Plant and Soil **144**: 167–176, 1992.

PLSO 9414

Effect of *Pseudomonas fluorescens* on the root exudates of two tomato mutants differently sensitive to Fe chlorosis

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Received 26 November 1991. Revised March 1992

Key words: iron nutrition, *Pseudomonas fluorescens*, rhizosphere microorganisms, tomato

Abstract

Plants with different Fe-mobilization properties are known to differ in the amount and kind of Fe-reducing and Fe-chelating compounds exuded by their roots. Although rhizosphere bacteria are known to affect the exudation of organic compounds by the plant roots, their effect on the root exudates of plants differing in Fe-mobilization properties is not known. We studied the effect of *Pseudomonas fluorescens*, on the exudation of sugars and organic and amino acids by roots of an iron chlorosis-resistant (T3238FER) and a chlorosis-susceptible (T3238fer) tomato mutant. Under sterile conditions two tomato mutants grew equally well and did not differ in the total amount of sugars and organic acid exuded by their roots. More amino acids, however, were exuded by the roots of T3238FER than T323fer. Mutants differed in the amount of oxalic acid and the amino acids Ala, Asp, Gaba, Gln, Gly, His, Hyl, Ile, Leu, Lys, Phe, Pro, and Val exuded by their roots into sterile rooting media. Addition of *P. fluorescens* to the rooting medium did not affect the growth of T3238FER but stimulated the root growth of chlorosis-susceptible T3238fer, reduced the amounts of glucose, arabinose and fructose but increased the amount of sucrose, reduced the amounts of fumaric, malic and oxalic acid but increased the amounts of citric and succinic acid in the rooting media of both mutants. *P. fluorescens* resulted in the following changes in the amino acids in the rooting media: reduced the amounts of Gly, Leu, and Lys in T3238FER, and of Asp, Gln, Hyp, and Ile in T3238fer, and increased the amounts of Cys, Glu, His, Hyp, Ile, Phe and Tyr in T3238FER and of Ala, Glu, His, Phe, and Ser in T323fer—in cases more than 40-fold. These differential effects of *P. fluorescens* in altering the pattern of organic and amino acids compounds with some Fe-chelating properties detected in the rooting medium of these two mutants may indicate that the differences in Fe-chlorosis susceptibility of these tomato mutants may be the result of, or modified by, the interactions between plant roots and rhizosphere microorganisms. We postulate that the Fe-chlorosis susceptibility in plants may be the product of the interactions between soil microorganisms and plant roots, and may not be solely related to the plant *per se*.

Introduction

Rhizosphere microorganisms and plant roots interact in several ways in affecting nutrient mobilization in the rhizosphere. For example, plants exude organic substances by their roots which could affect the availability of inorganic plant nutrients such as Mn (Jauregui and Reisenauer, 1982), P (Barea et al., 1976) and Fe (Boyer et al., 1989; Elgala and Amberger, 1988; Marschner et al., 1987; Mozafar, 1991; Takagi et al., 1984). Microorganisms present in the rooting media may stimulate the exudation of organic substances by plant roots (Barber and Lynch,

1977; Gardner et al., 1983; Hale and More, 1979; Kraffczyk et al., 1984; Laheurte et al., 1990; Martin, 1977; Prikryl and Vancura, 1980; Schwab et al., 1983), in cases by a factor 2–7 (see Gardner, Barber and Parbery, 1983), or increase the decomposition of organic root exudates (Kraffczyk et al., 1984). Microorganisms, such as *Pseudomonas fluorescens*, may themselves exude organic substances (siderophores) into the rhizosphere which may in turn affect the availability or uptake of certain mineral nutrients, especially that of iron (Hemming, 1986; Jurkevitch et al., 1988; Loper and Buyer, 1991; Lynch, 1982).

Studies on Fe mobilization have been mostly concerned with the measurement of the total amount of Fe-reducing phenolics (Olsen and Brown, 1980) and Fe-chelating phytosiderophores (Takagi, 1984) exuded by the plant roots. The interactions between rhizosphere microorganisms and Fe-chlorosis susceptibility in plants, especially with respect to the role of microorganisms in changing the composition of root exudate, however, have been very little investigated.

