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Proferrorosamines and phytopathogenicity in *Erwinia* spp.*

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Proferrorosamine A (pFR A) of the plant pathogenic bacterium *Erwinia rhapontici* was shown to inhibit growth of wheat and cress seedlings at the ⩾ 10 ppm level. When the seeds were continuously exposed to 100 ppm pFR A, the germination of cress and wheat seeds was inhibited up to 90% and 80%, respectively. The inhibition could be reversed through addition of equimolar amounts of ferrous iron, which indicates that the strong iron chelating capability of pFR A is responsible for the observed effect. The Fe(II) in the corresponding iron complex, ferrorosamine A, was found to be remarkably resistant towards oxidation by hydrogen peroxide and therefore redox-cycling in the Haber–Weiss cycle. It is thus conceivable that pFR A may also attenuate the generation of reactive hydroxyl radicals during the resistant and wound reaction. The apparent correlation between proferrorosamine production and virulence in erwiniae was further corroborated through the analysis of *Erwinia persicinus*, a newly described species. Using electrospray ionization mass spectrometry, *E. persicinus* was shown to produce pFR A and pFR B, and preliminary evidence for the phytopathogenicity of *E. persicinus* was found in cress. Inhibition of wheat seedlings by *E. persicinus* could not be demonstrated, but this may be due to technical difficulties or different host specificities. Taken together, our results indicate that the phytopathogenicity of *E. rhapontici* and *E. persicinus* may, as least in part, be due to the release of proferrorosamines.

Keywords: bacterial diseases, metabolic profiling, phytotoxicity, pigments, siderophores

Introduction

Proferrorosamine A (pFR A; L-2-(2-pyridyl)-1-pyrroline-5-carboxylic acid) (Figure 1) was first described as a metabolite of *Pseudomonas roseus fluorescens* (alias *Bacillus roseus fluorescens*) (Pouteau-Thouvenot *et al*. 1965) and *Pseudomonas* GH (Shiman & Neilands 1965), later as a metabolite of some strains of *Serratia marcescens* biogroup A4 (Grimont & Grimont 1984) and the plant pathogen *Erwinia rhapontici* (Feistner *et al*. 1983). *Pseudomonas roseus fluorescens* was also found to produce proferrorosamine B (Pouteau-Thouvenot *et al*. 1968), which at low pH is dehydrated to anhydroproferrorosamine B and at high temperature gives rise to proferrorosamine C of unknown structure (Pouteau-Thouvenot *et al*. 1970). Other proferrorosamine-like molecules, so-called siderochelins, were discovered in *Nocardia* (Liu *et al*. 1981) and *Actinomyces* (Mitscher *et al*. 1984) species.

Proferrorosamines belong to the family of the rare microbial iron(II) chelators (Vande Woestyne *et al*. 1991). As a group, proferrorosamines can most readily be identified in form of their iron complexes (ferrorosamines; FRs) since the latter have a characteristic electronic absorbance spectrum (λ_{max} at 556 nm, shoulder at 510 nm). In principle, differentiation of the various ferrorosamines should be straightforward based on their different molecular masses. However, ferrorosamines are very thermolabile compounds, and in the past, molecular mass

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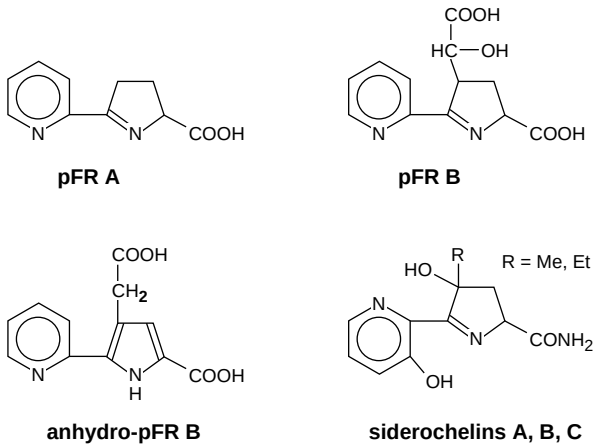

Figure 1. Structures of proferrorosamines and siderochelins.

determination of ferrorosamines has been difficult. Even 'soft' ionization with field desorption mass spectrometry (FD–MS) was only successful when co-desorption with tartaric acid or mannitol was used (Feistner *et al*. 1983).

A physiological function for the proferrorosamines is not yet known with certainty. However, recent transposon mutagenesis studies on *Pseudomonas* GH revealed that at least some of the corresponding biosynthetic genes are located on the chromosome, indicating that proferrorosamines have a useful function (Vande Woestyne & Verstraete 1992). The iron-complexing ability of proferrorosamines furthermore suggests a possible role in microbial iron uptake and probably endows pFR-producing bacteria with a competitive advantage in microbial communities (Liu *et al*. 1981). It has also been proposed that pFR A in *Pseudomonas* GH may be essential for siderophore (iron(III)-chelator) production (Vande Woestyne *et al*. 1991). However, neither the chemical nature of these iron(III)-chelators is known, nor do either pFR A or the putative iron(III)-chelators seem to be necessary for growth at very low or very high oxygen concentrations (Vande Woestyne *et al*. 1991).

