Defense Responses of *Fusarium oxysporum* to 2,4-Diacetylphloroglucinol, a Broad-Spectrum Antibiotic Produced by *Pseudomonas fluorescens*

Alexander Schouten,¹ Grardy van den Berg,¹ Véronique Edel-Hermann,² Christian Steinberg,² Nadine Gautheron,² Claude Alabouvette,² C. H. (Ric) de Vos,³ Philippe Lemanceau,² and Jos M. Raaijmakers¹

¹Laboratory of Phytopathology, Plant Sciences Department, Wageningen University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands; ²UMR Microbiologie et Géochimie des Sols, Institut National de la Recherche Agronomique (INRA), 17 rue Sully, 21065 Dijon Cedex, France; ³Plant Research International, P.O. Box 16, 6700AA Wageningen, The Netherlands

Submitted 20 April 2004. Accepted 5 July 2004.

A collection of 76 plant-pathogenic and 41 saprophytic *Fusarium oxysporum* strains was screened for sensitivity to 2,4-diacetylphloroglucinol (2,4-DAPG), a broad-spectrum antibiotic produced by multiple strains of antagonistic *Pseudomonas fluorescens*. Approximately 17% of the *F. oxysporum* strains were relatively tolerant to high 2,4-DAPG concentrations. Tolerance to 2,4-DAPG did not correlate with the geographic origin of the strains, formae speciales, intergenic spacer (IGS) group, or fusaric acid production levels. Biochemical analysis showed that 18 of 20 tolerant *F. oxysporum* strains were capable of metabolizing 2,4-DAPG. For two tolerant strains, analysis by mass spectrometry indicated that deacetylation of 2,4-DAPG to the less fungitoxic derivatives monoacetylphloroglucinol and phloroglucinol is among the initial mechanisms of 2,4-DAPG degradation. Production of fusaric acid, a known inhibitor of 2,4-DAPG biosynthesis in *P. fluorescens*, differed considerably among both 2,4-DAPG-sensitive and -tolerant *F. oxysporum* strains, indicating that fusaric acid production may be as important for 2,4-DAPG-sensitive as for -tolerant *F. oxysporum* strains. Whether 2,4-DAPG triggers fusaric acid production was studied for six *F. oxysporum* strains; 2,4-DAPG had no significant effect on fusaric acid production in four strains. In two strains, however, sublethal concentrations of 2,4-DAPG either enhanced or significantly decreased fusaric acid production. The implications of 2,4-DAPG degradation, the distribution of this trait within *F. oxysporum* and other plant-pathogenic fungi, and the consequences for the efficacy of biological control are discussed.

Additional keyword: detoxification.

The genus *Pseudomonas* contains not only plant, animal, and human pathogens, but also accommodates species of significant environmental interest, including those that promote plant growth, degrade xenobiotics, or control plant diseases. Numerous antibiotics have been isolated from various *Pseudomonas* strains, including phenazines, lipodepsipeptides, and phloroglucinols (Chin-A-Woeng et al. 2003; de Souza et al. 2003b,c; Nielsen et al. 2002; Raaijmakers et al. 2002). Phloro-

glucinols are phenolic compounds produced by *Pseudomonas* spp., but also by plants and algae (de Souza et al. 2003a). More than 60 phloroglucinol derivatives have been described and were reported to have antiviral, antibacterial, antifungal, antihelminthic, phytotoxic, antitumor, and plant growth-regulating activities (de Souza et al. 2003a). The broad-spectrum activity of 2,4-diacetylphloroglucinol (2,4-DAPG) produced by *Pseudomonas fluorescens* strains recently has drawn attention in the medical area due to its bacteriolytic activity against multi-drug-resistant *Staphylococcus aureus* (Kamei and Isnansetyo 2003). Over the past decade, 2,4-DAPG has been shown to play a key role in the biological control of various plant pathogens, including *Fusarium oxysporum* (Duffy et al. 2004; Duffy and Défago 1997; Keel et al. 1996; Thomashow and Weller 1996).

