Effect of fusaric acid and phytoanticipins on growth of rhizobacteria and *Fusarium oxysporum*

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Abstract: Suppression of soilborne diseases by biocontrol agents involves complex interactions among biocontrol agents and the pathogen and between these microorganisms and the plant. In general, these interactions are not well characterized. In this work, we studied (*i*) the diversity among strains of fluorescent *Pseudomonas* spp., *Bacillus* spp., and *Paenibacillus* sp. for their sensitivity to fusaric acid (FAc) and phytoanticipins from different host plants, (*ii*) the diversity of pathogenic and nonpathogenic *Fusarium oxysporum* isolates for their sensitivity to phytoanticipins, and (*iii*) the influence of FAc on the production of pyoverdine by fluorescent *Pseudomona*s spp. tolerant to this compound. There was a great diversity in the response of the bacterial strains to FAc; however, as a group, *Bacillus* spp. and *Paenibacillus macerans* were much more sensitive to FAc than *Pseudomonas* spp. FAc also affected production of pyoverdine by FAc-tolerant *Pseudomonas* spp. strains. Phytoanticipins differed in their effects on microbial growth, and sensitivity to a phytoanticipin varied among bacterial and fungal strains. Biochanin A did not affect growth of bacteria, but coumarin inhibited growth of *Pseudomonas* spp. strains and had no effect on *Bacillus circulans* and *P. macerans*. Conversely, tomatine inhibited growth of *B. circulans* and *P. macerans.* Biochanin A and tomatine inhibited growth of three pathogenic isolates of *F. oxysporum* but increased growth of three nonpathogenic *F. oxysporum* isolates. Coumarin inhibited growth of all pathogenic and nonpathogenic *F. oxysporum* isolates. These results are indicative of the complex interactions that can occur among plants, pathogens, and biological control agents in the rhizosphere and on the root surface. Also, these results may help to explain the low efficacy of some combinations of biocontrol agents, as well as the inconsistency in achieving disease suppression under field conditions.

Key words: biocontrol, pyoverdines, fluorescent *Pseudomonas* spp., *Bacillus* spp., *Paenibacillus* spp., plant–microbe interactions.

Résumé : L'élimination des infections transmises par le sol par l'utilisation d'agents de biocontrôle suppose des interactions complexes entre les agents de biocontrôle et le pathogène et entre ces microorganismes et la plante-hôte. De façon générale, ces interactions ne sont pas bien caractérisées. Notre étude a porté sur : (*i*) la diversité parmi des souches de *Pseudomonas* spp. fluorescentes, de *Bacillus* spp. et de *Paenibacillus* sp. provenant de différentes plantes-hôtes quant à leur sensibilité à l'acide fusarique (FAc) et les phytoanticipines, (*ii*) les différences de sensibilité aux phytoanticipines d'isolats de *Fusarium oxysporum* pathogènes ou non-pathogènes et (*iii*) l'influence de FAc sur la production de pyoverdine par les *Pseudomonas* spp. fluorescents tolérants à ce composé. On a noté une grande différence dans la réponse des souches bactériennes à la FAc mais, comme groupes, les *Bacillus* spp. et *Paenibacillus macerans* étaient beaucoup plus sensibles à la FAc que les *Pseudomonas* spp. La FAc affectait aussi la production de pyoverdine chez les souches de *Pseudomonas* spp. tolérantes à la FAc. Les phytoanticipines avaient des effets différents sur la croissance microbienne et la sensibilité à ces phytoanticipines variait entre les souches bactériennes et fongiques. La biochanine A n'affectait pas la croissance des bactéries mais la coumarine inhibait la croissance des souches de *Pseudomonas* spp. et par contre n'avait pas d'effet sur *Bacillus circulans* et *P. macerans*. À l'inverse, la tomatine inhibait la croissance de *B. circulans* et de *P. macerans*. La biochanine A et la tomatine inhibaient la croissance de trois isolats pathogènes de *F. oxysporum*, mais favorisaient la croissance de trois isolats de *F. oxysporum* non-pathogènes. La coumarine inhibait la croissance de tous les isolats de *F. oxysporum* pathogènes ou non-pathogènes. Les résultats de cette étude confirment la complexité des interactions qui peuvent survenir entre les plantes, les pathogènes et les agents de contrôle biologique dans la rhizosphère et à la surface des racines. Ces résultats pourraient aussi aider à expliquer la faible efficacité de certaines combinaisons d'agents de biocontrôle et le manque de réussite dans l'élimination des maladies dans les conditions retrouvées au champ.

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Mots clés : biocontrôle, pyoverdines, *Pseudomonas* spp. fluorescents, *Bacillus* spp., *Paenibacillus* spp., interaction plante–microorganisme.

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Introduction

Rhizobacteria (such as fluorescent *Pseudomonas* spp. and *Bacillus* spp.) and fungi (such as nonpathogenic *Fusarium oxysporum* Schlechtend.:Fr.) are potential biocontrol agents for the suppression of soilborne diseases. Over the last decade, there have been many studies on the interactions between microbes and plants, as well as among microbes themselves; some of these are involved in disease suppression, which enabled the identification of microbial and plant metabolites playing a role in these complex interactions (Cook et al. 1995; Van Loon et al. 1998). Among those metabolites, much emphasis has been placed on pyoverdines and phytoalexins. Pyoverdines are the siderophores produced by fluorescent pseudomonads. Several studies have stressed the role played by pyoverdine-mediated iron competition in the microbial antagonism performed by biocontrol strains against some pathogens (Duijff et al. 1999; Lemanceau et al. 1993; Loper and Buyer 1991). Also, pyoverdine is involved in the systemic resistance induced by *Pseudomonas fluorescens* in tobacco (*Nicotiana tabacum* L.) (Maurhofer et al. 1994). Other studies have underlined the involvement of pyoverdine-mediated iron uptake in the ecological fitness of different strains of fluorescent pseudomonads (Höfte et al. 1992; Mirleau et al. 2000; Raaijmakers et al. 1995).

Phytoalexins are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms (Dakora and Phillips 1996; VanEtten et al. 1994; Van Peer et al. 1991). Excluded from this definition are low molecular weight, antimicrobial compounds that are present in plant tissue prior to microbial infection or that are produced after infection solely from pre-existing constituents. These latter antimicrobial compounds are known as phytoanticipins (Dakora and Phillips 1996; VanEtten et al. 1994). Recently, the phytoalexin concept was expanded because many isoflavonoids phytoalexins (the most widely studied class of phytoalexins) were shown to serve as signal molecules during infection of plant roots by symbiotic microbes. Also, mutualistic bacteria and arbuscular mycorrhizal fungi were shown to enhance the production or exudation of phytoalexins (reviewed in Dakora and Phillips 1996). In addition, some biocontrol agents were shown to induce production of phytoalexins. For example, inoculation of carnation (*Dianthus caryophylium* L.) with a biocontrol strain of fluorescent pseudomonads accelerated accumulation of phytoalexins, which accounted for the systemic resistance induced by the bacteria (Van Peer et al. 1991). Similarly, inoculation of cucumber (*Cucumis sativus* L.) with *Pseudomonas putida* protected the plant against *Pythium aphanidermatum* (Edson) Fitzp. as result of the systemic induction of phytoalexins (Ongena et al. 2000). Also, production of phytoalexins was demonstrated to increase after prior inoculation of chickpea (*Cicer arietinum* L.) seedlings with nonpathogenic isolates of *F. oxysporum* (inducers), and this was correlated with a delay on the onset of symptoms and reduction of Fusarium wilt development (Cachinero 1996; Cachinero et al. 2002). Phytoalexins and phytoanticipins produced by plants are likely to be released from roots into soil or sloughed from root border cells (Dakora and Phillips 1996), and this may influence the rhizospheric microflora. In fact, Stevenson et al. (1997), Cachinero (1996), and Cachinero et al. (2002) showed that phytoalexins produced by chickpea after root infection by *F. oxysporum* Schlechtend.:Fr. f.sp. *ciceris* (Padwick) Matuto & K. Sato, the Fusarium wilt agent, or by nonpathogenic *F. oxysporum* isolates were present in, or released from, the root.

Fusaric acid (FAc) is a toxic compound produced by plant pathogenic and nonpathogenic isolates of *Fusarium* spp. FAc produced by pathogenic isolates in plants behaves as a nonspecific phytotoxin that could contribute to wilt and root rot diseases of various crops caused by *F. oxysporum* (e.g., Chakrabarti and Basu Chaudhary 1980; Kern 1972; Remotti and Löfter 1996; Toyoda et al. 1988). Similarly, production of FAc by soilborne nonpathogenic isolates of *Fusarium* spp. suggests that it may play a role in the interactions among soil- and rhizosphere-inhabiting microorganisms. Actually, FAc was shown to impair the metabolism of the biocontrol strain *P. fluorescens* CHA0. In fact, FAc repressed bacterial synthesis of the antibiotic 2,4-diacetylphloroglucinol, which is a key factor of the microbial antagonism determined by this strain (Duffy and Défago 1997*a*; Schnider-Keel et al. 2000).