Tomato (*Lycopersicon esculentum* Mill.) mutants T3238FER and T3238fer are chlorosis-resistant and -susceptible plants, respectively, which in a non-sterile rooting medium differ in their ability to reduce rhizosphere pH and exudation of Fe-reducing compounds by their roots (Camp, Jolley and Brown, 1987; Olsen et al., 1981). We cultured the roots of these mutants and noted that under sterile conditions they possess similar Fe uptake properties. Addition of *P. fluorescens* to the culture medium was noted to differently affect the Fe uptake by the roots of these mutants, an observation which was interpreted to be due to the effect of siderophores exuded by the bacteria on the uptake of Fe by the plant roots (Duss et al., 1986). No information is available, however, on whether the presence of these bacteria in the rooting media of these plants may also affect the exudation of Fe-mobilizing compounds by the plant roots themselves, thereby affecting the Fe efficiency of the plants. Thus, we studied the effect of *P. fluorescens* on the composition of root exudates of two tomato mutants differing in Fe-chlorosis susceptibility. Since the presence of microorganisms on the surface of plant leaves is also suspected to affect the exudation of organic compounds from plant roots (Hale and Moore, 1979), we conducted these experiments by growing the plants in specially constructed glass chambers under fully enclosed sterile conditions.

Material and methods

Tomato seeds were surface-sterilized by a 30-second pretreatment with 80% ethanol, soaking the seeds under mild vacuum and gentle shaking in a 1% Ca hypochlorite solution containing 0.001% Tween 80, and rinsing the seeds with three portions of sterile distilled water. Seeds were then allowed to germinate at 25°C on a 0.8% Difco-Bacto agar for 5–7 days. Sterile seedlings were then transferred to the glass growth chambers for further growth for 6–8 weeks.

Ten glass growth chambers as shown in Figure 1 were constructed which could be autoclaved and reused in a series of experiments. They consisted of top (shoot) and bottom (root) chambers made of Duran glass (Schott Ruhrglas, Bayreuth, Germany) which could be connected to each other with quick-release clamps. The root chamber consisted of a flat-flange beaker (neck inside diameter of 10 cm, height of 27 cm, and two-liter volume) to which three 10-mm-diameter threaded glass tubings were mounted on the sides. These ports were used for a) sampling of the nutrient solution (for sterility tests), b) addition of sterile distilled water or nutrient solution to replenish water loss through evapotranspiration, and c) aeration of the nutrient solution. Threaded glass tubings were closed with screw caps (having an aperture in the center) and Teflon-protected septa. Sterile aeration (50 mL m^{-1} chamber) of nutrient solution was achieved by inserting a glass tubing (with a Teflon tube at its end) through one of the septa, and connecting the tubing to 0.2 μ-diameter hydrophobe Millipore filter. The top chamber was a flat-bottom flat-flange flask (neck diameter of 10 cm, height of 26 cm and volume of 6 liter) to which one threaded glass tube was connected to the side to act as an exit port for the air introduced into the nutrient solution. The air exit port was also connected to a 0.2 μm hydrophobe