The suggestion that pFR A might be a microbial virulence factor was first made in connection with its original identification in *E. rhapontici* (Feistner *et al*. 1983). According to this hypothesis, *E. rhapontici* may induce iron deficiency, and consequently growth inhibition, chlorosis, and impaired energy production, in its host plants via the release of the strongly iron-chelating proferrorosamines. In this regard it is worth mentioning that 2,2′-dipyridyl, which binds ferrous iron via the same diimine structure as proferrorosamines, is known to inhibit chlorophyll biosynthesis (Johanningmeier 1988, Oster *et al*. 1991) and the respiration of young wheat roots (James 1956).

E. rhapontici is endemic to at least Japan, North America, Europe, and the Middle East, and can be found in water (Pouteau-Thouvenot *et al*. 1965), soil (Feistner *et al*. 1983), and on plant surfaces (Sellwood & Lelliott 1978). *E. rhapontici* derives its name from rhubarb (*Rheum rhaponticum*), on which it causes crown rot (Millard 1924, Metcalfe 1940, Letal 1976). It can also cause rot in citrus (Volcani 1955), hyacinth (Sellwood & Lelliott 1978), onion (Ohuchi *et al*. 1983), wasabi (Goto & Matsumoto 1986), and tomato (Volcani 1955, Shaban *et al*. 1991). In wheat (*Triticum aestivum*) (Howe & Simmonds 1937, Campbell 1958, Luisetti & Rapilly 1967, Roberts 1974, McMullen *et al*. 1984) and pea (*Pisum sativum*) (Huang *et al*. 1990), infection by *E. rhapontici* causes pink and shrivelled seeds that do not germinate well. The pink discoloration is presumably due to ferrorosamines. However, a direct link between proferrorosamines and germination inhibition has not yet been established. Interestingly, *Pseudomonas* GH has meanwhile been reclassified as a strain of *E. rhapontici* (De Vos *et al*. 1993), and it seems likely that by today's criteria, *Pseudomonas roseus fluorescens* may also be misidentified, since macrorestriction analysis showed no similarities to *Pseudomonas fluorescens* (Grothues & Rudolph 1991).

We initiated this study to prove that proferrorosamines are capable of inhibiting seedling growth and thus contributing to phytopathogenicity. While this study was under way, we became aware of the description of the new species, *Erwinia persicinus* (alias *Serratia rubefaciens*), which is closely related to *E. rhapontici* and also produces a water-soluble pink pigment (Hao *et al*. 1990). We therefore tested *E. persicinus* for the production of proferrorosamines, using a novel soft-ionization technique, electrospray ionization mass spectrometry (ESI–MS). When pFR production was confirmed, we speculated that *E. persicinus* would also be plant pathogenic. Preliminary evidence for phytopathogenicity was indeed found.

Materials and methods

Bacterial strains

The type strains *E. rhapontici* ATCC 29283, *Erwinia persicinus* ATCC 35998, and *Erwinia herbicola* ATCC 33243, and two additional strains of *E. rhapontici*, GSPB 454 and 455, were used. Except when proferrorosamine

production was to be stimulated (see below), the bacteria were grown on standard media such as King B, Luria-Bertani (LB), yeast extract/glucose (Rudolph 1990), or MMB minimal medium as indicated. The composition of the MMB medium is as follows: 2 gl^{-1} $K_2HPO_4 \cdot 3H_2O$, 0.1gl^{-1} $MgSO_4 \cdot 7H_2O$, 0.4 gl^{-1} $(NH_4)_2SO_4$, 2 gl^{-1} sucrose, 100 μgl^{-1} nicotinic acid, and ferric citrate in a concentration of 1×10^{-8} M.

Proferrorosamine A for inhibition studies

Erwinia rhapontici ATCC 29289 was used to produce milligram quantities of pFR A for seedling inhibition studies as described (Feistner *et al*. 1983).

Effect of pFR A on the germination of wheat and cress seeds

In the first set of experiments, pFR A was tested in con-centrations of 4, 20, and 100 $\mu g\ ml^{-1}$. Seeds of the spring wheat variety 'Kolibri' and cress seeds of the commercial variety 'Kröbel' were used. Seeds were either: (a) pre-soaked for one hour (35 wheat seeds in 1 ml or 70 cress seeds in 0.5 ml of each pFR A solution) and then spread onto moist filter paper in 5 cm internal diameter (I.D.) Petri dishes (filter paper wetted with 1 ml water, 10 wheat seeds or 20 cress seeds per filter paper); or (b) dry seeds were spread onto filter paper moistened with 1 ml of one of the three pFR A test solutions. Experiments were performed in triplicate, and appropriate controls with water instead of pFR A solutions were included. After incubation in the dark at 20°C for five days, the lengths of stem and longest root of each wheat seedling and the total length of each cress seedling were measured. The lengths of all seeds in a given Petri dish were summed, and the mean 'total length' and the corresponding standard deviation of three parallel experiments were determined.

When the above tests confirmed a growth inhibitory action of pFR A, the reversibility of the inhibition by $FeSO_4$ and the influence of light (3000 lux for 14 h per day) were investigated. These studies were performed at the 100 ppm ($\mu g\ ml^{-1}$) pFR A level with 'dry' cress seedlings. Two $FeSO_4$ concentrations were tested: 145 and 725 ppm. Other conditions were as described above.