The four compounds referred to above, i.e., pyoverdines, phytoalexins, phytoanticipins, and FAc, may have a toxic effect on the rhizosphere microflora, including biocontrol agents. These deleterious effects can influence both the population density and activity of the biocontrol agents and, thus, impair their efficacy in disease suppression. To be efficient, biocontrol agents should optimally be tolerant to toxic compounds produced either by the soil microflora, such as pyoverdine and FAc, or by the host plant, such as phytoalexins.

The aims of this study were to assess (*i*) the diversity among strains of fluorescent *Pseudomonas* spp., *Bacillus* spp., and *Paenibacillus* sp. for their sensitivity to FAc and phytoanticipins (biochanin A, coumarin, and tomatine), (*ii*) the diversity of *F. oxysporum* isolates for their sensitivity to phytoanticipins (biochanin A, coumarin, and tomatine), and (*iii*) the influence of FAc on the synthesis of pyoverdine by fluorescent pseudomonads strains tolerant to this compound.

Materials and methods

Bacterial isolates and inoculum production

Thirty-four bacterial isolates, representing a range of species comprising biocontrol agents were used in this study. Of these 34 isolates, 29 are species and biovars of fluorescent *Pseudomonas* spp., 4 are *Bacillus* spp., and 1 is *Paenibacillus*

Table 1. Bacterial strains used in this study, listed by source, location, and year of isolation.

Isolate*	Source	Location†	Year
P. fluorescens bv. II C7	flax rhizoplane	Châteaurenard	1986
P. fluorescens bv. II C7R12‡	—	—	—
P. putida Li	—	—	—
P. putida WCS 358§	potato rhizosphere	—	—
P. fluorescens bv. VI A6	bean rhizosphere	—	—
P. putida bv. A DS 131	soil	Dijon	1992
Pseudomonas (IT) DS 824¶	soil	Dijon	1992
P. putida bv. A DS 1026	soil	Dijon	1992
P. putida bv. A DLR 223	flax rhizosphere	Dijon	1992
P. putida bv. A DLR 228	flax rhizosphere	Dijon	1992
P. fluorescens bv. IV DLR 426	flax rhizosphere	Dijon	1992
P. fluorescens bv. IV DLRP 214	flax rhizoplane	Dijon	1992
P. fluorescens bv. IV DLE 411 J	flax endophytic	Dijon	1992
P. putida bv. A DLE 3216	flax endophytic	Dijon	1992
P. chlororaphis bv. V DTR 133	tomato rhizosphere	Dijon	1992
P. fluorescens bv. IV DTR 335	tomato rhizosphere	Dijon	1992
P. putida bv. A DTRP 621	tomato rhizoplane	Dijon	1992
P. putida bv. A CS 111	soil	Châteaurenard	1994
P. putida bv. A CS 413	soil	Châteaurenard	1994
P. fluorescens bv. II CS 611	soil	Châteaurenard	1994
P. fluorescens bv. II CLR 711	flax rhizosphere	Châteaurenard	1994
P. fluorescens bv. II CLRP 812	flax rhizoplane	Châteaurenard	1994
P. fluorescens bv. II CLE 513	flax endophytic	Châteaurenard	1994
P. fluorescens bv. II CTR 212	tomato rhizosphere	Châteaurenard	1994
P. fluorescens bv. II CTR 1015	tomato rhizosphere	Châteaurenard	1994
P. fluorescens bv. II CTRP 112	tomato rhizoplane	Châteaurenard	1994
P. fluorescens bv. V RGAF 19	chickpea rhizosphere	Santaella (A)	1993
P. fluorescens bv. IV RG 22	chickpea rhizosphere	Santaella (A)	1993
P. fluorescens bv. II RG 26	chickpea rhizosphere	Santaella (A)	1993
B. circulans RGAF 6a	chickpea rhizosphere	Santaella (A)	1993
B. circulans RGAF 6b	chickpea rhizosphere	Santaella (A)	1993
B. megaterium RGAF 12	chickpea rhizosphere	Santaella (A)	1993
B. megaterium RGAF 51	chickpea rhizosphere	Santaella (A)	1993
Paenibacillus macerans RGAF 101	chickpea rhizosphere	Santaella (B)	1993

Note: *P.*, *Pseudomonas*; *B.*, *Bacillus*.

*Strains of *Pseudomonas* spp. had been previously characterized to species and biovars (Lemanceau et al. 1995), and those of *Bacillus* spp. and *Paenibacillus* spp. were characterized to species by Landa et al. (1997).

†Soils from Châteaurenard and Dijon (France) are suppressive (Alabouvette et al. 1987) and conducive (Lemanceau et al. 1995), respectively, to Fusarium wilt diseases. Soils A and B from Santaella (Spain) have been used for Fusarium wilt resistance screening for more than 15 years. Soil A is infested with different races of *F. oxysporum* f.sp. *ciceris*. Soil B has been under barley monoculture for the same time period (Landa et al. 1997).

‡Spontaneous rifampicin-resistant mutant of *P. fluorescens* bv. II C7 [21].

§*Pseudomonas putida* WCS 358 was kindly provided by Dr. P.A.H.M. Bakker, University of Utrecht, Netherlands.

¶Intermediate type *P. fluorescens–P. putida*.

macerans (formerly *Bacillus*). The isolates were obtained from rhizosphere and bulk soils sampled from a Fusarium wilt suppressive soil (10 isolates) located at Châteaurenard, France, as well as two Fusarium wilt conducive soils located at Dijon, France (12 isolates), and Santaella, Spain (8 isolates) (Alabouvette et al. 1987; Eparvier et al. 1991; Jiménez-Díaz et al. 1989; Landa et al. 1997). *Pseudomonas putida* WCS 358 is a reference strain kindly provided by Dr. P.A.H.M. Bakker (University of Utrecht, Netherlands). The 34 bacterial isolates and their characteristics are listed in Table 1. Bacterial cells produced in Luria–Bertani (LB) broth were stored in 25% glycerol at –80°C. Active cultures were obtained by streaking bacteria from stock cultures onto potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich., U.S.A.) (*Bacillus* and *Paenibacillus* isolates) slants or on King's B (KB) agar (*Pseudomonas* isolates) slants and incubating at 28°C for 2 days.

Inocula were obtained by pipetting 5 mL of sterile distilled water (SDW) onto KB agar or PDA slants and gently shaking them to wash cells free from the agar surface. For each isolate, 400 µL of the cell suspension was transferred to 50 mL of succinate medium (SM), pH 7.0, (Meyer and Abdallah 1978) for *Pseudomonas* isolates or potato dextrose broth (PDB, Difco Laboratories) for *Bacillus* spp. and

Table 2. Isolates of *Fusarium oxysporum* used in this study, listed by pathogenicity, source, and location.

Isolate	Pathogenicity	Source	Location*
F. oxysporum Fo 47	nonpathogenic	soil	Châteaurenard
F. oxysporum Fo 9009	nonpathogenic	chickpea roots	Santaella
F. oxysporum Fo 90105	nonpathogenic	chickpea roots	Santaella
F. oxysporum f.sp. *ciceris* Foc 7802	pathogenic, race 0	chickpea	Santaella
F. oxysporum f.sp. *ciceris* Foc 8012	pathogenic, race 5	chickpea	Santaella
F. oxysporum f.sp. *lycopersici* Fol 32	pathogenic	FPFS†	—

*Soils from Châteaurenard and Dijon (France) are suppressive (Alabouvette et al. 1987) and conducive (Lemanceau et al. 1995), respectively, to Fusarium wilt diseases. Soils A and B from Santaella (Spain) have been used for Fusarium wilt resistance screening for more than 15 years. Soil A is infested with different races of *Fusarium oxysporum* f.sp. *ciceris*. Soil B has been under barley monoculture for the same time period (Landa et al. 1997).

†Flore Pathogéne Faune du sol culture collection, Institut National de la Recherche Agronomique (INRA), Dijon, France.

Paenibacillus isolates in 150-mL Erlenmeyer flasks. Cultures were incubated on a rotary shaker at 150 rpm and 25°C for 24 h. Cells were harvested and washed twice in SDW by centrifugation (11 000 × *g*, 20 min) to remove residual metabolites and any traces of nutrients and resuspended in 10 mL of SDW. The number of cells per millilitre was determined by measuring absorbance at 600 nm using standard curves.

Fusarium oxysporum isolates and inoculum production

Six monoconidial isolates of *F. oxysporum* were used in this study (Table 2). Of the six isolates, two (Fo 9009 and Fo 90105) are nonpathogenic *F. oxysporum* shown effective in the suppression of Fusarium wilt of chickpea (Hervás et al. 1997, 1998), one (Fo 47) is nonpathogenic to tomato (*Lycopersicum esculentum* Mill.) (Alabouvette et al. 1987; Fuchs et al. 1999), two (*F. oxysporum* f.sp. *ciceris* Foc 7802 and Foc 8012) are pathogenic to chickpea (Jiménez-Díaz et al. 1989; Trapero-Casas and Jiménez-Díaz 1985), and one (*F. oxysporum* Schlechtend.:Fr. f.sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans. Fol 32) is pathogenic to tomato. *Fusarium oxysporum* isolates were stored in 50% glycerol at –80°C or in sterile soil tubes at 4°C. Active cultures were obtained by placing small drops or soil aliquots from stock cultures onto PDA and incubating for 5 days at 25°C and a 12-h photoperiod of fluorescent and near-UV light at 36 $\mu E \cdot m^{-2} \cdot s^{-1}$.