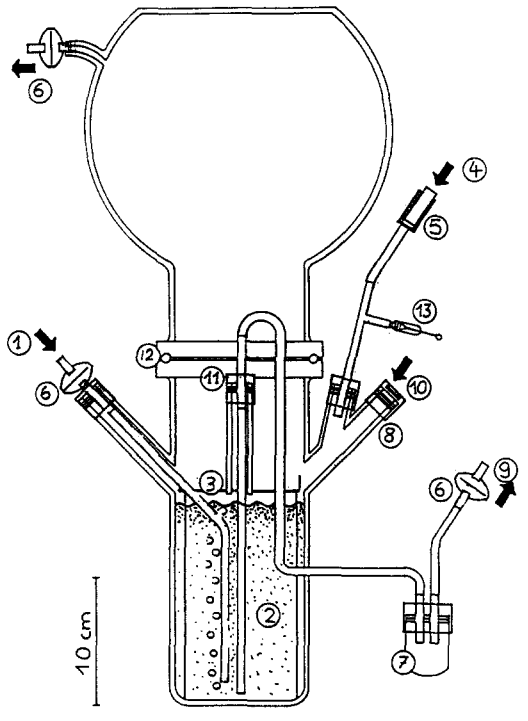

Fig. 1. Glass chamber used for growing the tomato plants. 1: aeration tube, 2: nutrient solution. 3: rack for holding the plants over the nutrient solution, 4: nutrient solution replenishment tube, 5: filter to sterilize the nutrient solution, 6: filter to sterilize the aeration gas, 7: container to take nutrient solution sample, 8: input port for adding bacterial culture, 9: to vacuum pump, 10: septum at the port of entry for bacterial culture, 11: exit port for taking nutrient solution samples, 12: clamp to fasten the top and bottom chambers, 13: safety valve to prevent the build-up of excess pressure.

Millipore filter to assure against the entry of microorganisms into the flask despite the presence of positive pressure inside the chambers caused by the aeration of the chambers. A pressure security valve (BCS/213, 1/4 inch, Serva Technik AG, Glattbrug, Switzerland) (set at 0.1–0.2 MPa) was connected to one of the bottom chamber ports to guard against the build up of excess pressure within the glass vessels. Chamber parts were autoclaved before use and all handlings were performed inside a sterile laboratory hood.

The root chambers were each filled with 1500 mL autoclave-sterilized nutrient solution with the following composition (μmol L^{-1}): KNO_3, 2500; $Ca(NO_3)_2$, 2500; $MgSO_4$, 1000; KH_2PO_4, 500; KCl, 50; Fe-EDTA [ethylenediaminetetraacetic acid], 20; H_3BO_3, 25; $MnSO_4$, 1; $ZnSO_4$, 1; $CuSO_4$, 0.5 and Na_2MoO_4, 0.5. A 9-cm-diameter stainless-steel screen mounted on top of a 17-cm-tripod placed into the root chamber acted as support for the plants to grow on. The screen stood ca. 5 mm above the level of the nutrient solution in the bottom chamber.

Five sterile seedlings were planted in the screen by passing their roots through the screen holes. As the plants grew, roots grew deeper into the nutrient solution and plants grew more or less upright by partly supporting each other or by leaning against the wall of the top chamber. Autoclaved nutrient solution was added to the chamber through a sterile filter as needed (2–3 times in 8 weeks) to replenish the water used through evapotranspiration. The glass chambers were placed in a walk-in growth chamber with a 16-hour photoperiod (from a combination of Osram tungsten and Sylvania cool white fluorescent lamps with photon flux density of 710 μE m^{-2} S^{-1}) and a day/night temperature of 25/15°C. Light intensity inside the top chamber was lower by 70 μE m^{-2} s^{-1} than the outside value. The temperature inside the chamber was, however, higher than the temperature outside, but never more than 3.1°C. This indicates that the rate of chamber aeration was enough to carry away the major portion of heat which was produced inside the glass chambers through the greenhouse effect. Experiments were replicated at least three times. The data was analyzed by Student's Multiple-Range Test.

Sterility of the nutrient solutions and growth of *P. fluorescens* in the nutrient solutions were tested by periodically withdrawing 10 mL of nutrient solution from the bottom chamber by applying vacuum (through a sterile air filter) to a sampling tube whose end was at the bottom of the chamber. The number of colony-forming units (cfu) was measured by using Merck standard nutrient agar No. 1 (pH = 7.5) with the following composition (g L^{-1}): special peptone, 15.6; yeast extract, 2.8; NaCl, 5.6; D-glucose, 1; agar, 12. Colonies were counted after incubating the plates at 25°C for 7 days.

Effect of *P. fluorescens* (La 2777, International Center for Information and Distribution of Type Cultures, Lausanne, Switzerland) on root exu-

date was studied by culturing the plants for 6–8 weeks under sterile conditions and then inoculating the rooting media of some chambers with the bacteria (about 5×10^6 per chamber). Plants were allowed to grow for another two weeks and then harvested, and the number of bacteria in the rooting media, number of bacteria in the rhizoplane, growth of the plants and the chemical composition of the rooting media for the organic root exudates were studied. Rhizoplane bacteria were determined, as described by Zogg and Jäggi, 1974. Root samples (10–15 mg dwt) were placed into 25 mL of 0.18% $Na_4P_2O_7$ and were shaken on a rotary shaker (150 rpm) for 15 minutes. The number of bacteria released from the root to this solution was determined as described above.