F. oxysporum contains opportunistic human pathogens, antagonists, and plant pathogens (Summerbell and Schroers 2002). Plant-pathogenic *F. oxysporum* causes substantial yield losses in many economically important crops and is considered to be the most important soil borne *Fusarium* spp. *F. oxysporum* produces an array of toxins (Chelkowski 1989), including mycotoxins, that have adverse effects on human and animal health (Gupta et al. 2000; Joffe 1986). During the saprophytic and parasitic stages of its life cycle, *F. oxysporum* is exposed to multiple toxic compounds, including antifungal metabolites produced by microorganisms that reside in the same microsites (Chin-A-Woeng et al. 2003; Lagopodi et al. 2002). Evidently, *F. oxysporum* must be able to tolerate or combat these toxic metabolites to propagate, colonize, and successfully infect plants.

Various mechanisms that enable microorganisms to resist toxic compounds have been described in the areas of medical microbiology, bioremediation, and plant-pathogen interactions, and include enzymatic degradation or inactivation of antibiotic compounds, alteration of the target sites, and active efflux (Alexander et al. 1999; De Waard 1997; Fleissner et al. 2002; Morrissey and Osbourn 1999; Schoonbeek et al. 2002; Steffens et al. 1996; VanEtten et al. 2001). Relatively little is known about the role of these mechanisms in fungal defense against microbial antagonism. Studies have shown that, within several fungal populations, there is variation in sensitivity to antifungal metabolites produced by antagonistic bacteria (Duffy et al. 2003). For example, various isolates of the take-all fungus differ considerably in their sensitivity toward 2,4-DAPG and phenazines produced by *Pseudomonas* spp. (Mazzola et al. 1995). With respect to defense responses

Corresponding author: A. Schouten; Telephone: +31 317 483131; Fax: +31 317 483412; E-mail: sander.schouten@wur.nl

in *F. oxysporum* to antibiotics produced by antagonistic *Pseudomonas* spp. Duffy and Défago (1997) elegantly demonstrated that fusaric acid, a pyridine-carboxylic acid, acts as a signal that specifically represses 2,4-DAPG biosynthesis in the antagonistic strain *P. fluorescens* CHA0. Fusaric acid was shown to act as a repressor of *phlA* expression both in vitro and in situ (Notz et al. 2002; Schnider-Keel et al. 2000).

Blocking fusaric acid production by the pathogen via zinc amendment relieved *phlA* repression and improved the bio-control activity of strain CHA0 (Duffy and Défago 1997). Recent studies have further shown that certain 2,4-DAPG-producing *Pseudomonas* strains are relatively insensitive to fusaric acid as a negative signal in 2,4-DAPG synthesis (Duffy et al. 2003, 2004).