To obtain inocula for experiments, isolates were grown on PDA slants incubated under the same conditions as above. Cultures were vortexed and microconidia suspended in SDW were filtered through a 40-µm mesh. Then, 400 µL of the microconidia suspension was added to 50 mL of minimal medium (MM) (Correll et al. 1987) in 150-mL Erlenmeyer flasks and incubated on a rotary shaker at 150 rpm and 25°C for 6 days. Cultures were filtered as previously described, and microconidia in the filtrate were pelleted, washed twice in SDW by centrifugation (11 000 × *g*, 10 min), and resuspended in 10 mL of SDW. The concentration of microconidia in the suspensions was determined with a haemocytometer (Blaubrand, Brand, Germany), adjusted with SDW, and stored at 4°C until use (less than 3 h).

Effect of fusaric acid on bacterial growth

FAc, or 5-butylpicolinic acid (Sigma-Aldrich Company Ltd., Dorset, U.K.), was used in this study. FAc was dissolved in SDW to a final concentration of 1 mg/mL and filtered through a sterile 0.22-µm Millipore filter (Millipore Corp., Bedford, Mass.).

The effect of FAc on growth of the fluorescent pseudomonad isolates was studied using sterile flat-bottom 96-well microtiter plates. Ten microlitres of appropriate FAc dilutions in SDW were added to wells containing 200 µL of a bacterial suspension of 10^6 colony forming units (CFU)/mL to obtain a final FAc concentration of 0, 12.5, 25, 50, 100, 200, 300, 400, and 500 µg/mL in a total volume of 210 µL. Wells with 10 µL of SDW and 200 µL of the bacterial cell suspension in sterile SM served as controls. Cultures were incubated at 25°C for 50 h. Bacterial growth in a well was monitored at time 0 and at 3-h intervals by measuring absorbance at 590 nm with a Thermomax microplate reader (Molecular Devices, Sunnyvale, Calif., U.S.A.). There were four replications (wells) for each bacterial isolate–FAc concentration combination. The experiment was repeated twice. From this experiment, FAc-tolerant isolates were selected and further screened at the following FAc concentrations: 0, 12.5, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/mL, as previously described. This experiment was repeated once.

The effect of FAc on growth of *Bacillus* spp. and *Paenibacillus* sp. isolates was studied with an experimental approach similar to that used for fluorescent pseudomonads but using cell suspensions (10^6 CFU/mL) in sterile PDB and lower concentrations of FAc (1–10 µg/mL). Cultures were incubated at 30°C for 65 h. The experiment was repeated twice.

Effect of culture media and fusaric acid on growth and production of pyoverdine by fusaric acid-tolerant fluorescent pseudomonads

Five *P. fluorescens* isolates, three *P. putida* isolates, and one *Pseudomonas chlororaphis* isolate capable of growing at FAc concentrations higher than 100 µg/mL were selected to study the effect of FAc on growth and siderophore production. *Pseudomonas fluorescens* PL1 was used as a nonsiderophore producer. This latter isolate is a pyoverdine-lacking mutant of *P. fluorescens* C7R12 obtained by random insertion of a Tn*5* transposon (Mirleau et al. 2000).

For each isolate, 400 µL of an adjusted cell suspension in SDW was transferred into 50 mL of sterile SM (pH 7.0) or KB broth (pH 7.0) in 150-mL Erlenmeyer flasks to obtain an inoculum concentration of 10^6 CFU/mL. Treatments with

FAc received appropriate amounts of a FAc solution in SDW to obtain a final concentration of 100 μg/mL of FAc. Controls received the same volume of SDW. Cultures were incubated at 25°C in a rotary shaker at 150 rpm for 48 h. Cells were harvested by centrifugation (11 000 × *g*, 20 min). The resulting supernatant was adjusted to pH 7.0 and membrane-filtered (pore size 0.22-μm, Millipore) to obtain a cell-free solution of crude pyoverdines. The concentration of pyoverdines was estimated by determining the absorbance of the filtrates at 400 nm using a molar extinction coefficient of 11 500 M^{-1} cm^{-1} of free pyoverdine absorption spectrum (Meyer and Abdallah 1978). Bacteria cell concentration in the suspension was determined using standard curves as previously described. There were two replications for treatments of *Pseudomona*s isolates grown in SM and three replications for those grown in KB. The experiments including cultures in KB and SM were repeated once and twice, respectively.

Effect of phytoanticipins on growth of bacteria and *F. oxysporum*

Eleven fluorescent *Pseudomonas* spp., *Bacillus* spp., and *Paenibacillus macerans* isolates (*P. chlororaphis* DTR 133; *P. putida* WCS 358; *P. fluorescens* RGAF 19, RG 22, RG 26, and C7R12; *Bacillus circulans* RGAF 6a and RGAF 6b; *Bacillus megaterium* RGAF 12 and RGAF 51; and *Paenibacillus macerans* RGAF 101) and six *F. oxysporum* isolates (*F. oxysporum* f.sp. *ciceris* Foc 7802 and Foc 8012, *F. oxysporum* f.sp. *lycopersici* Fol 32, and nonpathogenic *F. oxysporum* Fo 9009, Fo 90105, and Fo 47) were used in the experiments.

Tomatine (lycopersicin), coumarin (1,2-benzopyrone), and the constitutive isoflavone biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) from Sigma-Aldrich were used as phytoanticipins in this study. Tomatine and coumarin were dissolved in 10 mM citrate–phosphate buffer and used at concentrations of 0, 25, 50, and 100 μg/mL. Biochanin A was dissolved in ethanol and used at concentrations of 0, 0.02, 0.01, and 0.05 mM; ethanol in the solutions was allowed to evaporate from suspensions of bacterial or fungal inocula before use.

For each bacterial isolate, 200 μL of a cell suspension (10^6 CFU/mL) in sterile SM (*Pseudomonas* spp.) or PDB (*Bacillus* spp. and *Paenibacillus macerans* RGAF 101) were mixed with 10 μL of a phytoanticipin solution in a well in sterile microtiter plates. Cultures of *Pseudomonas* spp., *Bacillus* spp., and *Paenibacillus macerans* RGAF 101 were incubated at 25 and 30°C for 50 h, respectively.

Microconidial suspensions of *F. oxysporum* isolates in SDW obtained as previously described were diluted in MM to a final inoculum concentration of 2.5×10^2 microconidia/mL. For each isolate, 200 μL of the conidial suspension was mixed with 10 μL of a phytoanticipin solution in a well of sterile microtiter plates to obtain a final concentration of 50 microconidia per well in a total volume of 210 μL. Cultures were incubated at 25°C for 120 h.

Controls for phytoanticipin treatments consisted of 10 μL of 10 mM citrate–phosphate buffer (coumarin and tomatine) or ethanol (biochanin A) mixed with 200 μL of a bacterial suspension in sterile SM (*Pseudomonas* spp.) or PDB (*Bacillus* spp. and *Paenibacillus macerans* RGAF 101) or a microconidial suspension in sterile MM (*F. oxysporum* isolates) was also used as a control. Growth of bacteria and *F. oxysporum* in phytoanticipins and control treatments was monitored at time 0 and at 3- to 5-h intervals by measuring absorbance at 590 nm (bacterial isolates) or at 650 nm (*F. oxysporum* isolates) as previously described. There were four replications (wells) for each microorganism–phytoanticipin combination. Experiments were repeated twice.

Data analysis

All experiments were conducted using a factorial treatment design arranged in randomized complete blocks. Absorbance data were plotted over time to develop absorbance increase curves (AICs). From these curves, the following variables were calculated: (*i*) $t_{0.1}$ = the number of hours taken for absorbance = 0.1, which was established as an estimate of the onset for absorbance increase or lag phase and calculated by interpolation from data; (*ii*) final absorbance (FA = absorbance determined at the end of the incubation period of cultures); and (*iii*) area under the AIC (AUAIC) calculated by trapezoidal integration method (Campbell and Madden 1990). These three variables were used since assessment of growth after short or prolonged incubation periods may suggest overstated effects or fail to detect differences in lag phases or growth rates, respectively, of the tested compounds on test microorganisms (Wyman and VanEtten 1978). Values of these variables were expressed in relative units (i.e., $Rt_{0.1}$, RFA, and RAUAIC) by dividing the value for a treatment in a replicate by the corresponding value in the control treatment of same replicate. These relative values aimed to minimize differences in absorbance values that might occur among different bacterial or fungal isolates because of pigmentation in culture. When no bacterial or fungal growth occurred for a treatment, the $t_{0.1}$ value for such a treatment used in statistical analysis was arbitrarily established as the duration time of the experiment.

The effects of FAc on growth of bacterial isolates and of phytoanticipins on growth of bacterial and *F. oxysporum* isolates were assessed by hierarchical cluster analyses. These analyses were performed using the Euclidean distance between treatments. The Ward's method was used in the CLUSTER procedure of SAS (Version 6.11; SAS Institute Inc., Cary, N.C.).