Proferrorosamine production in E. persicinus

To prove that *E. persicinus* is capable of proferrorosamine synthesis, strain ATCC 35998 was grown in the following pFR-biosynthesis stimulating minimal medium: 4 gl^{-1} $K_2HPO_4 \cdot 3\ H_2O$, 0.2 gl^{-1} $MgSO_4 \cdot 7\ H_2O$, 2 gl^{-1} $(NH_4)_2SO_4$, 2 gl^{-1} sucrose, and 2 gl^{-1} asparagine. The bacteria were grown in a 500 ml batch for 5 days at 20°C under vigorous stirring using a Teflon-coated magnetic stir bar.

Proferrorosamine extraction

Cells of *E. persicinus* were removed by centrifugation at 17 $700 \times g$ and filtration through Nalgene 0.45 μm filter units (Fisher Scientific Co., Pittsburgh, Pennsylvania). Sterile filtration was slow due to the presence of copious amounts of bacterial slime. The filtrate (100 ml) was passed over a strong cation exchange column (Dowex AG 50WX8 (Bio-Rad Laboratories, Hercules, California), 20–50 mesh, H^+-form, 18×2 cm), the column washed with water (100 ml) and the pFRs eluted with 10% aqueous NH_3 (100 ml). The eluate was concentrated to 5 ml via rotary evaporation (at < 40°C) to remove most of the ammonia, then analyzed by reversed-phase high-performance liquid chromatography (RP–HPLC), RP–LC–electrospray ionization mass spectrometry (RP–LC–ESI–MS), and tandem mass spectrometry (RP–LC–ESI–MS/MS).

RP–HPLC analysis

The presence of pFRs in the cation exchange extract was corroborated by treatment with a dilute ferrous sulfate solution until no more ferrorosamine was formed, and subsequent analysis by RP–HPLC (C_{18}, 220×2.1 mm column plus 30 mm precolumn) and peak-controlled on-the-fly acquisition of absorbance spectra (190–590 nm). A Hewlett Packard (Palo Alto, California) 1090M workstation with diode array absorbance detector, a gradient of 0–50% acetonitrile in 0.1% TFA over 50 min, a flow rate of 0.2 ml min^{-1}, and an injection volume of 25 ml were used. Two ferrorosamines were detected at retention times 24.7 (FR A) and 27.4 min (new FR), respectively.

RP–LC–ESI–MS analysis

Mass spectrometric analysis of the bacterial culture supernatant was performed using a microgradient LC–ESI–MS system that has been described elsewhere (Feistner *et al*. 1993, Davis *et al*. 1995). Briefly, the LC–MS system consisted of a self-constructed capillary column (250 µm I.D. × 15 cm, packed with 5 µm 300 Å C_{18}), a home-built low-pressure gradient mixer, an ISCO (Lincoln, Nebraska) 100D high pressure syringe pump, an on-line variable wavelength absorbance detector (Kratos Spectroflow 757), an Analytica (Branford, Connecticut) ESI source, and a Finnigan (San Jose, California) TSQ700 triple stage quadrupole mass analyzer. Samples were loaded at 20 µl and chromatographed at 2 min/µl. Injection volumes were of the order of a few microliters. For the ferrous sulfate-treated extract, iron hydroxide/oxide particles had to be thoroughly removed by centrifugation to avoid clogging of the capillaries. A linear gradient of 1–61% acetonitrile in 0.1% TFA over 45 min was used. ESI was assisted with a gas sheath of nitrogen (30 psi) and a liquid sheath of 2-methoxy-ethanol (ME) (2 µl min^{-1}). Collision activation was achieved with 2 mtorr argon using a collision energy of 10–15 eV.

Pathogenicity of E. rhapontici *and* E. persicinus *towards wheat and cress*

In the first experiments, spring wheat seeds, variety 'Penawawa', were punctured twice in their groove with a

25G5/8 needle. To also test a different kind of injury, some seeds were scratched (1–2 mm) on their back; other seeds were left intact as controls. Ten seeds each were placed in 9 cm I.D. Petri dishes on filter paper that had previously been soaked with 1 ml of various bacterial inocula (see below) or just water (control for iron-depletion study) or 10^{-5} M ferric citrate (control for iron-supplementation study). Inocula were prepared as follows: *E. rhapontici*, *E. persicinus*, and *E. herbicola* (type strains) were grown overnight in either MMB (for iron-depletion study) or LB medium (for iron-supplementation study), upon which they reached cell densities (OD_{620}) of 0.22, 0.36, 0.10 (MMB) and 1.91, 2.02, 1.93 (LB), respectively. Parallel batches on MMB medium were allowed to grow for 5 days (final OD_{620} of 0.44, 0.39, 0.59, respectively), then analyzed for proferrioxamines (pFOs) and proferrorosamines (pFRs). While the culture supernatant of *E. herbicola* contained the expected pFOs, neither *E. rhapontici* nor *E. persicinus* had produced pFOs or pFRs under these culture conditions (low oxygen concentration). Any FRs detected in our seedling assay must therefore have been produced *in situ*. Bacteria were pelleted at $2000 \times g$, washed twice with 10 ml water, and resuspended in appropriate volumes of water (iron-deficient, MMB grown cultures) or 10^{-5} M ferric citrate (iron-replete, LB grown cultures) to achieve inocula with an OD_{620} of 0.2–0.3 throughout. Each experimental condition was tested in triplicate. The Petri dishes were wrapped with Parafilm to keep the seeds moist, and germination was monitored daily until day five, when the lengths of roots and stems were measured. The lengths of all stems, all longest roots, and all roots in each Petri dish were summed to give a 'total length', and the mean 'total length' and its standard deviation for the triplicate experiments were determined.