Table 1. Strains of *Fusarium oxysporum* analyzed in this study^a

Strain	Formae speciales	Isolated from	Country	Year	IGS type ^b	2,4-DAPG tolerance ^c	Source, reference ^d
Foln3	<i>lini</i>	Flax	France	1981	12	–	FPFS
Fol8	<i>lycopersisci</i>	Tomato	France	1971	23	–	FPFS
Fol15	<i>lycopersisci</i>	Tomato	Tunisia	1968	25	–	FPFS
Fol24	<i>lycopersisci</i>	Tomato	France	1977	23	–	FPFS
Fol27	<i>lycopersisci</i>	Soil	France	1980	25	–	FPFS
Fol28	<i>lycopersisci</i>	Soil	France	1980	25	–	FPFS
Fol29	<i>lycopersisci</i>	Tomato	France	1981	25	–	FPFS
Fol32	<i>lycopersisci</i>	Tomato	France	1985	23	–	FPFS
Fol33	<i>lycopersisci</i>	Tomato	United States	1987	25	–	FPFS
Fol34	<i>lycopersisci</i>	Tomato	United States	1987	25	–	FPFS
Fol35	<i>lycopersisci</i>	Tomato	Japan	NA	ND	–	FPFS, 880621a-1 (T. Arie)
Fom3	<i>melonis</i>	Melon	France	1962	ND	–	FPFS
Fom7	<i>melonis</i>	Melon	France	1964	ND	–	FPFS
Fom15	<i>melonis</i>	Melon	France	1965	ND	–	FPFS
Fom20	<i>melonis</i>	Melon	France	1965	ND	–	FPFS
Fom24	<i>melonis</i>	Melon	France	1968	21	–	FPFS
Fom28	<i>melonis</i>	Melon	Netherlands	1970	ND	+	FPFS
Fom31	<i>melonis</i>	Melon	France	1970	ND	–	FPFS
Fom32	<i>melonis</i>	Melon	France	1970	ND	–	FPFS
Fom35	<i>melonis</i>	Melon	France	1985	ND	–	FPFS
Fom37	<i>melonis</i>	Melon	France	1985	ND	+	FPFS
Fom38	<i>melonis</i>	Melon	United States	NA	ND	++	FPFS, MC6-8S (T. R. Gordon)
Fom39	<i>melonis</i>	Melon	United States	NA	ND	–	FPFS, KNH (T. R. Gordon)
Fom117	<i>melonis</i>	Melon	Turkey	1962	ND	–	FPFS
Fom1127	<i>melonis</i>	Melon	United States	1987	ND	++	FPFS, Fom 1127 (T. R. Gordon)
Focub1	<i>cubense</i>	Banana	Taiwan	NA	ND	++	FPFS
Focub2	<i>cubense</i>	Banana	Taiwan	NA	ND	++	FPFS
Focub3	<i>cubense</i>	Banana	Taiwan	NA	ND	–	FPFS
Focub6	<i>cubense</i>	Banana	Taiwan	NA	ND	–	FPFS
Focub7	<i>cubense</i>	Banana	Taiwan	NA	ND	–	FPFS
Focub9	<i>cubense</i>	Banana	Taiwan	NA	ND	–	FPFS
Focub13	<i>cubense</i>	Banana	Taiwan	NA	ND	++	FPFS
Focub16	<i>cubense</i>	Banana	Taiwan	NA	ND	–	FPFS
Fob2	<i>basilicum</i>	Basilicum	France	1994	ND	–	FPFS
Fob3	<i>basilicum</i>	Basilicum	Italy	NA	ND	+	FPFS
Fod1	<i>dianthi</i>	Soil	France	1987	ND	+	FPFS
Fod2	<i>dianthi</i>	Carnation	Netherlands	NA	50	–	FPFS, WCS816 (R. P. Baayen)
Fod3	<i>dianthi</i>	Carnation	Argentina	NA	ND	–	FPFS, CBS214.49
Fod4	<i>dianthi</i>	Carnation	Argentina	NA	ND	–	FPFS
Fod8	<i>dianthi</i>	Carnation	Netherlands	NA	64	–	FPFS, NAKS3 (R. P. Baayen)
For1	<i>raphani</i>	Radish	Netherlands	NA	ND	–	FPFS, WCS600
For2	<i>raphani</i>	Radish	Unknown	NA	ND	–	FPFS
For3	<i>raphani</i>	Radish	Germany	NA	ND	–	FPFS
For4	<i>raphani</i>	Radish	France	NA	ND	–	FPFS
Foci1	<i>ciceri</i>	Chick pea	Spain	1990	ND	–	FPFS
Foci2	<i>ciceri</i>	Chick pea	Tunisia	1991	ND	–	FPFS
Foci4	<i>ciceri</i>	Chick pea	Spain	1980	ND	–	FPFS
Focy1	<i>cyclamini</i>	Cyclamen	France	1994	ND	–	FPFS
Focy2	<i>cyclamini</i>	Cyclamen	France	1994	ND	–	FPFS
Foe1	<i>elaidis</i>	Oil palm	Ivory Coast	NA	ND	–	FPFS
Foa1	<i>albedinis</i>	Date palm	Morocco	1966	ND	–	FPFS
Foa2	<i>albedinis</i>	Date palm	Algeria	1972	ND	+	FPFS
Foa3	<i>albedinis</i>	Date palm	Algeria	1973	ND	–	FPFS
Foa4	<i>albedinis</i>	Date palm	Morocco	1984	ND	–	FPFS

(continued on next page)

^a NA = not available and ND = not determined.

