar tendencies (data not shown).

Effect of Fe-EDDHA and Fe-DTPA on the colonization of roots by Fod strain WCS816 and Pseudomonas sp. strain WCS417r. Seven days after inoculation with either *Pseudomonas* sp. strain WCS417r or *Fod* strain WCS816, root colonization did not significantly differ, at different concentrations of both iron chelators in the nutrient solution (Fig. 3A). In contrast, when both strains were co-inoculated, the number of propagules of *Fod* strain WCS816 was reduced at concentrations lower than 10^{-5} M Fe-EDDHA but was not reduced when Fe-DTPA was used. Colonization by *Pseudomonas* sp. WCS417r, however, was similar at all concentrations of Fe-EDDHA (Fig. 3B).

In vitro growth and siderophore production of Pseudomonas sp. strain WCS417r and Fod. strain WCS816. With decreasing iron concentrations from 10^{-4} M Fe-EDDHA or Fe-DTPA downwards, concentrations of the *Fod* siderophore fusarine increased (Fig. 4). Fusarine concentrations were significantly higher at concentrations of Fe-EDDHA lower than 10^{-5} M compared to equal concentrations of Fe-DTPA. Fusarine could not be detected at $10^{-4.5}$ M Fe-EDDHA/DTPA, while growth of *Fod* strain WCS816 was still increasing. Growth of *Fod* did not significantly differ between media with Fe-EDDHA and those with Fe-DTPA. Siderophore production by *Pseudomonas* sp. strain

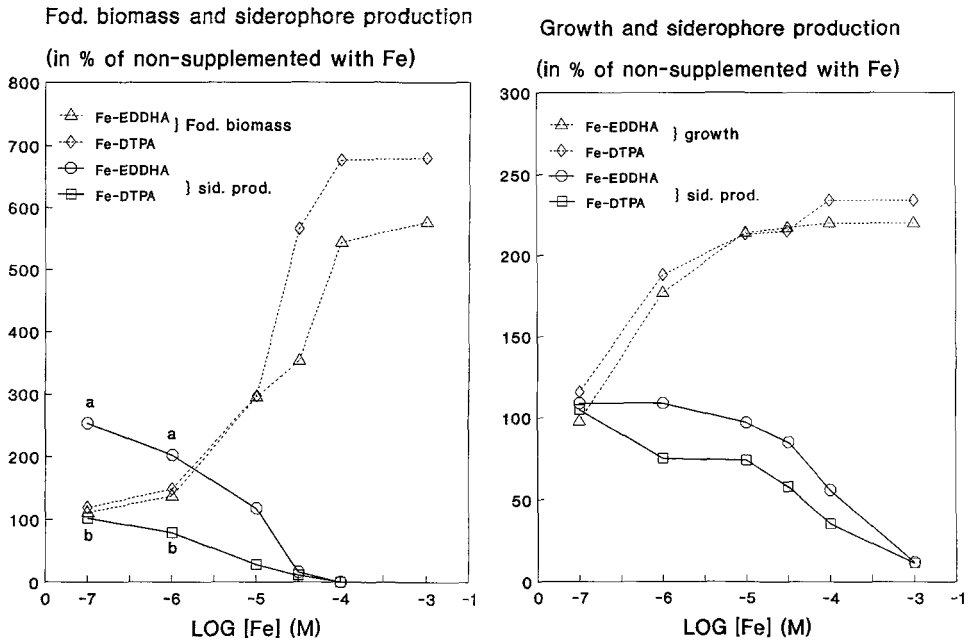

Fig. 4. (Left). Growth and siderophore (fusarine) synthesis of *Fusarium oxysporum* f.sp. *dianthi* (*Fod*) strain WCS816 in media with different concentrations of Fe-EDDHA or Fe-DTPA. Growth and fusarine production were measured as biomass (dry weight in mg) and absorbance at 440 nm, respectively and were expressed as a percentage of the treatment without additional iron. Each value is the mean of four replicates.

Fig. 5. (Right). Growth and siderophore production of *Pseudomonas* sp. strain WCS417r in media with different concentrations of Fe-EDDHA or Fe-DTPA. Growth and siderophore production was measured as the absorbance at 660 nm and 400 nm, respectively, and was expressed as a percentage of the treatment without additional iron. Each value is the mean of four replicates.