In a second series of experiments, winter wheat seeds (15 g, cv. 'Astron' from Strube Co., Söllingen) and cress seeds (1 g, cv. 'Kröbel') were incubated for 3.5 h in 70 ml bacterial suspension each in 500 ml Erlenmeyer flasks on a rotary shaker at 20°C and 160 rpm. Silica beads (Merck Co., no. 1925, particle size 1–3 mm; (Merck KG, Darmstadt, Germany) were added to cause injury in the presence of the bacteria. Inocula were prepared from the cell growth at 20 h on equal numbers of Petri dishes (nutrient glucose agar or King B-agar; Rudolph 1990) by suspending the bacteria in sterile distilled water to give a final OD_{660} of 0.36–0.42. Following inoculation, the seeds were placed on wet filter paper in 9 cm I.D. Petri dishes (30 cress seeds and 20 wheat seeds, respectively, per plate). All experiments were carried out in triplicate. Seedling development was evaluated after 4 days (cress) and 7 days (wheat) by measuring the total length of cress seedlings, and the lengths of the stems and the longest roots of the wheat seedlings, respectively.

In a third experiment, experiment series 2 was repeated but without the injury-causing silica beads. Other conditions were largely identical, except that the bacteria were grown for 20 h in yeast extract/glucose liquid medium (Rudolph 1990), pelleted by centrifugation, and resuspended in deionized water to an OD_{600} of 0.3, which was determined to be equivalent to $3–4 \times 10^8$ CFU/ml, and that the seeds were incubated in 100 ml bacterial suspension for 3 h at 130 rpm.

Results

Effect of proferrorosamine on seed germination

pFR A clearly inhibited germination and seedling development in both wheat (Figure 2) and cress (Figure 3). The effect was concentration dependent and entirely reversible with the addition of equi-

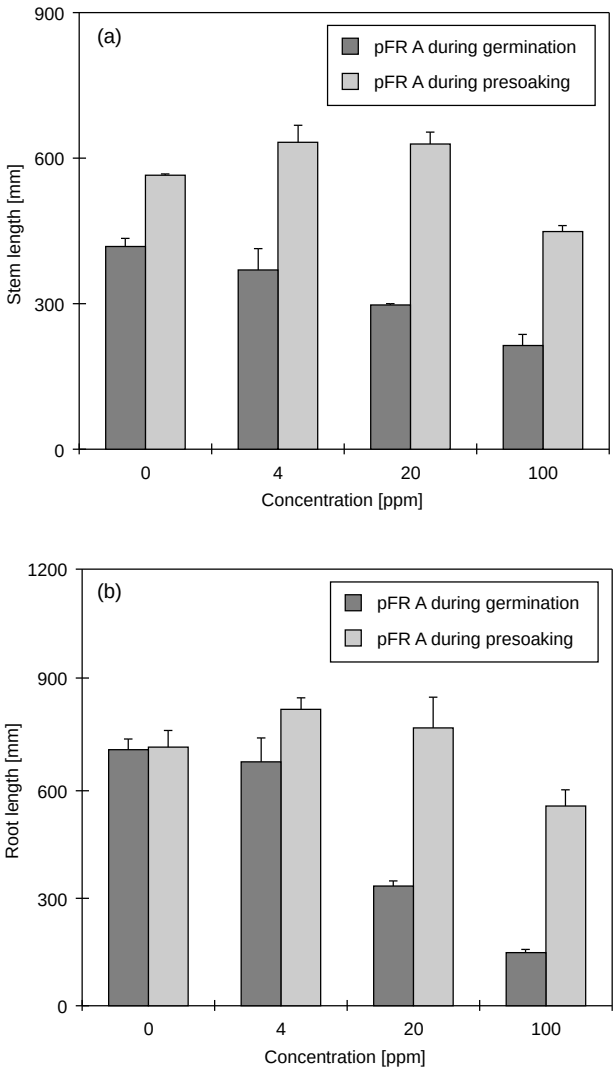

Figure 2. Effect of pFR A on the germination of wheat. Shown is the mean total (summed) length of: (a) 10 wheat seedling stems; and (b) 10 wheat seedling roots as a function of the pFR A concentration. pFR A was applied either during the entire germination period or only during a presoaking step. The error bars refer to the standard deviation of three parallel experiments.

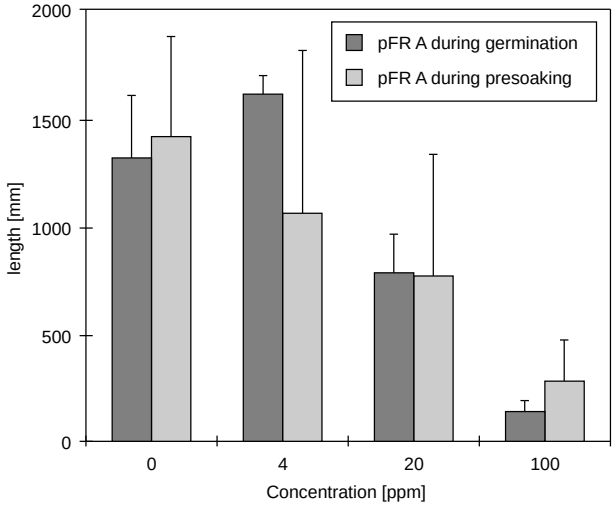

Figure 3. Effect of pFR A on the germination of cress. Shown is the mean total (summed) length of 20 cress seedlings as a function of the pFR A concentration. pFR A was applied either during the entire germination period or only during a presoaking step. The error bars refer to the standard deviation of three parallel experiments.