^b Restriction patterns revealed with seven restriction enzymes for each intergenic spacer (IGS) type were described previously (Edel et al. 1995, 1997, 2001; Lori et al. 2004).

^c Tolerant strains were divided into three categories depending on their radial growth with 2,4-diacetylphloroglucinol (2,4-DAPG) at 400 µg/ml: strains for which the radial growth was reduced by more than 90% (+), strains for which the radial growth was reduced by 70 to 90% (++), and strains for which the radial growth was reduced by 50 to 70% (+++).

^d FPFS: Collection of Flore Pathogène et Faune du Sol, UMR Microbiologie et Géochimie des Sols (MGS), Institut National de la Recherche Agronomique (INRA), Dijon, France. WU-Phyto: Department of Phytopathology, Wageningen University, Wageningen, The Netherlands.

In this study, a large collection of *F. oxysporum* strains obtained from various host plants, soil, and geographical locations and representing multiple intergenic spacer (IGS) groups was screened for sensitivity toward 2,4-DAPG. Strains tolerant to 2,4-DAPG were further analyzed at the biochemical level with respect to the nature of their defense mechanism against 2,4-DAPG. Finally, the effect of 2,4-DAPG on fusaric acid production in several *F. oxysporum* strains was monitored.

RESULTS

Genotypic diversity of *F. oxysporum*.

A subsample of 32 pathogenic strains was characterized by RFLP analysis of the ribosomal IGS. The 32 pathogenic strains were assigned to eight IGS types (Table 1). The 40 non-pathogenic strains were representative of 17 IGS types (Edel et al. 1995, 1997, 2001). Two identical IGS types were found

Table 1. (continued from preceding page)