Root exudates in the nutrient solutions were obtained by first filtering 200 mL of nutrient solution through a 0.2-μm filter to remove possible root-cell debris. Solutions were then frozen with a acetone-dry ice mixture (−80°C) and then freeze-dried under vacuum. These dried root exudates (fraction A) were then stored at −20°C till analyzed. For the analysis of root exudates, a given amount of fraction A as needed (60 mg for the amino acid and 600–1000 mg for the sugar and organic acids) was first dissolved in sufficient volume of distilled water (1.5 mL for amino acids and 6 mL for sugar and organic-acids determinations), extracted for 30 minutes in a water bath at 60°C and then centrifuged for 20 minutes at 5000 rpm. The supernatant (fraction B) was used directly for the analysis of amino acids or was used for the determination of sugars and organic acids, as follows.

The amino acid content of fraction B was measured using an automatic amino acid analyzer (Kontron, Liquimat III) having a Pierce Dionex DC 6A column and the method described by Amado et al. (1983). Prior to analysis, the pH of the extracts was reduced to 2.3 with 6 *N* HCl. A 0.1-mL aliquot was then injected into the analyzer. The buffer flow was 20 mL h^{-1} and the ninhydrin flow was 10 mL h^{-1}. Since the protein content of the extract was less than 0.8% (w/v) it did not interfere with the separation of amino acid, and thus no precipitation of proteins was undertaken.

Sugar and organic acids in the fraction B were first separated with the water/chloroform mixture as described by Pertzsch et al. (1981). The aqueous fraction was vacuum-dried and then dissolved in 80% (v/v) ethanol/water and passed through Analytichem Bond Elut extraction columns (ITC, Basel, Switzerland) containing strong cation (type SCX) and anion (type SAX) resins mounted in series. The neutral fraction (fraction C) passing through the anion exchange resin contained the sugars. The organic acid fraction (fraction D) was eluted from the anion exchange resin with a 1 mol L^{-1} HCl solution. The fractions C and D were first vacuum-dried and then used for analysis of their contents.

Sugars in fraction C were measured by gas chromatography as described by Zürcher et al. (1975), first through siliation of the sugars by adding 0.25 mL pyridine, 0.4 mL STOX, 0.2 mL BSTFA and 0.05 mL 60 mmol L^{-1} NDA (nanodecanoic acid methyl ester) in acetone at 70°C for 30 minutes. The column used was the same as used for the determination of organic acids. Organic acid content of fraction D was measured by the gas chromatography method, as described by Tanaka and Hine (1982) after a 2-h siliation of dry exudates with 0.2 mL BSTFA plus 0.5 mL acetone and 0.05 mL of 6 mmol L^{-1} NDA in acetone as internal standard. One μL of siliated extract was injected into a 0.53-mm-diameter and 10-m-long capillary fused silica column filled with OV-1701 used with a Perkin Elmer Sigma 1 chromatogram having a FID detector.

Results

Growth and root exudates under sterile conditions

Glass growth chambers proved to be suitable for growing tomato under fully sterile conditions for a relatively long period of time. In a total of more than 40 runs, only in two cases were the nutrient solutions in the chambers found to be contaminated as determined by dilution plate counting. Growth of tops and roots of tomato mutants did not differ under sterile conditions (Table 1).

Under sterile condition, tomato mutants did

Table 1. Growth of plant tops and roots (mg plant^{-1}) and exudation of sugars, organic acids and amino acids (μg (mg dry root)$^{-1}$) by two tomato mutants (T3238FER and T3238fer) grown under fully sterile conditions for 8 weeks or under sterile conditions for 6 weeks and 2 weeks in the presence of *P. fluorescens* in the rooting medium

	T3238fer		T3238FER	
	Sterile	+*P. flu.*	Sterile	+*P. flu.*
Tops	914.7a*	953.7a	912.5a	843.4a
Roots	49.7a	67.5b	46.7a	45.8a
Sugars	14.0b	11.5a	14.2b	12.2a
Organic acids	10.1b	8.6a	10.4b	10.6b
Amino acids	0.27a	0.26a	0.30a	0.32a