To describe the effects of FAc concentrations (FAcQ) on $Rt_{0.1}$ and RAUAIC of FAc-tolerant fluorescent pseudomonads, the expanded exponential [$Rt_{0.1}$ (FAcQ) = $C\exp(r \times \text{FAcQ}) + K$] and negative asymptotic models {RAUAIC (FAcQ) = $C_1 - [C_2/(1 + \exp[b - r \times \text{FAcQ}])]$}, respectively, were fitted to data by nonlinear regression analyses. In these models, C, C_1, and C_2 are constants; r is a rate; K is the asymptote; and b is a parameter related to the threshold inhibitory concentration. Analyses were carried out using the least-squares program for nonlinear models procedure of SAS. The coefficient of determination (R^2), the mean square error, and the asymptotic standard error associated with the estimated parameter were used to evaluate the appropriateness of a model to describe the data. The standard errors of parameters obtained from regression analysis were used to compare the threshold inhibitory concentration of FAc among the bacterial isolates (Campbell and Madden 1990).

Table 3. Growth of fluorescent *Pseudomonas* spp. strains in succinate medium amended with fusaric acid (FAc).

	FAc concentration (μg/mL)								
Isolate	0	12.5	25	50	100	200	300	400	500
P. fluorescens bv. IV DTR 335	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–
P. fluorescens bv. IV DLR 426	+/+	+/+	+/+	+/–	–/–	–/–	–/–	–/–	–/–
P. putida bv. A DS 1026	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–
P. putida bv. A DLR 223	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–
P. putida bv. A DS 131	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–
P. putida bv. A DLR 228	+/+	+/+	+/+	+/+	+/–	–/–	–/–	–/–	–/–
Pseudomonas (TI) DS 824	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. putida bv. A CS 413	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. VI A6	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. II CS 611	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. II CLRP 812	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. II C7R12	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. II C7	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. IV DLRP 214	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. fluorescens bv. II CTR 212	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
P. putida bv. A DLE 3216	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–
P. fluorescens bv. IV DLE 411J	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–
P. fluorescens bv. II CTRP 112	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–
P. fluorescens bv. II CTR 1015	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–
P. fluorescens bv. II CLE 513	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–
P. putida bv. A CS 111	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–
P. fluorescens bv. II RG 26	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
P. fluorescens bv. IV RG 22	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
P. fluorescens bv. II CLR 711	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/–	–/–
P. putida bv. A DTRP 621	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/–
P. chlororaphis DTR 133	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
P. putida Li	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
P. putida WCS 358	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
P. fluorescens bv. V RGAF 19	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

Note: Cultures were incubated at 25°C for 50 h. Bacterial growth (+) was assessed by measuring absorbance at 590 nm with a microplate reader. Results indicate two replicated experiments. (–), no growth indicated by absorbance <0.05.

Data on the effect of FAc on growth and pyoverdine production of fluorescent pseudomonads were also analyzed by standard analysis of variance using the general linear model procedure of SAS. Population values were log-transformed before analysis. Mean comparisons among treatments were performed using Fisher's protected LSD test (P = 0.05). Similarity among experiments tested by preliminary analysis of variance using experimental runs as blocks and Barlett's test of equal variances allowed to combine data for analyses.

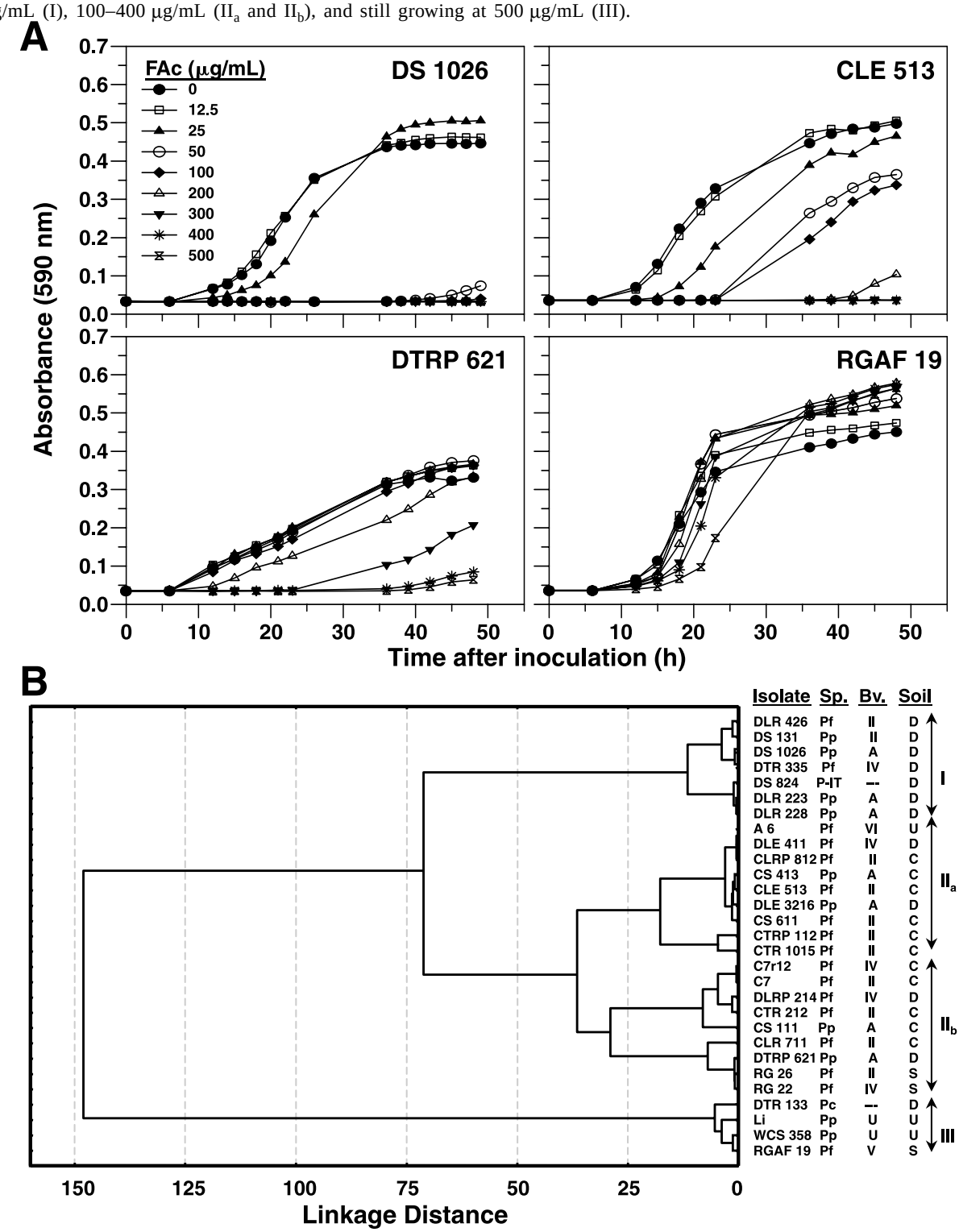
Results

Effect of fusaric acid on bacterial growth

The 29 isolates of fluorescent pseudomonads differed greatly in their ability to grow over a range of FAc concentration and showed a continuum of growth responses to increasing amounts of FAc in the medium. Thus, some strains (*P. fluorescens* DTR 335, *P. fluorescens* DLR 426, *P. putida* DLR 223, *P. putida* DLR 228, *P. putida* DS 131, and *P. putida* DS 1026) were inhibited at FAc concentrations <100 μg/mL (Table 3); most strains were inhibited at FAc concentrations between 100 and 400 μg/mL; and only four strains (*P. chlororaphis* DTR 133, *P. putida* Li, *P. putida* WCS 358, and *P. fluorescens* RGAF 19) still grew at the highest concentration (500 μg/mL) of FAc (Table 3). Figure 1A shows AICs of four selected *Pseudomonas* strains (*P. putida* DS 1026, *P. fluorescens* CLE 513, *P. putida* DTRP, and *P. fluorescens* RGAF 19) in SM amended with FAc, representing the range of the responses found (from the most sensitive to the most tolerant strains). Using the AICs obtained for each FAc concentration for each strain, three variables related to growth parameters expressed in relative units ($Rt_{0.1}$, RFA, and RAUAIC) were calculated to characterize the effect of FAc on bacterial growth. The cluster analysis allowed differentiation of the 29 fluorescent pseudomonads into four main clusters (I, II_a, II_b, and III) (Fig. 1B), which comprised strains ordered from the most sensitive to FAc to the most tolerant. The four bacterial strains presented in Fig. 1A show the general growth response obtained in each cluster. Each cluster includes strains belonging to different species and biovars isolated from plants or from soil and from different geographic areas. The four bacterial isolates in cluster III, which were the most FAc-tolerant isolates, were markedly different from the rest of the isolates (Fig. 1B).