Strain	Formae speciales	Isolated from	Country	Year	IGS type ^b	2,4-DAPG tolerance ^c	Source, reference ^d
Foa5	<i>albedinis</i>	Date palm	Morocco	1985	ND	–	FPFS
Foap2	<i>asparagi</i>	Asparagus	Netherlands	1990	ND	+	FPFS, CWB6 (Wim Blok)
For11	<i>radicis lycopersici</i>	Tomato	France	1991	32	–	FPFS
For12	<i>radicis lycopersici</i>	Tomato	France	1991	25	–	FPFS
For16	<i>radicis lycopersici</i>	Tomato	France	1993	25	–	FPFS
For17	<i>radicis lycopersici</i>	Tomato	France	1994	25	–	FPFS
For18	<i>radicis lycopersici</i>	Tomato	France	1995	32	–	FPFS
For19	<i>radicis lycopersici</i>	Tomato	France	1995	32	–	FPFS
For110	<i>radicis lycopersici</i>	Tomato	France	1995	32	–	FPFS
For112	<i>radicis lycopersici</i>	Tomato	Morocco	1995	25	–	FPFS
For114	<i>radicis lycopersici</i>	Tomato	France	1992	23	–	FPFS
For115	<i>radicis lycopersici</i>	Tomato	France	1994	3	–	FPFS
For119	<i>radicis lycopersici</i>	Tomato	France	1985	25	–	FPFS
For122	<i>radicis lycopersici</i>	Tomato	France	1985	25	–	FPFS
For123	<i>radicis lycopersici</i>	Tomato	France	1986	25	–	FPFS
For125	<i>radicis lycopersici</i>	Tomato	France	1986	25	–	FPFS
For131	<i>radicis lycopersici</i>	Tomato	France	1997	32	–	FPFS
For143	<i>radicis lycopersici</i>	Tomato	France	1998	32	–	FPFS
For150	<i>radicis lycopersici</i>	Tomato	France	1998	25	–	FPFS
For160	<i>radicis lycopersici</i>	Tomato	Canada	NA	23	–	FPFS
For168	<i>radicis lycopersici</i>	Tomato	France	1998	32	–	FPFS
For170	<i>radicis lycopersici</i>	Tomato	Japan	NA	ND	–	FPFS, SUF959 (T. Arie)
Fo47	...	Soil	France	1978	22	–	FPFS, Edel et al. 2001
Fo5A4	...	Soil	France	1984	5	–	FPFS, Edel et al. 2001
91002	...	Soil	France	1991	1	–	FPFS, Edel et al. 1997
91004	...	Soil	France	1991	4	–	FPFS, Edel et al. 1997
91014	...	Soil	France	1991	14	+	FPFS, Edel et al. 1997
91015	...	Soil	France	1991	11	++	FPFS, Edel et al. 1997
91069	...	Soil	France	1991	3	++	FPFS, Edel et al. 1997
91121	...	Soil	France	1991	2	–	FPFS, Edel et al. 1997
91142	...	Soil	France	1991	8	–	FPFS, Edel et al. 1997
91248	...	Soil	France	1991	9	–	FPFS, Edel et al. 1997
91426	...	Soil	France	1991	15	+++	FPFS, Edel et al. 1997
92008	...	Soil	France	1992	16	–	FPFS, Edel et al. 1997
92015	...	Soil	France	1992	7	–	FPFS, Edel et al. 1997
92018	...	Soil	France	1992	10	–	FPFS, Edel et al. 1997
91B2	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B3	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B9	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B12	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B13	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B18	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B50	...	Wheat	France	1991	4	++	FPFS, Edel et al. 1997
91B51	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B52	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B53	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B54	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
91B56	...	Wheat	France	1991	4	–	FPFS, Edel et al. 1997
92B11	...	Wheat	France	1992	4	–	FPFS, Edel et al. 1997
92T1	...	Wheat	France	1992	4	–	FPFS, Edel et al. 1997
92T6	...	Wheat	France	1992	4	–	FPFS, Edel et al. 1997
92T11	...	Tomato	France	1992	11	+	FPFS, Edel et al. 1997
92T60	...	Tomato	France	1992	11	–	FPFS, Edel et al. 1997
Carq1	...	Soil	France	1996	5	–	FPFS, Edel et al. 2001
Carq2	...	Soil	France	1996	3	–	FPFS, Edel et al. 2001
Carq6	...	Soil	France	1996	37	++	FPFS, Edel et al. 2001
Carq38	...	Soil	France	1996	14	–	FPFS, Edel et al. 2001
Carq290	...	Soil	France	1996	23	–	FPFS, Edel et al. 2001
Chat3	...	Soil	France	1996	5	–	FPFS, Edel et al. 2001
Chat7	...	Soil	France	1996	3	+	FPFS, Edel et al. 2001
Chat59	...	Soil	France	1996	20	–	FPFS, Edel et al. 2001
Chat214	...	Soil	France	1996	23	–	FPFS, Edel et al. 2001
R23	...	Wheat rhizosphere	Netherlands	2002	ND	+++	WU-Phyto

among both pathogenic and nonpathogenic *F. oxysporum* strains; therefore, a total of 23 IGS types were found. The genetic relationships between the IGS types detected among the strains analyzed are illustrated in Figure 1.

Sensitivity of *F. oxysporum* for 2,4-DAPG.

Sensitivity of the 117 *F. oxysporum* strains for 2,4-DAPG was tested on one-fifth-strength PDA plates supplemented with 2,4-DAPG at concentrations ranging from 0 to 400 µg/ml. The radial growth was measured after 5 days of incubation at 25°C. Mycelial growth of many strains was completely inhibited at 2,4-DAPG concentrations of 25 and 50 µg/ml. These strains were considered 2,4-DAPG sensitive (Table 1; Fig. 2). Eleven pathogenic and nine nonpathogenic strains were able to grow at 2,4-DAPG concentrations of up to 400 µg/ml, although growth for most strains was reduced compared with the control (Fig. 2). These strains were considered 2,4-DAPG tolerant and included pathogenic *F. oxysporum* strains Fom28,

Fom37, Fom38, Fom1127, Focub1, Focub2, Focub3, Focub13, Fob3, Fod1, Foa2, and Foap2, and nonpathogenic *F. oxysporum* strains 91014, 91015, 91069, 91426, 91B50, 92T11, Carq6, Chat7, and R23. On average, 2,4-DAPG-tolerant strains accounted for approximately 17% of the total number of strains tested.

Tolerant *F. oxysporum* strains degrade 2,4-DAPG.