* Values on the same line followed by different letters are significantly different at the 0.05 level.

not differ in the total amounts of sugars and organic and amino acids exuded by their roots (Table 1). Mutants also did not differ in the kinds of sugars and organic acids (with the exception of oxalic acid) exuded by their roots (Figs. 2, 3). Mutants, however, differed considerably in the kind of amino acids exuded by their roots (Fig. 4). In general, T3238FER excluded more Ala, Asp, Hyl, Hyp, Leu, Lys, Phe, Pro, and Ser, but less Cys, Ile, Gln, Gly, His, and Val than did T3238fer. Some of the more pronounced differences were, as follows. T3238FER exuded (μg mg root^{-1}) 21 times more Phe (6.3 vs. 0.3), six times more Lys (7.5 vs. 1.22), five times more Hyp (57.3 vs. 10.9), and four times more Gaba (14.6 vs 3.14), but 50% less Gly (68.8 vs 128.4), three times less Val (8.4 vs. 26.0) and Gln (5.3 vs. 16.6) than did T3238fer.

Effect of bacteria on growth and composition of root exudates

Addition of *P. fluorescens* to the rooting medium did not affect the growth of T3238FER but significantly increased root growth of the chlorosis-susceptible T3238fer. The number of suspended bacteria in the nutrient solution was not affected by the type of tomato mutant growing in the solution. The rhizoplane of T3238fer, however, contained significantly more bacteria than that of T3238FER (Table 2).

P. fluorescens reduced the amount of glucose and fructose and increased the amount of sucrose in the rooting media (Fig. 2), which may be due to a possible conversion of glucose and fructose to sucrose by these bacteria. Addition of *P. fluorescens* to the rooting media significantly reduced the total amount of organic acids in the rooting media of T3238fer (Table 1), which was

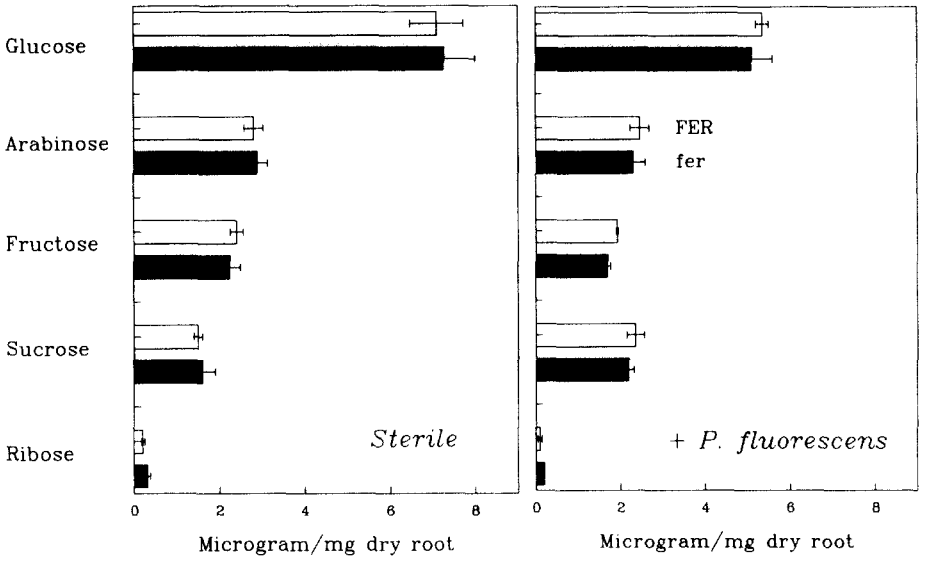

Fig. 2. Sugars detected in the nutrient solution of two tomato mutants T3238FER and T3238fer (FER and fer, respectively) grown under sterile conditions or in the presence of *P. fluorescens*.