WCS417r was detected at all concentrations of both iron chelators (Fig. 5). Concentrations of the siderophore produced by *Pseudomonas* sp. strain WCS417r were higher in the Fe-EDDHA treatments than in Fe-DTPA treatments, but did not differ significantly. Growth of this strain increased with increasing iron concentrations, but was not significantly different between Fe-EDDHA and Fe-DTPA treatments.

Interaction of the siderophore of WCS417r with Fe-EDDHA and Fe-DTPA and its effects on germ tube length. In the first set of experiments, iron concentrations were varied at constant concentrations of the chelators and siderophores (20 μM). Germ tube lengths in treatments with or without addition of the siderophore of *Pseudomonas* sp. strain WCS417r in the nutrient solution were significantly lower at all Fe concentrations of 16 μM or lower when EDDHA was used instead of DTPA (Fig. 6). When iron was omitted from the nutrient solution, differences between the chelators were most pronounced, but there was no significant effect of the addition of the Pseudomonas siderophore of strain WCS417r. This indicates a toxic effect of EDDHA on germ tube

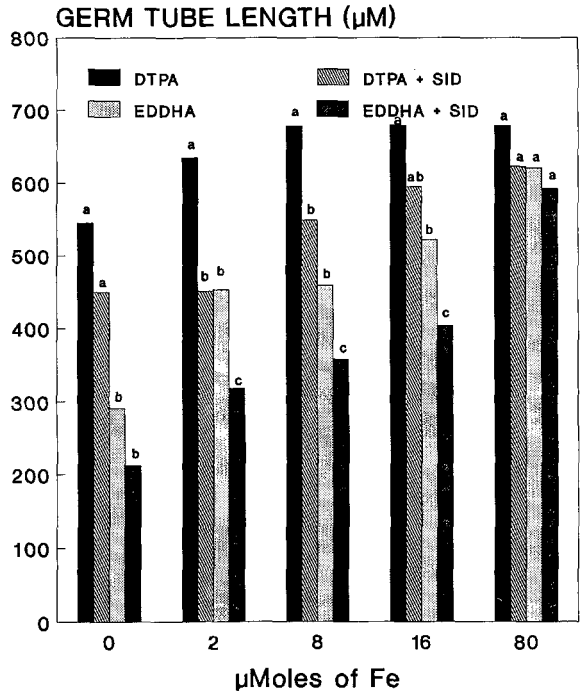

Fig. 6. Germ tube length of microconidia of *Fusarium oxysporum* f.sp. *dianthi* strain WCS816 at constant concentrations of the chelators EDDHA or DTPA (20 μM) with or without 20 μM of the siderophore of *Pseudomonas* sp. WCS417r. Iron saturation of the chelators was varied by the addition of $FeCl_3$. Significant differences ($P \leq 0.05$) for each concentration of iron added are indicated by different letters.

length. In the second set of experiments, concentrations of the des-ferri-siderophore of WCS417r were varied at constant concentrations of the iron-chelators (80% saturated with $FeCl_3$). When the siderophore of WCS417r was added to the Fe-EDDHA treatment a significant reduction of germ tube lengths was already measured at 10 μM Fe-EDDHA compared to e measured at a similar concentration of Fe-DTPA (50 % saturation of the iron-chelator) (Fig. 7). In addition, when 40 μM of the siderophore of *Pseudomonas* sp. strain WCS417r was added, significantly different germ tube lengths were found as well, again indicating that EDDHA is toxic for *Fod* and not only competing for iron.

Discussion

The severity of wilting of carnations grown on rockwool caused by *Fusarium oxysporum* f.sp. *dianthi* could be controlled by lowering the iron availability of the nutrient solution using Fe-EDDHA instead of Fe-DTPA as sole iron source. The increased production of the *Fod* siderophore fusarine, when Fe-EDDHA instead of Fe-DTPA was used, indicates that the iron availability for *Fod* is decreased by Fe-EDDHA (Fig. 4). Although no such significant increase was found at the levels of Fe-chelators used in the disease