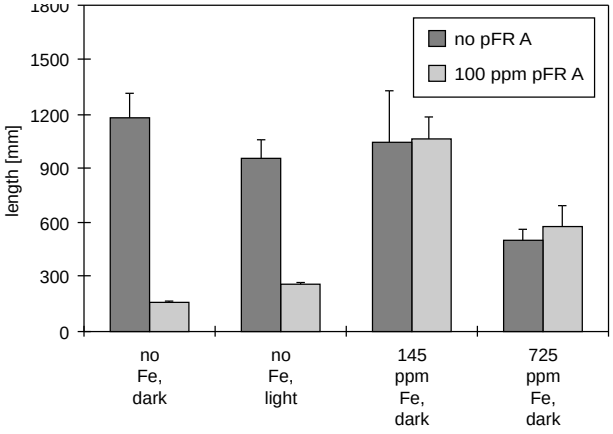

Figure 4. Influence of light and iron supplementation on pFR A-induced growth inhibition. Shown is the effect on the mean total (summed) length of 20 cress seedlings; error bars indicate the standard deviation of three parallel experiments.

molar or larger concentrations of ferrous sulfate (Figure 4). Inhibition was less in light than in the dark (Figure 4). Maximum inhibition was observed when pFR A was present during the entire experiment, i.e. in those experiments where dry seeds germinated on pFR A solution. Presoaking with pFR A clearly inhibited germination of cress, but caused only little inhibition of wheat.

Specifically, at the 100 ppm level, there was a 50% growth inhibition for wheat stems and 80% growth inhibition for the longest wheat roots, when seedlings grew on pFR A, compared with only 21 and 23% reductions in stem and root growth, respectively, when the seeds were presoaked with pFR A. The growth inhibition observed for presoaked cress was of the order of 80%, whereas cress seedlings growing in the presence of 100 ppm pFR A showed a 90% growth inhibition. The threshold level for inhibition of both wheat and cress seemed to be about 10 ppm. In the repeat experiment (Figure 4), the growth inhibition of cress at the 100 ppm pFR A level was found to be 86% when seeds were kept in the dark, and 72% when the seeds were exposed to light. There was no growth inhibition due to pFR A in the presence of 145 or 725 ppm $FeSO_4$.

Ferrorosamines are resistant to hydrogen peroxide

A solution of FR A, freshly prepared from dilute ferrous sulfate and pFR A, gave no immediate visible reaction when treated with H_2O_2. In the control experiment with only ferrous sulfate, immediate oxidation and precipitation of ferric hydroxide/oxide occurred, as expected. That is not to say that pFR A was entirely stable against hydrogen peroxide; decomposition was noticable after several hours. Nevertheless, the protection of Fe(II) from oxidation by pFRs seems sufficient to significantly slow down the generation of reactive hydroxyl radicals via the Haber–Weiss cycle (Cadenas 1989).

E. persicinus *produces proferrorosamines*

Upon addition of ferrous sulfate, the cation extract of the culture filtrate of *E. persicinus* turned pink red. The corresponding pigment could be separated by RP–HPLC into two components, both of which showed the typical visible absorbance spectrum of FRs (λ_{max} 556). Molecular mass analysis by RP–LC–ESI–MS established the earlier eluting pigment as FR A ($[3\ pFR\ A + (Fe - 2H) + H]^+$ *m/z* 625) (Figure 5a). The second FR was characterized by a singly charged molecular ion of nominal mass 755 (Figure 5b); a possible composition is suggested below. Molecular mass information was much easier to obtain by ESI–MS than by FD–MS. Interestingly, the ESI spectra showed not only the singly and doubly ($[3\ pFR + (Fe - 2H) + 2\ H]^{2+}$ *m/z* 313 and 378) charged molecular ions, but also strong $[M + H + n\ ME]^{2+}$ ions (M = 3 pFR + (Fe – 2H); ME = 2-methoxyethanol; *n* = 2, 4; *m/z* 389, 465, 454, 530). The preferred adduction of even numbers of matrix molecules seems to indicate some unique