At a 2,4-DAPG concentration of 400 µg/ml, the antibiotic partly precipitates in the agar medium, resulting in opaque plates. Visual and microscopic inspection of plates amended with 2,4-DAPG at 400 µg/ml revealed that all but two tolerant *F. oxysporum* strains (91015 and 91B50) showed a clear zone (halo) underneath and, in many cases, up to 3 mm in front of the growing mycelium (Fig. 3). These results indicated that the 2,4-DAPG crystals had disappeared. To determine the fate of 2,4-DAPG, an extraction was performed on three different zones followed by high-performance liquid chromatography

Fig. 1. Dendrogram showing the restriction site differences in the intergenic spacer (IGS) region of *Fusarium oxysporum* strains representing 23 IGS types. The IGS types marked by an asterisk include 2,4-diacetylphloroglucinol-tolerant strains of *F. oxysporum*. The scale indicates the number of restriction site differences.

Fig. 2. In vitro assay to determine the sensitivity of the *Fusarium oxysporum* strains for different concentrations of 2,4-diacetylphloroglucinol (2,4-DAPG) present in one-fifth-strength potato dextrose agar plates. Displayed are three different strains. Fo47 is considered a sensitive strain; growth is completely inhibited at 2,4 DAPG concentrations of less than 50 µg/ml. Focub13 and 91426 are considered tolerant strains. Radial growth is never completely inhibited, even at 2,4-DAPG concentrations of 400 µg/ml.

(HPLC) analysis and photodiode array spectroscopy (Fig. 4). In the extract of zone A, two compounds were visible: peak 1, eluting at 37 min, represents the antioxidant BHT added during extraction; peak 2, at 25.7 min, represents 2,4-DAPG based on both retention time and spectral characteristics with absorption maxima at 270 and 330 nm (Fig. 4A). Based on integration of the peak area, 2,4-DAPG was present in zone A at a concentration of 427 $\mu\text{g/ml}$. In the extract from zone B, 2,4-DAPG also was detected at concentrations ranging from 300 to 345 $\mu\text{g/ml}$, representing, on average, 76% of the concentration detected in zone A (Fig. 4B). In the extract from zone C, 2,4-DAPG still could be detected at concentrations of 2.7 to 40 $\mu\text{g/ml}$, representing 0.6 to 9% of the initial 2,4-DAPG concentration as detected in zone A (Fig. 4C). These results demonstrated that most tolerant *F. oxysporum* strains were capable of metabolizing high concentrations of 2,4-DAPG.

Degradation of 2,4-DAPG through deacetylation.

The identification of 2,4-DAPG degradation intermediates may facilitate the determination of which mechanism or mechanisms are used by tolerant *F. oxysporum* strains to metabolize 2,4-DAPG. For most strains, compounds in addition to BHT (peak 1) and 2,4-DAPG (peak 2) emerged in zones B and C (Fig. 4B and C). Based on retention time and spectral analysis, peak 4 matched with fusaric acid. Peak 5 could not be characterized by photodiode array spectroscopy at first instance. Only for strain R23, an additional compound (peak 3) was detected by HPLC with a retention time of 18.5 min and with an absorption maximum at 285 nm (Fig. 5A and B). The spectral characteristics and retention time matched those of monoacetylphloroglucinol (MAPG) (Fig. 5B), suggesting that 2,4-DAPG is metabolized to MAPG via deacetylation. To further verify whether strain R23 is capable of metabolizing 2,4-DAPG by deacetylation, resulting in generation of MAPG and possibly phloroglucinol, accurate mass liquid chromatography–mass spectrometry (LC-MS) was performed on metabolites extracted from zones B and C. For all compounds analyzed, the observed mass was within 5 ppm of the expected mass, thus confirming the identity of 2,4-DAPG, MAPG, and fusaric acid (Table 2). Peak 5 represented a compound with a m/z of 127.0390 and deduced elemental composition of

Fig. 3. Two consecutive enlargements of a plate containing 2,4-diacetylphloroglucinol (2,4-DAPG) at 400 $\mu\text{g/ml}$ and the tolerant *Fusarium oxysporum* Focub13. At this concentration, 2,4-DAPG is partly precipitated (see also black particles in inset, indicated by black arrows), resulting in an opaque plate. The precipitated 2,4-DAPG disappears in the vicinity of the growing mycelium, resulting in the formation of a halo (indicated by white arrows). Indicated are the zones (A, B, and C) typically used for 2,4-DAPG concentration and metabolization analysis by methanol extraction followed by high-performance liquid chromatography analysis.