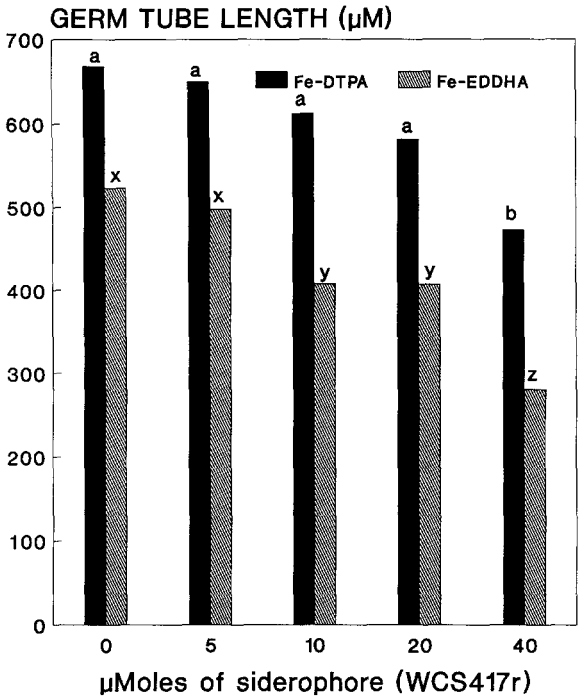

Fig. 7. Germ tube length of microconidia of *Fod* strain WCS816 at constant concentrations of iron chelators EDDHA or DTPA (20 μM) that were 80% saturated with iron (16 μM $FeCl_3$). Concentrations of the siderophore of *Pseudomonas* sp. WCS417r were varied. Significant differences ($P \leq 0.05$) for each chelator between concentrations of siderophore added are indicated by different letters.

suppression tests, we suppose that because of the presence of pseudomonads on the root surface, which are competing for Fe(3+) as well, actual levels on the root surface will be lower than the amount added to the nutrient solution. Germ tube lengths of *Fod* strain WCS816 were significantly less with Fe-EDDHA at all concentrations lower than 16 μM of added iron (Figs 6 and 7). It appeared however, that the reduction is not (completely) due to iron deprivation for the the fungus. The chelator EDDHA also exhibits a toxic effect for *Fod* (Fig. 7). This toxic effect could *e.g* be demonstrated when 40 μM siderophore was added to the solution containing either 20 μM EDDHA or DTPA (both complexed with 16 μM $FeCl_3$) (Fig. 7). All iron present in the solution then becomes bound to the siderophores, leaving 24 μM uncomplexed siderophore and 20 μM of either EDDHA or DTPA in the solution. It appeared. Despite the same concentration of chelators in the solution, significantly different germ tube lengths were recorded (Fig. 7). The toxic effect of EDDHA may be explained by the chelation of other ions in the nutrient solution, that are in chelated form toxic to the fungus. It was demonstrated before that EDDHA can bind to other ions than iron as well (Lindsay, 1979; Simeoni et al., 1987)

No differences between Fe-EDDHA and Fe-DTPA were demonstrated in their effects on the number of viable propagules of *Fod* strain WCS816 in the rhizosphere,

seven days after addition of different concentrations of the Fe-chelators and *Fod* (WCS816) to the nutrient solution with carnations. Differences are possibly to be expected at a later stage. It appeared that, during this first week, the numbers of isolates inhibiting *Fod* on KB (antagonistic to *Fod*), are very low and only start to increase thereafter (Table 1, Fig. 2B). This hypothesis is supported by the observation that when high numbers of the selected strain WCS417r were simultaneously inoculated with *Fod*, numbers of viable propagules were indeed significantly lower one week later (Fig. 3B). Apparently, the effect of Fe-EDDHA on disease development compared to that of Fe-DTPA is the result of both the inhibition of germ tube growth and the reduction in propagules numbers of *Fod* strain WCS816. In the presence of competing Pseudomonads, the iron deprivation is intensified as these pseudomonads are better equipped than Fusarium to utilize iron from Fe-EDDHA. Their population is thus able to grow more rapidly than that of *Fod* strain WCS816.