Eight fluorescent pseudomonad isolates in clusters II_b and III, which were characterized as highly tolerant to FAc (growth up to FAc = 300 μg/mL), were selected to further study the effect of a broad range of FAc concentrations on

Fig. 1. (A) Absorbance increase curves (AICs) of four pseudomonad strains (*Pseudomonas putida* DS 1026, *Pseudomonas fluorescens* CLE 513, *P. putida* DTRP 621, and *P. fluorescens* RGAF19) in response to fusaric acid (FAc), representative of the four groups (below) derived from cluster analysis. AICs were obtained by measuring the absorbance (590 nm) at periodic time intervals with a microplate reader as described in Materials and methods. (B) Dendrogram representing relative similarities among 29 isolates of fluorescent pseudomonads in tolerance to FAc. Sp., isolate species (Pc, *Pseudomonas chlororaphis*; Pf, *P. fluorescens*; Pp, *P. putida*; P-IT, intermediate type *P. fluorescens–P. putida*); Bv., biovar. Soil source: U, unknown; C, Châteaurenard (France); D, Dijon (France); S, Santaella (Spain). I, II_a, II_b, and III identify categories of tolerance to FAc; strains were inhibited at FAc concentrations <100 μg/mL (I), 100–400 μg/mL (II_a and II_b), and still growing at 500 μg/mL (III).



bacterial growth. The variation in $Rt_{0.1}$ and RAUAIC with the increase in FAc concentration, which relates to the effect of FAc on bacterial growth, was adequately described by the expanded exponential and negative asymptotic models, respectively. $Rt_{0.1}$ increased exponentially with the increase in FAc concentration (Fig. 2A) irrespective of bacterial isolates. $Rt_{0.1}$ values for isolates DTR 133, RGAF 19, Li, and WCS 358 (cluster III) at FAc concentrations higher than 100 µg/mL were always lower than those of isolates in cluster II_b. Conversely, at FAc concentrations higher than an inhibitory threshold, the RAUAIC diminished asymptotically with the increase in FAc concentration (Fig. 2B). The *b* parameter in the negative asymptotic model, which is correlated with the threshold inhibitory concentration, became higher as the FAc concentration increases. The *b* value differed significantly ($P < 0.05$) among the bacterial isolates and was highest for *P. fluorescens* RGAF 19 and *P. putida* WCS 358, the isolates most tolerant to FAc (data not shown).

As expected, *Bacillus* spp. and *Paenibacillus macerans* RGAF 101 were highly sensitive to the addition of FAc in the culture medium as compared with *Pseudomonas* spp. fluorescent isolates. *Bacillus circulans* RGAF 6a (Fig. 3A) and RGAF 6b grew only at a FAc concentration of 1 µg/mL and constituted one single group in the cluster analysis (Fig. 3B); whereas *B. megaterium* RGAF 12 (Fig. 3A) and RGAF 51 and *Paenibacillus macerans* RGAF 101 grew at FAc concentrations up to 5 µg/mL and were grouped together in a separate cluster (Fig. 3B).

Effect of culture media and fusaric acid on growth and production of pyoverdine by fusaric acid-tolerant fluorescent pseudomonads

Growth and pyoverdine production of FAc-tolerant *Pseudomonas* isolates were assessed in two different media (KB and SM), either amended or not amended with 100 µg/mL of FAc. Both culture media and FAc influenced pyoverdine production and growth of FAc-tolerant *Pseudomonas* isolates. The effect of FAc on bacterial growth differed according to the bacterial strain and media. For all strains, bacterial growth was higher in KB (i.e., a rich medium) than in SM (Table 4). Growth of *Pseudomonas* isolates in KB was not affected by FAc. On the contrary, in SM, four strains (*P. fluorescens* C7R12 and its siderophore-defective mutant PL1 and *P. fluorescens* RG 22 and RG 26) grew significantly ($P < 0.05$) better in the presence of FAc (100 µg/mL) (Table 4).

Although total pyoverdine production (log(mol/L)) was higher in KB than in SM, the relative pyoverdine production (log(mol/CFU)) was similar in both media (Table 4). FAc affected the production of pyoverdine. All bacterial isolates (except for *P. fluorescens* RGAF 19 and RG 22 grown in KB medium and *P. chlororaphis* DTR 133) produced significantly ($P < 0.05$) less pyoverdine (total Log(mol/L) and relative Log(mol/CFU) production) in KB and SM amended with FAc compared with the unamended medium (Table 4).

Effect of phytoanticipins on growth of bacteria and *F. oxysporum*

Three variables related to growth parameters expressed in relative units ($Rt_{0.1}$, RFA, and RAUAIC), derived from the AICs from each of phytoanticipin concentration and bacterial strain or fungal isolate, were used to characterize the global effect of phytoanticipins on bacterial and fungal growth. Biochanin A, coumarin, and tomatine influenced both bacterial and fungal growth. Growth of *Pseudomonas*, *Bacillus*, and *Paenibacillus macerans* isolates were differentially affected by the three phytoanticipins (Fig. 4). For biochanin A and coumarin, *Pseudomonas* isolates were grouped in one cluster and *Bacillus* spp. and *Paenibacillus macerans* isolates in a separate cluster. Second order clusters grouped bacterial isolates according to the effect of each particular phytoanticipin on the onset ($Rt_{0.1}$) and extent of bacterial growth (RAF and RAUAIC) (Figs. 4A and 4B). In general, tomatine did not appear to influence growth of *B. megaterium* isolates or *Pseudomonas* spp. (except for *P. fluorescens* RGAF 19) but delayed and inhibited growth of *Paenibacillus macerans* RGAF 101, *B. circulans* RGAF 6a and RGAF 6b, and *P. fluorescens* RGAF 19 (Fig. 4C). Overall, biochanin A did not inhibit bacterial growth (no effect on RFA, RAUAIC, or $Rt_{0.1}$). Rather, the presence of biochanin A in culture media appeared to enhance growth of *P. fluorescens* RGAF 19, RG 22, and RG 26, as indicated by higher values of RFA and RAUAIC and delayed growth of

Fig. 2. Effect of fusaric acid (FAc) concentration in succinate medium on the time to the growth onset ($t_{0.1}$) and the area under the curve of absorbance increase over time (AUAIC) of *Pseudomonas chlororaphis* DTR 133, *Pseudomonas fluorescens* CLR 711, *P. fluorescens* RGAF 19, *P. fluorescens* RG 22, *P. fluorescens* RG 26, *Pseudomonas putida* Li, *P. putida* DTRP 621, and *P. putida* WCS 358. Values of $t_{0.1}$ and AUAIC for each FAc concentration were expressed in relative units by dividing the actual value by that in the control (0 µg/mL of FAc) for each bacterial isolate. Lines represent the predicted curves calculated by the expanded exponential model (A) or the negative asymptotic model (B).

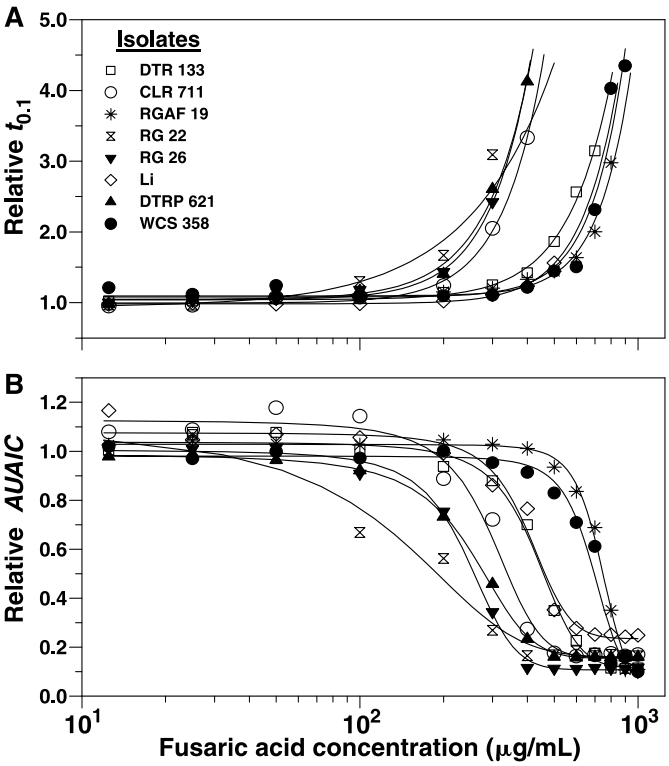
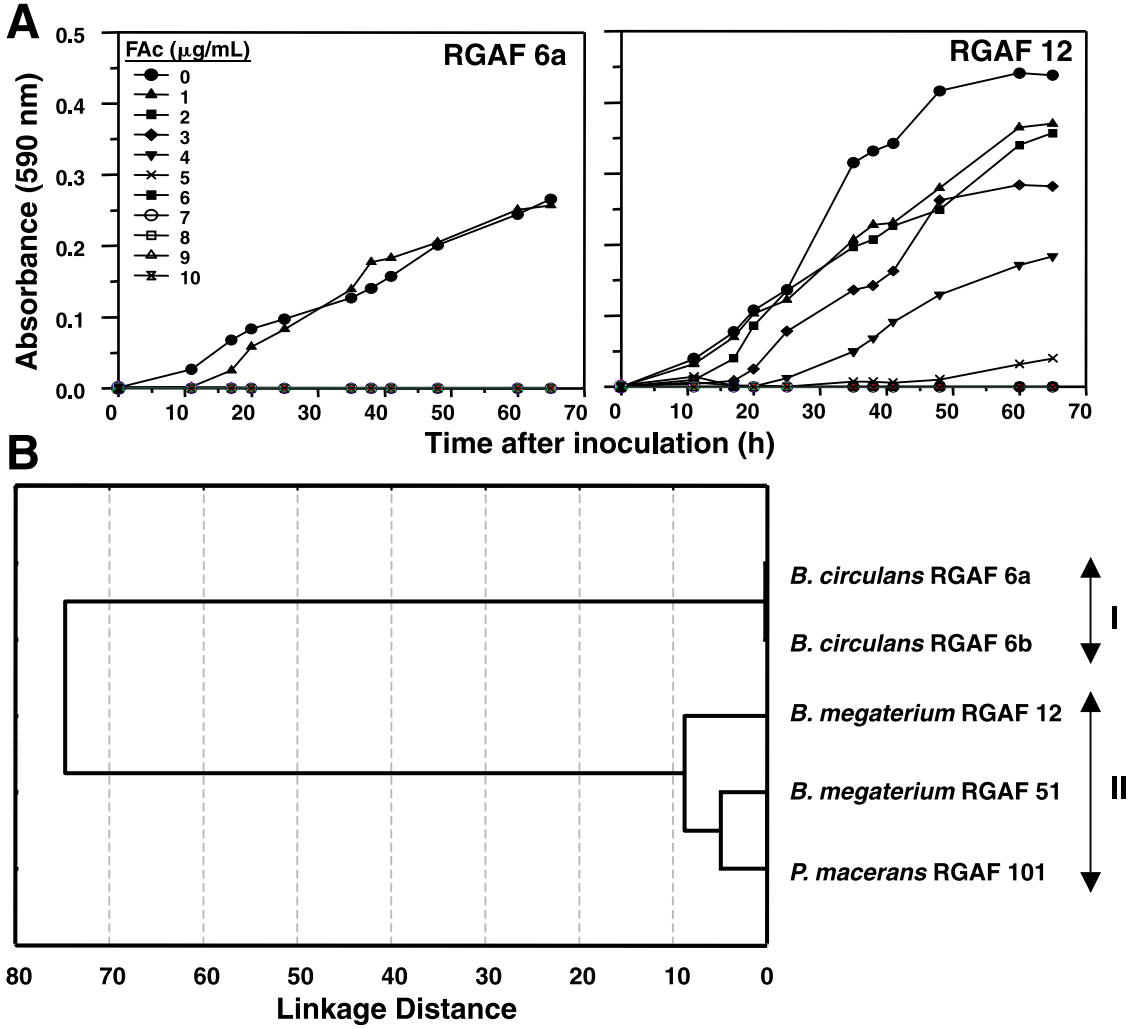