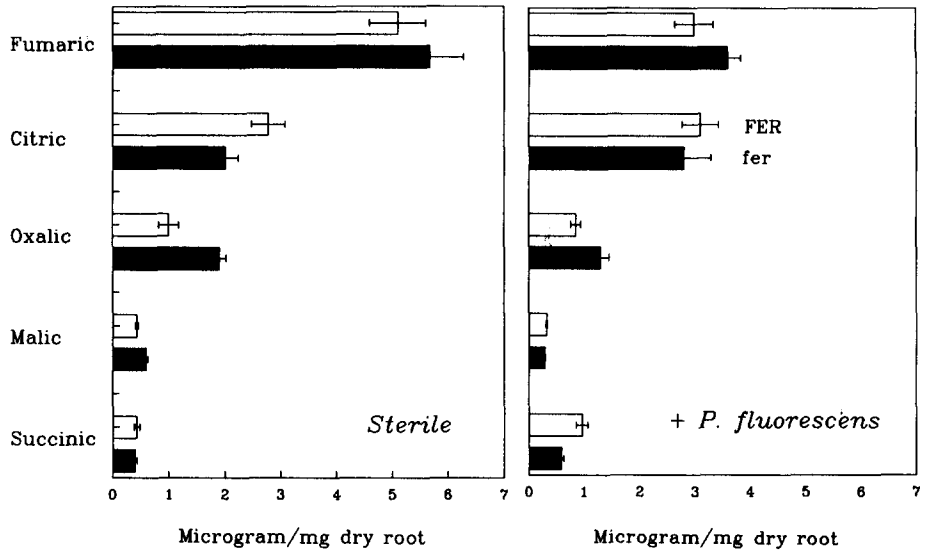

Fig. 3. Organic acids detected in the nutrient solution of two tomato mutants T3238FER and T3238fer (FER and fer respectively) grown either under sterile conditions or in the presence of *P. fluorescens*.

Table 2. Growth of *P. fluorescens* (log cfu mL^{-1}) in the nutrient solution (suspended bacteria) and in the rhizoplane of tomato mutants two weeks after the inoculation of the rooting media

Mutant	Suspended	Rhizoplane
T3238FER	7.65a*	5.45a
T3238fer	8.86a	7.83b

* Values in each column followed by different letters are significantly different at 0.05 level.

mainly due to reduction in the amounts ($\mu g\, mg\, root^{-1}$) of fumaric acid (5.1 vs. 3.6), oxalic acid (1.9 vs. 1.3) and malic acid (0.6 vs. 0.3). Succinic acid was the organic acid whose content was most differently affected by the presence of *P. fluorescens* in the rooting media of these two mutants differently susceptible to Fe chlorisis. Thus, although *P. fluorescens* increased the amount ($\mu g\, mg\, root^{-1}$) of succinic acid by only 50% (0.4 vs. 0.6) in the rooting media of chlorosis-susceptible T3238fer, it nearly doubled (0.43 vs. 0.94) the amount of succinic acid in the rooting media of chlorosis-resistant T3238FER (Fig. 3).

The pattern of amino acids detected in the rooting media was strongly affected by the presence of *P. fluorescens* in the rooting media which was more pronouncedly observed in the rooting media of T3238FER (Fig. 4). Thus, for example, addition of bacteria reduced the amounts of Gly, Leu, and Lys, but increased the amounts of Cys, Glu, His, Hyp, Ile, Phe, and Tyr in the rooting media of T3238FER. Addition of *P. fluorescens* to the rooting media of T3238fer, however, reduced the amounts of Asp, Gln, Hyp, and Ile but increased the amounts of Ala, Glu, His, Phe, and Ser (Fig. 4). Some more pronounced effects of the presence of bacteria on amino acid composition of rooting media are, as follows. Addition of bacteria to the rooting medium of T3238FER reduced the amount ($ng\, mg\, root^{-1}$) of Leu by more than forty times (8.12 vs. 0.17), but increased the amount of Ile by two (5.8 vs. 12.1) and of Tyr by more than four times (2.45 vs. 11.4). The addition of bacteria to the rooting medium of chlorosis-susceptible T3238fer, however, reduced the amount ($ng\, mg\, root^{-1}$) of Asp by about 50% (8.7 vs. 4.4), of Ile by more than three times (0.2 vs. 2.5), but increased the amount of Glu by two times (6.0 vs. 12.7), and that of Phe by more than eight times (0.3 vs. 2.6).