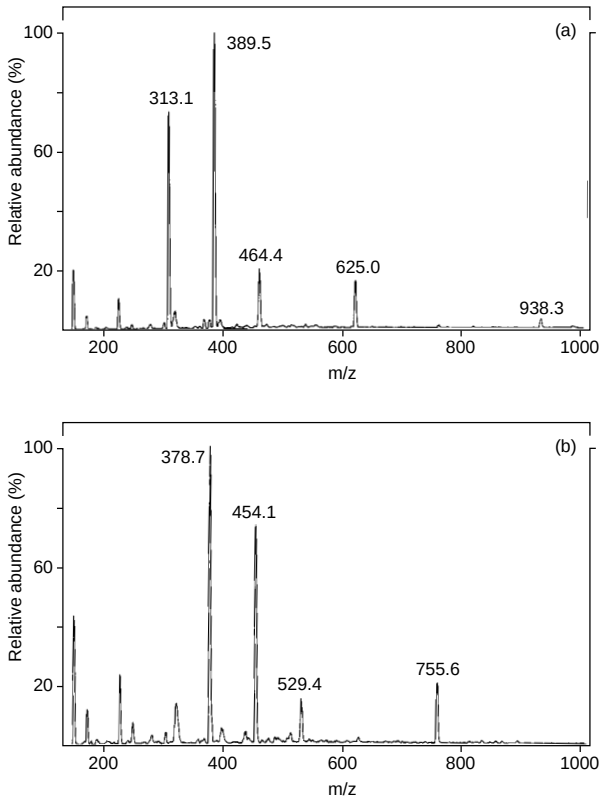

Figure 5. ESI–MS spectra of FRs from *E. persicinus*. (a) FR A and (b) a novel FR. Spectra were obtained by on-line RP–LC–ESI–MS analysis at low mass resolution (peaks are 1–2 u wide). The labeled peaks correspond to the singly (*m/z* 625 and 755) and doubly charged (*m/z* 313 and 378) molecular ions, to a cluster ion consisting of a singly plus a doubly charged molecular ion (313 + 625 = 938 u), and to the adduction of 2-methoxy-ethanol to the doubly charged molecular ions (*m/z* 389, 465, 454, and 530; see text).

complexation chemistry; however, its significance is not clear. The adduction of matrix molecules was confirmed through collisional activated dissociation (CAD), upon which the ME-adduct ions fell apart to yield mostly doubly charged ferrorosamines.

RP–LC–ESI–MS analysis of the non-iron treated *E. persicinus* extract provided evidence for $[M + H]^+$ ions (*m/z* 191 and 265) of free pFR A and pFR B, with pFR B eluting later than pFR A. Consistent with the presence of carboxylic acid functions, the corresponding tandem mass spectra showed $[M + H - H_2O - CO]^+$ ions (loss of 46 u) at *m/z* 145 and 219, respectively. In addition the CAD spectrum of *m/z* 191 showed a product ion with *m/z* 118, whereas the CAD spectrum of *m/z* 265 showed a product ion with *m/z* 120; the compositions of these ions still need to be determined. CAD of the doubly charged molecular ion for the ferrorosamine with mass 754 led to fragment ions of *m/z* 247, 219, and 120, where *m/z* 247 formally corresponds to the protonated molecular ion for anhydro-pFR B. The ferrorosamine of mass 754 may thus consist of one molecule each of pFR A, anhydro-pFR B, and pFR B (190 + 246 + 264 + (Fe – 2 H) + H = 755 u), although this needs to be confirmed by further experiments. Nevertheless, the capability of *E. persicinus* to produce proferrorosamines A and B is established.

Phytopathogencity of Erwinia spp.

Other than expected from the pFR inhibition assay (Figure 2), no inhibition of wheat root growth was observed in any of the three different inhibition tests with live erwiniae. In contrast, the inhibitory effect of pFR A had been largest on the roots of wheat seedlings. Infection with erwiniae had an inhibitory effect on the growth of wheat stems, but consistent with the observed effect of pFR A on wheat stems, only a moderate inhibition was observed. The reason for this tissue specificity towards infection is not known but may perhaps be due to the fact that stems provide a stimulating environment for proferrorosamine synthesis whereas roots do not.

Specifically, in the first series of experiments, where wheat seeds were injured with a needle and external iron was withheld, some stem growth inhibition was seen with *E. rhapontici* (26% inhibition) (Figure 6), and some of the seeds actually turned pink, indicating that the positive control was working. This compares to a stem growth inhibition of 30% in the presence of 20 ppm pFR A (Figure 2). Since in this study the erwiniae intentionally had not been primed to produce pFRs, perhaps no larger effect was to be expected. Under iron supplementation conditions, seeds infected with *E. rhapontici* showed less growth inhibition (14% inhibition compared to the uninfected control), thus corroborating our presumption that the virulence mechanisms of *E. rhapontici* involve iron deprivation.

Under iron-replete conditions, wheat seeds infected with *E. persicinus* did not show any sign of growth inhibition. *E. herbicola*, which was included in the experiment as negative control, actually seemed to stimulate growth. A slight growth inhibition was seen with *E. persicinus* under iron-deprived conditions, but this was not significant, since *E. herbicola*-infected seeds showed the same degree of inhibition. Also, none of the seeds infected with *E. persicinus* turned pink. Because *E. persicinus* caused neither a signficant growth inhibition nor pink discoloration of the wheat seeds, we concluded