Fig. 3. (A) Absorbance increase curves (AICs) of two *Bacillus* strains (*Bacillus circulans* RGAF 6a, and *Bacillus megaterium* RGAF 12) in response to fusaric acid (FAc), representative of the two groups (below) derived from cluster analysis. The AICs were obtained by measuring the absorbance (590 nm) at periodic time intervals with a microplate reader as described in Materials and methods. (B) Dendrogram representing relative similarities among four *Bacillus* spp. isolates and a *Paenibacillus macerans* isolate in tolerance to FAc. I and II identify the categories of tolerance to Fac; strains were inhibited at Fac concentrations <1 μg/mL (I) or <5–6 μg/mL (II).



B. megaterium RGAF 12 and RGAF 51 and *Paenibacillus macerans* RGAF 101 (higher values of $Rt_{0.1}$). Conversely, coumarin inhibited (lower values of RFA and RAUAIC) and delayed (higher values of $Rt_{0.1}$) growth of most *Pseudomonas* isolates, but it had no effect on growth of *B. circulans* RGAF 6a and RGAF 6b and *Paenibacillus macerans* RGAF 101 (Fig. 4B).

Biochanin A and tomatine had similar effects on growth of *F. oxysporum* isolates (Figs. 5A and 5C), but these effects differed depending on the pathogenic (*F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *lycopersici*) or nonpathogenic (*F. oxysporum* nonpathogenic) nature of isolates. Indeed, increased concentrations of biochanin A and tomatine significantly ($P < 0.05$) inhibited (lower values of RFA and RAUAIC) and delayed (higher values of $Rt_{0.1}$) growth of *F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *lycopersici* isolates. However, growth of nonpathogenic isolates increased with increasing concentrations of both phytoanticipins (Figs. 5A and 5C). Although coumarin inhibited growth of all *F. oxysporum* isolates (Fig. 5B), the extent of inhibition was higher for pathogenic isolates compared with that of nonpathogenic ones. Thus, for all the three phytoanticipins, *F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *lycopersici* (pathogenic isolates) were grouped in one cluster and nonpathogenic *F. oxysporum* isolates comprised a different cluster (Fig. 5).

Discussion

There have been many studies aimed at the application of rhizobacteria for the suppression of soilborne diseases. Although encouraging results have been obtained, in practice, the efficacy of biocontrol agents in disease suppression frequently suffers from inconsistency. One of the reasons for such a lack of consistency is the complexity of interactions taking place between the pathogenic or antagonistic microorganisms and the plant. These interactions are far from being well characterized. Some studies have pointed out a role for secondary metabolites, such as FAc produced by *F. oxysporum* and pyoverdine produced by the fluorescent *Pseudomonas* spp., in the interactions between these organisms

Table 4. Bacterial growth (CFU/mL) and production of pyoverdine (mol/L and mol/CFU) by fluorescent *Pseudomonas* spp. isolates in King's B (KB) and succinate media (SM) amended (100 μg/mL) or not amended (0 μg/mL) with fusaric acid.

	KB						SM					
	Log (CFU/mL)		–Log (mol/L)		–Log (mol/CFU)		Log (CFU/mL)		–Log (mol/L)		–Log (mol/CFU)	
Isolate	0	100	0	100	0	100	0	100	0	100	0	100
P.c. DTR 133	10.22	10.24	3.71	3.72	16.94	16.95	9.49	9.63	4.48*a*	4.92*b*	16.98*a*	17.55*b*
P.f. C7r12	10.14	10.12	3.75*a*	3.84*b*	16.89*a*	16.95*b*	8.67*b*	9.07*a*	4.06*a*	4.19*b*	15.73*a*	16.25*b*
P.f. PL1	10.04	10.03	—	—	—	—	9.20*b*	9.36*a*	—	—	—	—
P.f. CLR 711	10.05	10.00	4.01*a*	4.23*b*	17.05*a*	17.24*b*	9.16	9.28	5.05*a*	4.09*b*	17.21*a*	17.05*b*
P.f. RGAF 19	10.05	10.07	3.74	3.76	16.79	16.83	9.23	9.25	3.97*a*	4.09*b*	16.20*a*	16.34*b*
P.f. RG 22	10.03	10.06	3.74	3.74	16.77	16.79	9.19*b*	9.39*a*	3.96*a*	4.05*b*	16.25*a*	16.34*b*
P.f. RG 26	10.20	10.19	3.75*a*	3.89*b*	16.95*a*	17.08*b*	9.38*b*	9.52*a*	4.04*a*	4.98*b*	16.42*a*	16.50*b*
P.p. Li	9.63	9.70	3.75*a*	3.77*b*	16.39*a*	16.48*b*	9.20	9.23	4.32*a*	4.50*b*	16.52*a*	16.73*b*
P.p. DTRP 621	10.09	10.10	3.73*a*	3.82*b*	16.82*a*	16.92*b*	9.24	9.29	4.36*a*	4.78*b*	16.60*a*	17.06*b*
P.p. WCS 358	9.99	9.99	3.73*a*	3.76*b*	16.73*a*	16.75*b*	9.33	9.30	3.86*a*	3.95*b*	16.18*a*	16.25*b*

Note: Bacterial growth was estimated by measuring the absorbance at 600 nm using standard curves. Pyoverdine concentration was estimated by measuring the absorbance of the cell-free filtrates at 400 nm, as described in Materials and methods. Means in a row within a culture medium with different letters are significantly different ($P < 0.05$) from the mean for the corresponding treatment at the other FAc concentration, according to Fisher's protected LSD. *P.c.*, *Pseudomonas chlororaphis*; *P.f.*, *Pseudomonas fluorescens*; *P.p.*, *Pseudomonas putida*; —, pyoverdine production below the detection limit ($A_{400} < 0.05$).