Addition of the selected siderophore producing antagonist WCS417r, significantly reduced the numbers of diseased carnations of cv. Pallas receiving Fe-EDDHA but not of those receiving Fe-DTPA. Fe-EDDHA as iron source did not enhance the numbers of WCS417r (Fig. 3A). The disease reduction after bacterization of carnations of cv. Pallas, therefore can not be explained as a result of a competitive advantage of *Pseudomonas* sp. strain WCS417r due to enhancement of its population by Fe-EDDHA. Germ tube length of microconidia of *Fod* was significantly reduced when the purified siderophore of WCS417r was added to microconidial suspensions of *Fod* containing either EDDHA or DTPA (Figs. 6 and 7). Therefore, it is tempting to conclude that a direct competition for Fe(3+) between siderophores of *Pseudomonas* sp. strain WCS417r and *Fod strain* WCS816 does play a role, at least partly, in the protection provided by *Pseudomonas* sp. strain WCS417r against Fusarium wilt caused by *Fod* WCS816. In addition, *in vitro*, WCS417r still produces siderophores at higher concentrations of iron than *Fod* strain WCS816 (Figs 4 and 5). This may imply that the bacterium rapidly creates an iron limitation for *Fod* strain WCS816 before the latter starts producing its own siderophores. In addition, a slower increase in siderophore production of *Fod* strain WCS816 than of*Pseudomonas* sp. strain WCS417r was demonstrated *in vitro* (R. van Peer, unpublished results). It has been postulated by Ahl et al. (1986) and Swinburne(1986), that not the iron limitation for the pathogen created by the Pseudomonas siderophore is responsible for the disease suppresion, but the iron-siderophore complex being toxic to the pathogen. In our model system this seems not to be the case. Growth of *Fod* was inhibited when purified siderophore of *Pseudomonas* sp. strain WCS417r (100 μM) was added to the KB agar plates, but growth was not inhibited when the iron saturated siderophore was added (R. van Peer, unpublished results).

Bacterization with *Pseudomonas* sp. strain WCS417r, of carnations grown with Fe-EDDHA, reproducably reduced the disease development of the moderately resistant cultivar Pallas, but not of the susceptible cultivar Lena (Fig. 1). This suggests that mechanisms other than competition for iron between Fusarium and pseudomonads and possibly related to the mechanism of disease resistance, are involved as well.

Samenvatting

Bescherming tegen Fusarium verwelkingsziekte bij anjer op steenwol door Pseudomonas sp. stam WCS417r en door Fe-EDDHA

Verwelkingsziekte in anjers op steenwol, veroorzaakt door *Fusarium oxysporum* f. sp. *dianthi* (*Fod*), werd gereduceerd indien het ijzer-chelaat Fe-EDDHA in plaats van Fe-DTPA werd toegevoegd aan de nutriëntenvloeistof. Bacterisatie met *Pseudomonas* sp. stam WCS417r had een additioneel effect bij de matig resistente cultivar Pallas maar niet bij de vatbare cultivar Lena. Toevoeging van Fe-EDDHA in plaats van Fe-DTPA aan planten als ijzerbron resulteerde op de wortels in hogere aantallen en percentages fluorescerende pseudomonaden, die *in vitro* antagonistisch waren ten opzichte van *Fod*. De verschillen waren echter alleen significant 56 dagen na planten voor de cultivar Lena en 14 en 28 dagen na planten voor de cultivar Pallas. Beide chelaten vertoonden bij verschillende concentraties geen effect op de kolonisatie van de wortel door beide micro-organismen. Echter, wanneer beide micro-organismen gezamelijk werden toegevoegd nam de wortelkolonisatie door *Fod* stam WCS816 af bij concentraties lager dan 10^{-5} M Fe-EDDHA. Er werd meer van het siderofoor fusarine door *Fod* stam WCS816 geproduceerd bij concentraties lager dan 10^{-4} M Fe indien Fe-EDDHA in plaats van Fe-DTPA als ijzerbron aan het cultuurmedium was toegevoegd. Er werd geen effect van beide chelaten gevonden op de siderofoorproduktie door WCS417r. Indien een overmaat van het gezuiverde siderofoor van WCS417r werd toegevoegd aan Fe-EDDHA werd een sterkere afname van de kiembuislengte gevonden dan toevoeging aan Fe-DTPA. De reductie van de kiembuislengte bleek niet volledig verklaard te kunnen worden door een afname van de ijzerbeschikbaarheid. Het chelaat EDDHA heeft ook een toxisch effect op conidiën van fusarium.

Wij concluderen, dat de waargenomen reductie van de verwelkingziekte door Fe-EDDHA een gevolg is van de afname van de ijzerbeschikbaarheid voor *Fod*. Dit wordt waarschijnlijk versterkt door de ontwikkeling van een antagonistische, fluorescerende *Pseudomonas*-populatie die sterk concurreren om ijzer. Het additioneel effect dat door bacterisatie met *Pseudomonas* sp. WCS417r van de met Fe-EDDHA behandelde matig resistente anjers ('Pallas') werd verkregen is voor een deel het gevolg van een directe concurrentie om ijzer tussen de sideroforen van *Fod* stam WCS816 en van *Pseudomonas* sp. stam WCS417r.

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