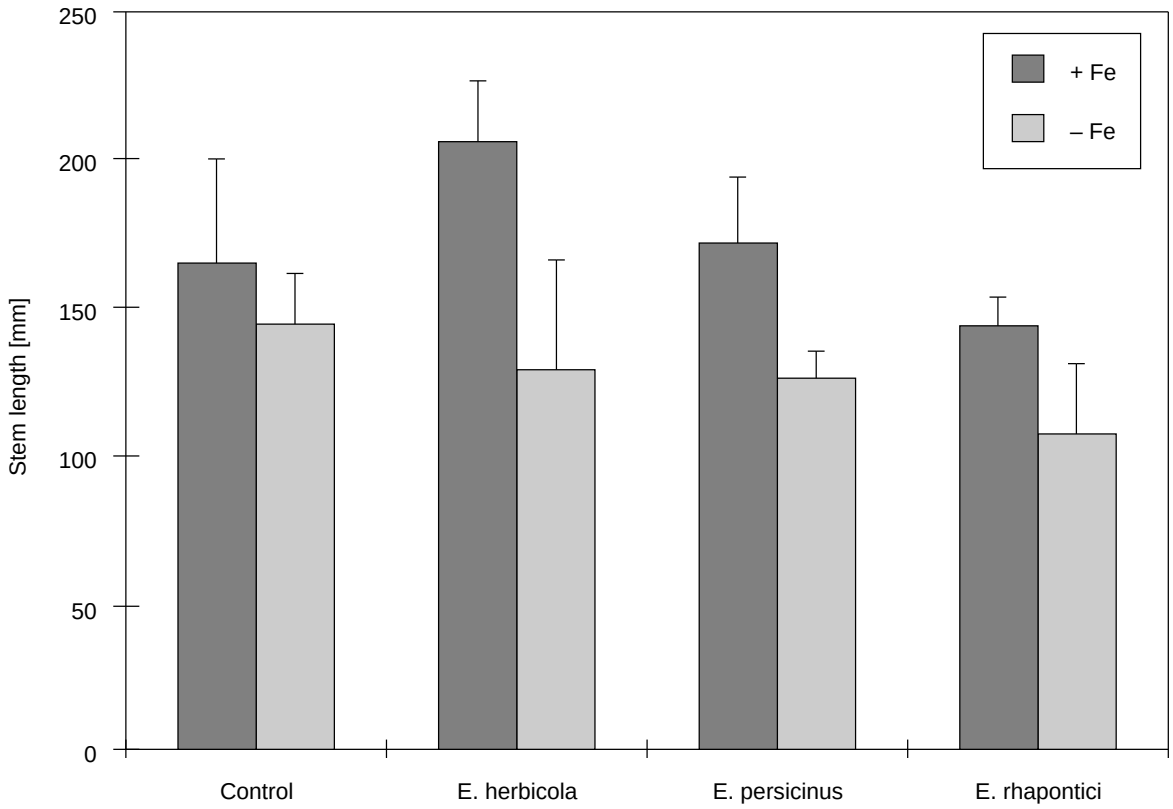

Figure 6. Effect of various erwiniae on the germination of wheat seeds. Shown is the mean total (summed) length of 10 seedling stems; root growth was not affected by the bacteria. The error bars refer to the standard deviation of three parallel experiments. +Fe, iron-replete; –Fe, iron-deplete.

that *E. persicinus* was not pathogenic towards wheat under the conditions of our experiment. All the above results were observed with groove-punctured seeds. Intact seeds or seeds that had been scratched on the back of the groove developed normally in the presence of *E. persicinus* (stem lengths of 108 and 106% of control seeds, respectively).

In the second series of experiments, where wheat and cress seeds were injured during inoculation by shaking the seeds in the presence of silica beads, the germination of wheat seeds was significantly retarded neither by *E. persicinus* nor by two strains of *E. rhapontici* (< 10% stem growth inhibition; not shown). For cress seedlings, the largest effect seen was for *E. persicinus* (11% inhibition); however, the injury by itself seemed to have a more deleterious effect than the infection (Figure 7).

In the third series of experiments, which was a repeat of series 2, but with uninjured seeds, the effect of infection on cress seedlings became more obvious. As Figure 7 shows, *E. persicinus* (26% inhibition) was even more virulent than *E. rhapontici* (16% inhibition). At the same time these results indicate that prior injury is not essential for pathogenicity towards cress. For reasons unknown, the growth of wheat seedling was apparently stimulated under these conditions (1–20% for the two *E. rhapontici* strains; 27–38% for *E. persicinus*, not shown).

Discussion

Our studies clearly reveal for the first time that proferrorosamines are phytotoxic since a remarkably high inhibition and retardation of germination was observed. The threshold concentration for phytotoxicity of 10 ppm is in the range of many non-host specific toxins (Rudolph 1976). That the growth inhibition by pFR A or *E. rhapontici* can be reversed by external iron confirms our hypothesis that the phytotoxic effect is due, at least in part, to deprivation of iron. The higher sensitivity of cress versus wheat seeds with respect to the chelators as well as the