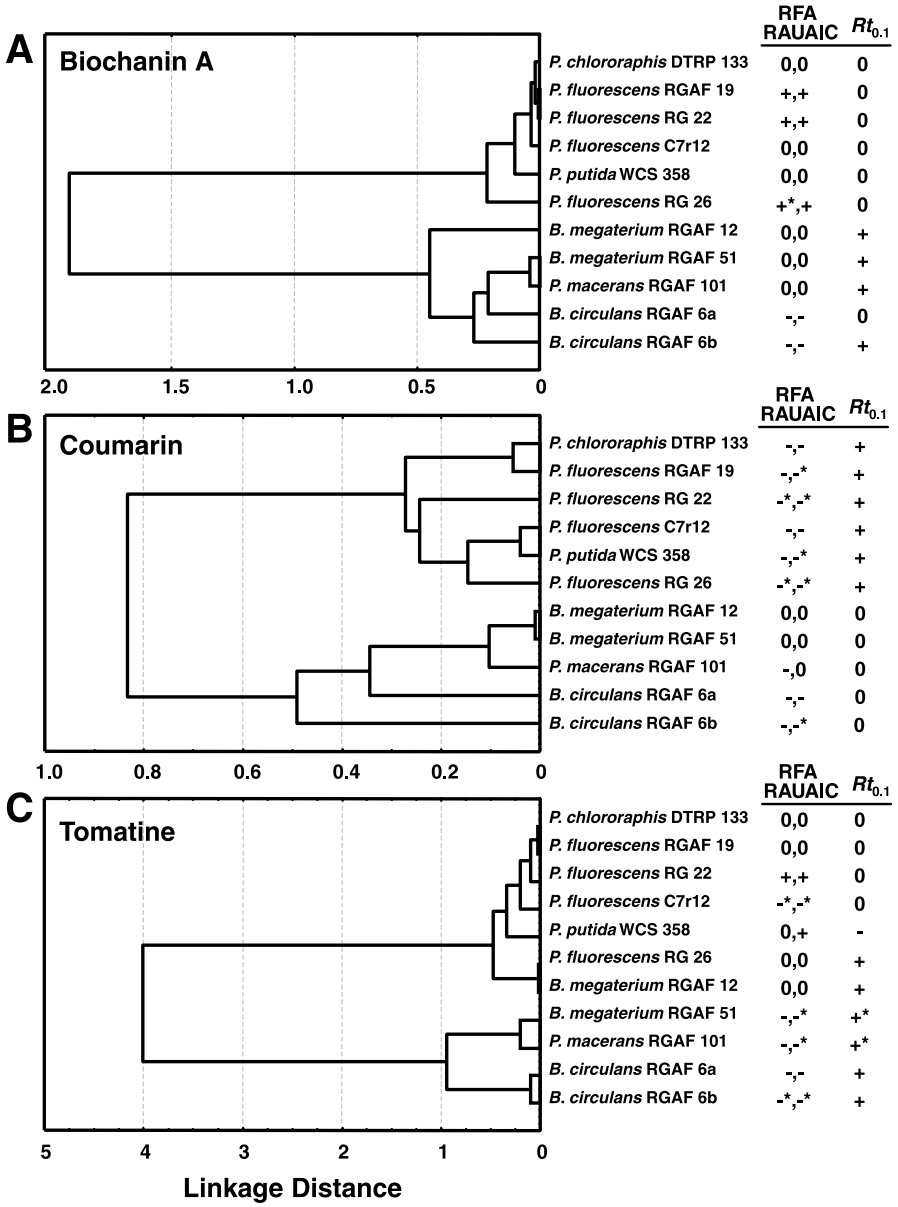
(Lemanceau et al. 1993; Loper and Buyer 1991; Toyoda et al. 1988) or in the antimicrobial effects of some phytoalexins (Gnanamanickam and Mansfield 1981; Wyman and VanEtten 1978). However, little attention has been given to the compatibility between different biological control agents and the plant by addressing the tolerance of each partner to the secondary metabolites produced by the other. The aim of this work was to assess the effect of some secondary metabolites produced by isolates of *F. oxysporum* and the plant on the growth of a collection of isolates of *F. oxysporum* (pathogenic and nonpathogenic), fluorescent *Pseudomonas* spp., *Bacillus* spp., and *Paenibacillus macerans*.

FAc is one of the secondary metabolites produced by most, and possibly all, isolates of *F. oxysporum*. The potential roles of FAc in relation to pathogenicity of *F. oxysporum* strains (Kern 1972) and to activity of *Ralstonia solanacearum* in the control of Fusarium wilts (Toyoda et al. 1988) were previously addressed. In this study, the impact of this fungal metabolite on growth of a collection of fluorescent *Pseudomonas* spp., *Bacillus* spp., and *Paenibacillus macerans* was evaluated in a culture medium amended with increasing concentrations of FAc. The results clearly showed a continuum among bacterial strains, from those sensitive to low concentrations of FAc to strains tolerant to high concentrations. However, as a group, *Pseudomonas* spp. strains were much less sensitive to FAc than *Bacillus* spp. and *Paenibacillus macerans*. Based on the literature, we chose to apply very low concentrations of FAc to characterize the behavior of *Bacillus* spp. and *Paenibacillus macerans* strains (growth of these bacteria were completely inhibited by 10^{-4} M of FAc; Gäumann 1957). The results showed that growth of *Bacillus* spp. and *Paenibacillus macerans* was inhibited at a concentration as low as 5 μg/mL, whereas some strains of *P. chlororaphis*, *P. fluorescens*, and *P. putida* were still able to grow at concentrations higher than 500 μg/mL. The high sensitivity of *Bacillus* spp. and *Paenibacillus macerans* to FAc found in our study agree with results from other authors (Gäumann 1957; Kuo and Scheffer 1964).

From the AICs, three variables related to growth parameters were calculated: $t_{0.1}$ representing the lag phase, FA representing the bacterial concentration at the plateau, and AUAIC representing the total biomass produced. These parameters were used to perform a cluster analysis that distinguished four clusters among *Pseudomonas* spp. strains and two clusters among *Bacillus* strains in relation to their tolerance to FAc. Each of these clusters includes bacterial strains belonging to different species and biovars isolated from plants or from soil and from different geographic areas. These data indicate a lack of correlation between tolerance to FAc and the origin or taxonomic position of the fluorescent pseudomonads in the study. Ability to grow in the presence of FAc seems, then, to be a characteristic of each individual strain rather than of a taxonomic group. The analyses of the growth curves of eight *Pseudomonas* spp. strains highly tolerant to FAc adequately fit $Rt_{0.1}$ and RAUAIC data to the expanded exponential and the negative asymptotic models, respectively. The asymptotic decrease of $Rt_{0.1}$ and increase of RAUAIC when FAc concentrations reach a given level indicate that the threshold concentration of FAc inhibiting bacterial growth differs greatly among bacterial strains. The diversity in the sensitivity of bacterial strains to FAc would suggest that the structure of indigenous populations of soilborne pseudomonads could vary according to the concentration or presence of FAc in their environment. These data could also account for the variable compatibility of fluorescent *Pseudomonas* spp. strains when combined with nonpathogenic *F. oxysporum* for the suppression of Fusarium wilt diseases (Lemanceau and Alabouvette 1991). Indeed, a FAc-sensitive strain of fluorescent pseudomonads would not be compatible with an isolate of *F. oxysporum* producing high quantities of FAc.

Besides bacterial growth, FAc may also affect bacterial metabolism (Duffy and Défago 1997*a*; Schnider-Keel et al. 2000). Because pyoverdine is a bacterial metabolite playing a major role in the interactions among fluorescent pseudomonads, the soil microflora, and the plant (Loper and Buyer 1991), we evaluated the possible impact of FAc on pyoverdine synthesis by FAc-tolerant strains of *Pseudomonas*

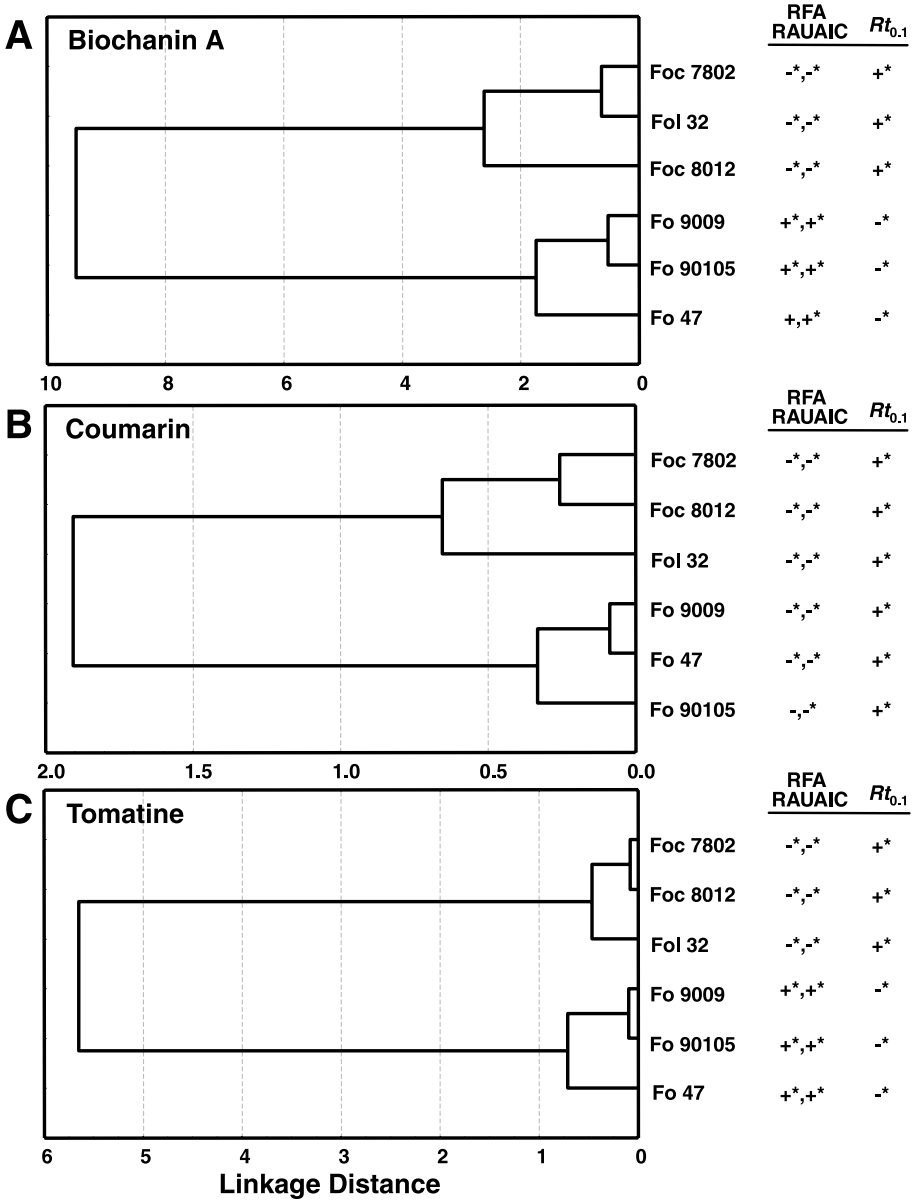
Fig. 4. Dendrogram representing relative similarities in tolerance to biochanin A (A), coumarin (B), and tomatine (C) among six fluorescent *Pseudomonas* spp. strains, four *Bacillus* spp. strains, and a *Paenibacillus macerans* strain. Bacterial growth was monitored by measuring the absorbance at 590 nm at periodic time intervals as described in Materials and methods. RFA, relative final absorbance; RAUAIC, relative area under the curve of absorbance increase over time; $Rt_{0.1}$, relative time to onset of bacterial growth. Increase or decrease in RFA, RAUAIC, and $Rt_{0.1}$ with the increase in phytoanticipin concentration is indicated by (+) and (–), respectively, while (0) indicates no effect. *, statistically significant compared with the control treatment at $P = 0.05$.