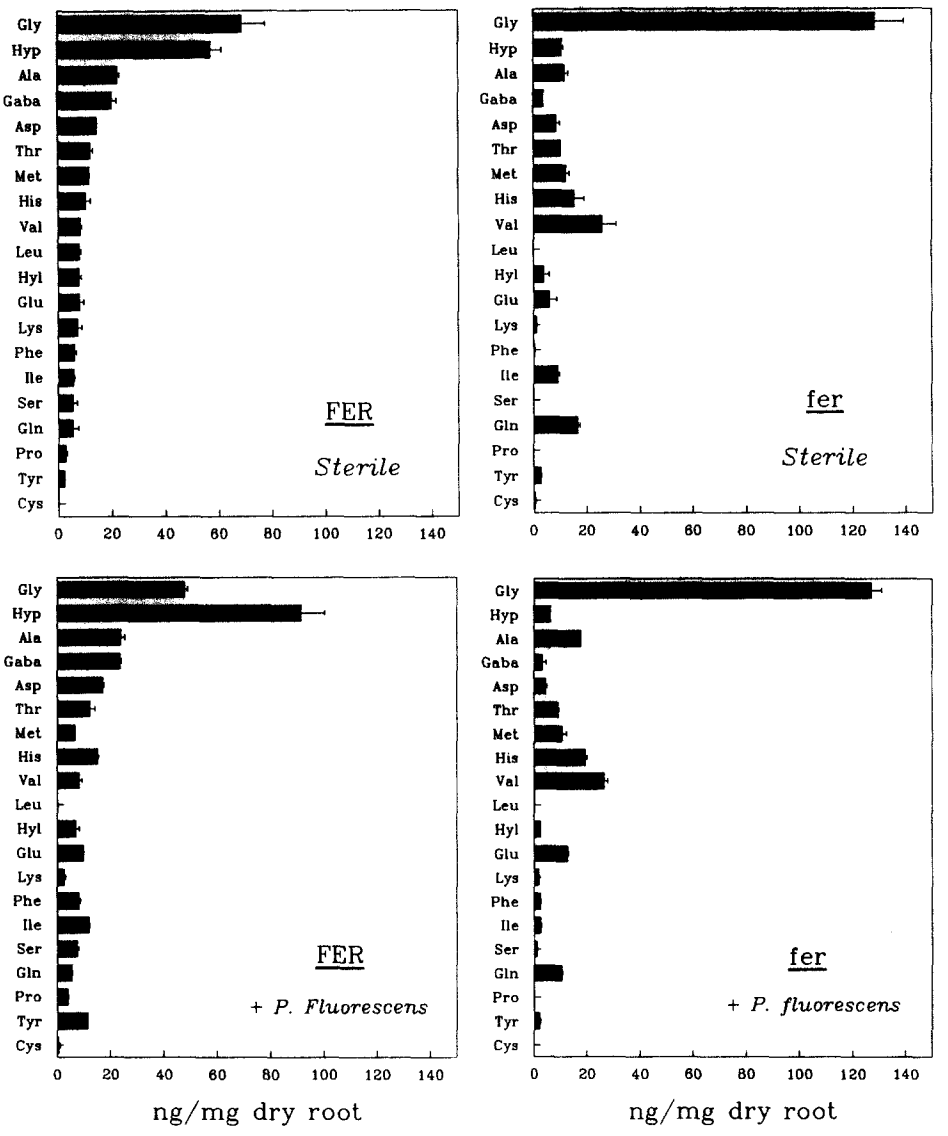

Fig. 4. Amino acid detected in the nutrient solution of two tomato mutants T3238FER and T3238fer (FER and fer, respectively) grown either under sterile conditions or in the presence of *P. fluorescens*. Amino acids are arbitrary arranged in the decreasing order of their amounts in the root exudates of mutant FER, when grown under sterile conditions. Amino acids tested were Gly (glycine), Hyp (hydroxyproline), Ala (alanine), Gaba (γ-butyric acid), Asp (aspartic acid), Thr (threonine), Met (methionine), His (histidine), Val (valine), Leu (leucine), Hyl (hydroxyalanine), Glu (glutamic acid), Lys (lysine), Phe (phenylalanine), Ile (isoleucine), Ser (serine), Gln (glutamine) Pro (proline), Tyr (tyrosine) and Cys (cysteine).