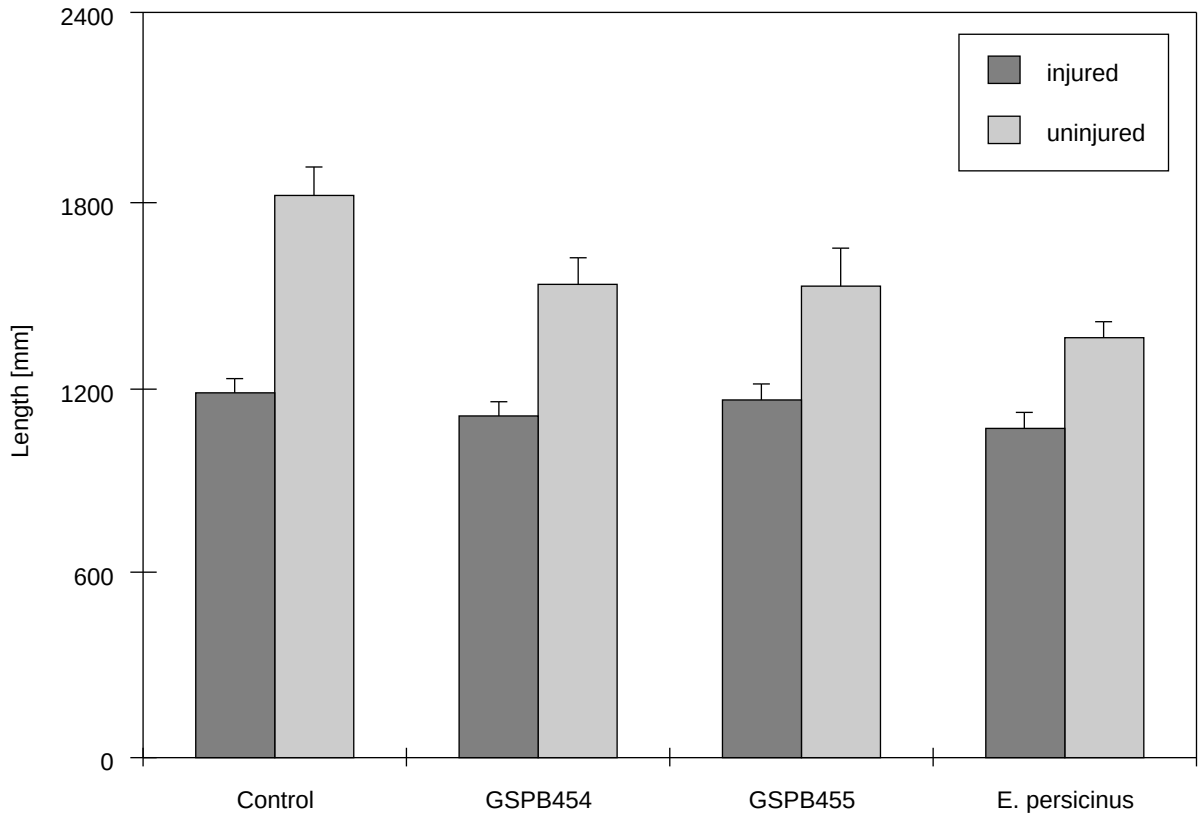

Figure 7. Effect of various erwiniae on the germination of cress seeds. Shown is the mean total (summed) length of 30 cress seedlings for each treatment. The error bars refer to the standard deviation of three parallel experiments. GSPB 454 and GSPB 455 are two strains of *E. rhapontici*.

producing bacteria may be due to a thinner seed coat or perhaps due to smaller amounts of stored iron.

A second possible mode of action of the proferrorosamines in the host/parasite interaction may be derived from the finding that FR A is relatively stable towards oxidation. The Haber–Weiss cycle and the accompanying release of reactive oxygen radicals are believed to be active during the hypersensitive response (Goodman and Novacky 1994), a defense reaction of infected or injured plants, as well as during active plant growth. By interfering with the Haber–Weiss cycle, pFR-producing bacteria may protect themselves from oxidative attack. Furthermore, since pFR production is critically dependent on oxygen concentration (Vande Woestyne *et al*. 1991), it would not be surprising if pFR synthesis were to be triggered by the oxidative defense reaction of the host plants.

Whereas *E. rhapontici* has been recognized as a plant pathogen for a wide variety of different plant species for decades (Millard 1924; Bradbury 1986), phytopathogenicity of *E. persicinus* was only speculated upon from its capacity to produce proferrorosamines (Feistner *et al*. 1992). Our data on the growth inhibition of cress seedlings by *E. persicinus* indicate a possible role as a phytopathogenic bacterium. Additional evidence for the phytopathogenicity of *E. persicinus* has come from an independent study (Brenner *et al*. 1994), which showed that *E. persicinus* is synonymous to *Erwinia nulandii*. *Erwinia nulandii* is a known, although rare, pathogen of bean (*Phaseolus vulgaris*) (Schuster *et al*. 1981). Further studies with a wide range of plant species at different growth stages will be required to unambiguously prove the phytopathogenicity of *E. persicinus* and to determine its host range.

Our inability to demonstrate growth inhibition of wheat seedlings by *E. persicinus*, and the rather small effects seen with *E. rhapontici* should be interpreted with care. Under natural conditions the developing seeds within the growing ears or fruits can be much more intensively colonized or even invaded by the bacteria than would have been possible in our experments. Impaired germination of wheat seeds by natural bacterial infestation with *E. rhapontici* has been reported by Luisetti and Rapilly (1967).

In the presence of iron, the well known epiphyte *E. herbicola*, which was used as a negative control in these experiments, seemed to stimulate the growth of wheat seedlings. This may perhaps be explained by the fact that plants are able to assimilate iron through the uptake of any ferrioxamines that are produced by *E. herbicola*.

The results of our studies are entirely consistent with the hypothesis that *E. rhapontici* exerts its growth inhibiting effect on wheat and peas by withholding essential iron via complexation to ferrorosamine, which presumably cannot be utilized by these plants. We thus consider proferrorosamines microbial virulence factors. Whether proferrorosamines are essential for pathogenicity remains to be shown by experiments with specific mutants of *E. rhapontici* and *E. persicinus* that are deficient in pFR synthesis (Vande Woestyne and Verstraete 1992).

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Chemical Abstracts Registry numbers

Proferrorosamine A, 3,4-dihydro-5-(2-pyridinyl)-2H-pyrrole-2-carboxylic acid [26927–08–2]; ferrorosamine A, tris[3,4-dihydro-5-(2-pyridinyl)-2H-pyrrole-2-carboxylato-N^1,N^5] hydrogen ferrate(1-) [80124–41–0]; siderochelin A & B, 3,4-dihydro-4-hydroxy-5-(3-hydroxy-2-pyridinyl)-4-methyl-2H-pyrrole-2-carboxamide [77550–88–0].

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