spp. At a concentration of 100 µg/mL, FAc affected the production of pyoverdine by all of the strains in the minimal succinate medium and by most of the strains in the rich KB medium. This effect on pyoverdine production was independent of bacterial growth, since FAc also affected the relative pyoverdine production. The deleterious effect of FAc on pyoverdine production is similar to that on production of the pyochelin siderophore and other bacterial metabolites described by Duffy and Défago (1997*a*). These authors demonstrated that FAc at a concentration as low as 0.12 µg/mL repressed the production of the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) in vitro by *P. fluorescens* CHA0, which is a key factor in the disease suppression by this strain. Duffy and Défago (1997*a*) also showed that production of pyoluteorin by *P. fluorescens* CHA0, but not that of hydrogen cyanide and protease, was also reduced in the presence of FAc. They concluded that FAc might affect biosynthesis of secondary metabolites at a regulatory level downstream of *gacA* and *apdA* genes. More recently, Schnider-Keel et al. (2000) also demonstrated that FAc strongly repressed the synthesis of monoacetylphloroglucinol and 2,4-DAPG and the expression of *phlA*, one of the 2,4-DAPG biosynthetic genes; this regulatory mechanism is mediated by PhlF, a transcriptional repressor.

Impairment of bacterial biosynthesis of metabolites by FAc may relate to the efficiency of bacteria as biocontrol

Fig. 5. Dendrogram representing relative similarities among nonpathogenic *Fusarium oxysporum* Fo 9009, Fo 90105, and Fo 47, pathogenic *F. oxysporum* f.sp. *ciceris* Foc 7802 and Foc 8012, and pathogenic *F. oxysporum* f.sp. *lycopersici* Fol 32 in minimal medium amended with different concentrations of biochanin A (A), coumarin (B), and tomatine (C). Fungal growth was monitored by measuring the absorbance at 650 nm at periodic time intervals as described in Materials and methods. RFA, relative final absorbance; RAUAIC, relative area under the curve of absorbance increase over time; $Rt_{0.1}$, relative time to onset of fungal growth. Increase or decrease in RFA, RAUAIC, and $Rt_{0.1}$ with the increase in phytoanticipin concentration is indicated by (+) and (–), respectively, while (0) indicates no effect. *, statistically significant compared with the control treatment at $P = 0.05$.



agents. Sharifi-Tehrani et al. (1998) compared a collection of 2,4-DAPG-producing *Pseudomonas* spp. belonging to three ARDRA (amplified 16S ribosomal DNA restriction analysis) groups for biocontrol activity against fusarium crown and root rot of tomato and pythium damping-off of cucumber. As a group, ARDRA group 2 strains were more effective than ARDRA group 1 strains. FAc blocks biosynthesis of 2,4-DAPG by ARDRA-1 strains (Duffy and Défago 1997*a*; Schnider-Keel et al. 2000) but not that of ARDRA-2 or ARDRA-3 strains (Duffy and Défago 1997*b*). This could explain, in part, why suppression of fusarium crown and root rot of tomato was less effective for ARDRA-1 strains compared with ARDRA-2 strains (Sharifi-Tehrani et al. 1998). These results (Duffy and Défago 1997*a*, 1997*b*, Schnider-Keel et al. 2000) and those of the present study indicate the need to address the inhibitory effect of FAc on growth and production of secondary metabolites by bacterial strains and nonpathogenic *F. oxysporum* isolates used for biological control of diseases caused by *Fusarium* spp. In doing so, better disease control could be obtained by using FAc-insensitive biocontrol strains or by using FAc-degrading strains (Duffy and Défago 1997*a*; Toyoda et al. 1988).

Strains of fluorescent *Pseudomonas* spp. and nonpatho-

genic isolates of *F. oxysporum* used for disease suppression should also be tolerant to toxic compounds produced by the host plant, such as phytoanticipins and phytoalexins that can reach high concentrations in root cells and soil (Armero 1996; Cachinero 1996; Cachinero et al. 2002; Dakora and Phillips 1996). Gram-negative bacteria were shown to be less sensitive to several phytoalexins than Gram-positive bacteria (Gnanamanickam and Mansfield 1981). Our results clearly show that (*i*) phytoanticipins differ in their effects on specific bacterial strains and (*ii*) the bacterial sensitivity to a phytoanticipin varies from one strain to the other. For example, biochanin A was neutral or stimulatory for *Pseudomonas* spp., but it delayed growth of *Bacillus* spp. In contrast, coumarin inhibited growth of *Pseudomonas* spp. but had no effect on *Bacillus* spp. and *Paenibacillus macerans*. These results agree with those of Wyman and VanEtten (1978), who showed that sensitivity of bacterial isolates to six selected isoflavonoid phytoalexins varied widely, with pseudomonads being more tolerant to phytoalexins than xanthomonads or *Achromobacter* isolates.

Several studies have indicated a relationship between greater virulence of fungal plant pathogens and increased tolerance to toxic compounds, such as phytoanticipins and phytoalexins produced by the host plant (reviewed in Suleman et al. 1996). However, other studies identified variation in phytoanticipin and phytoalexin tolerance among fungal species or isolates of a pathogen (Dakora and Phillips 1996; Suleman et al. 1996). Our results indicate that effects of biochanin A, coumarin, and tomatine phytoanticipins on fungal growth differed among nonpathogenic and pathogenic *F. oxysporum* isolates. The three pathogenic isolates of *F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *lycopersici* tested were highly sensitive to the three phytoanticipins, with increased lag phase and reduced growth. Conversely, the three nonpathogenic *F. oxysporum* isolates grew better in the presence of biochanin and tomatine and were inhibited by coumarin, although to a lesser extent compared with the pathogenic isolates. Thus, our results suggest that nonpathogenic *F. oxysporum* isolates are less sensitive to phytoanticipins than the pathogenic ones. The nonpathogenic *F. oxysporum* isolates involved in the suppression of Fusarium wilts are known to invade the root cortex of the host plant where they induce defense reactions (Olivain and Alabouvette 1997). Cachinero (1996) and Cachinero et al. (2002) demonstrated that prior inoculation of chickpeas with nonpathogenic *F. oxysporum* isolates caused a brown discoloration at surfaces of roots, cotyledonary node, and cotyledons and increased production of maackiain and medicarpin phytoalexins in the plant roots. Suleman et al. (1996) demonstrated that virulence of isolates of *F. oxysporum* f.sp. *lycopersici* was correlated with tolerance to rishitin phytoalexin and to a lesser extent with tolerance to tomatine phytoanticipin. However, they also found that nonpathogenic *F. oxysporum* isolates displayed, in general, the highest sensitivity to tomatine but not to rishitin. Lairini et al. (1997) found that a tomatine concentration of 20 mM inhibited mycelial growth of all 10 formae speciales of *F. oxysporum* investigated, with *F. oxysporum* f.sp. *ciceris* being the most sensitive to this phytoanticipin. All these results suggest that great variability may occur in the response of bacteria and fungi to phytoalexins and phytoanticipins regardless of the pathogenic or nonpathogenic nature of the species or isolate.

Our study, though conducted in vitro, involved the effects of metabolites produced by microbes and plants on plant pathogens and biocontrol agents that may eventually be released into the rhizosphere and affect the growth and (or) metabolism of these microorganisms. Our results are indicative of the complex interactions that can occur at the root surface and in the rhizosphere among the plant, the pathogen, and two types of antagonistic microorganisms used for biological control. A major challenge for the future would be to assess the effect of FAc and phytoanticipins on the survival and the activities of biocontrol agents in the rhizosphere using reporter genes.

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