Discussion

Studies on the composition of root exudates have been mostly performed by growing plants in test tubes containing agar or a small volume of nutrient solution (Boutler et al., 1966; Kreutzer and Baker, 1975) which, often due to technical limitations, were not aerated (Boulter et al., 1966; Kraffczyk et al., 1984) and thus could not have been conducted for a longer period of time. Long-term sterile experiments for studying plant root exudates are thus very limited in number

(Barber, 1967; Häggquist et al., 1984; Liljeroth et al., 1990; Richter et al., 1968; Trofymov et al., 1980). The glass chamber system we have designed enables long-term studies with whole plants under fully sterile condition. The possibility of sterile aeration of the plant roots, sampling of the nutrient solution during plant growth, and replenishment of water lost by evapotranspiration, without having to open the system, are the advantages of this system as compared to some of the methods used by others.

The increased root growth of chlorosis-susceptible T3238fer, but not of chlorosis-resistant T3238FER, by *P. fluorescens* reported here (Table 1) and the previously reported stimulating effect of this bacterium on the uptake of Fe by the roots of both tomato mutants (Duss et al., 1986) may or may not be related phenomena. *P. fluorescens* in the rooting medium altered the amounts of numerous organic and amino acids detected in the medium. These alterations may be the result of stimulation of root exudation, decomposition and/or consumption of some components of root exudates by the bacteria, or exudation of some compounds by the bacteria themselves, the contribution of each of these factors being hard to judge at this time. The effect of *P. fluorescens* in increasing the concentration of succinic acid, but decreasing that of malic acid, in the rooting media of both mutants, and increasing the concentrations of Hyp, Ile, Tyr in the rooting media of T3238FER, but not of T3238fer, deserves particular attention, especially with respect to their role in determining the degree of susceptibility of these mutants to Fe chlorosis. We note that a stimulating effect of some other strains of *Pseudomonas* on the growth of several other plants, including tomato, has been assumed to be partly due to the effect of root bacterization with *Pseudomonas* on suppressing the activity of deleterious rhizobacteria (Peer, 1990).

Iron mobilization by dicotyledonous plants is presently attributed to the ability of their roots to a) lower the rhizosphere pH, b) reduce Fe, c) exude Fe-reducing and Fe-chelating compounds into the rhizosphere (Brown and Jolly, 1989; Römheld, 1987). The capability to lower the soil pH was, however, observed not to be closely related to the Fe solubilization in the soil close to the roots, and it was therefore concluded that root exudates and/or microbial activity are more important in Fe solubilization than are pH changes in the rhizosphere (Youssef and Chino, 1990). Organic as well as amino acids possess Fe-mobilizing properties (Chaberek and Martell, 1959) and their addition to soil was observed to increase the availability of soil iron (Omran et al., 1982). The calculations of Inskeep and Comfort (1986) show that amino acids and organic acids in the root exudates could make a significant contribution to the concentration of total soluble Fe(III) in the rhizosphere. Thus, the data presented here on the differential effect of *P. fluorescens* on the kind of amino acid and organic acids exuded by tomato mutants differently susceptible to Fe chlorosis may indicate that the degree of Fe-chlorosis susceptibility in tomato may be the end-product of interactions between plant roots and soil microorganisms and may not be fully predetermined by the plants per se.

The mechanism by which the presence of *P. fluorescens* in the nutrient solution increased root growth of only T3238fer is hard to explain, unless these bacteria exuded some 'growth factors' which were stimulating root growth of chlorosis-susceptible, but not of chlorosis-resistant tomato plants. The larger number of bacteria estimated to be present in the rhizoplane of T3238fer as compared to that of T3238FER points so such a possibility which needs to be studied.

Acknowledgements

This work was funded by the Swiss National Fund (Project No. 3.096-0.81). Seeds of tomato mutants were kindly supplied by E V Wann, US Department of Agriculture, Vegetable Laboratory, Charleston, South Carolina.

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