$\text{C}_6\text{H}_6\text{O}_3$, which corresponds to phloroglucinol. The accumulation of phloroglucinol and MAPG could not be detected in control plates (without fungal strains) amended with 2,4-DAPG (data not shown). For the other 2,4-DAPG-tolerant *F. oxysporum* strains, the intermediates MAPG and phloroglucinol could not be detected either. This may suggest another

Fig. 4. Chromatogram, recorded at 270 nm, of the methanol extract of the typical agar plate zones of 2,4-diacetylphloroglucinol (2,4-DAPG)-tolerant *Fusarium oxysporum* R23. In all three panels, butylated hydroxytoluene, the antioxidant present in the methanol extraction solvent, is visible (peak 1). Both the peak height and surface area underneath each peak is indicative for the concentration of each particular compound. **A**, Chromatogram of the opaque zone, which is indicative for the initial 2,4-DAPG concentration present in the plate (peak 2). **B**, Chromatogram of the zone around the halo. The height of peak 2 is reduced and other peaks emerge (peaks 3, 4, and 5). **C**, Chromatogram of the halo zone. Peak 2 has vanished almost completely, indicating that there is almost no 2,4-DAPG present in this zone. Peaks 3, 4, and 5 further increased in height. Based on retention time, spectral analysis and accurate mass spectrometry, peak 3 was identified as monoacetylphloroglucinol, peak 4 as fusaric acid, and peak 5 as phloroglucinol.

pathway of 2,4-DAPG degradation or a highly efficient processing of MAPG into phloroglucinol. This latter hypothesis was supported by mass spectrometry analysis for the 2,4-DAPG-tolerant strain 91426, showing trace elements of MAPG (m/z 169.0498) and the accumulation of phloroglucinol (m/z 127.0390). Collectively, these results strongly suggest that deacetylation is, at least for some of the 2,4-DAPG-tolerant *F. oxysporum* strains, one of the initial steps in the degradation of 2,4-DAPG.

Fusaric acid production varies significantly among *F. oxysporum* strains.

As was demonstrated previously (Duffy and Dfago 1997, Notz et al. 2002), fusaric acid negatively affects the biosynthesis of 2,4-DAPG in several strains of *P. fluorescens*. This

finding may suggest that 2,4-DAPG-sensitive strains of *F. oxysporum* rely on the production of high fusaric acid levels to keep 2,4-DAPG accumulation in their habitat to a minimum, whereas the production of fusaric acid may not be as important for the 2,4-DAPG-tolerant *F. oxysporum* strains. Therefore, we determined the fusaric acid production of all 117 strains in an in vitro assay (Fig. 6). Among both 2,4-DAPG-sensitive and -tolerant strains, the fusaric production levels varied considerably, ranging from 1,362 mg/g of biomass dry weight for Fo132 to nondetectable levels for 22 of the 117 strains. Subsequently, the effect of 2,4-DAPG on fusaric acid production in *F. oxysporum* strains was studied. Six strains were included in this assay: three 2,4-DAPG-sensitive strains varying significantly in their fusaric acid production levels (Fo47, 92T6, and For11) and three 2,4-DAPG-tolerant strains, also varying significantly in their fusaric acid production levels (Focub2, 91426, and Fom1127). For each strain, 28 individual liquid cultures were grown; 2,4-DAPG was added to 14 cultures at a final concentration of 25 $\mu\text{g/ml}$, which is not lethal for all the strains tested. 2,4-DAPG concentrations were measured every 24 h and, if necessary, readjusted to concentrations of 25 $\mu\text{g/ml}$. In the case of strains Fo47, 92T6, For11, and Fom1127, the presence of 2,4-DAPG did not result in a statistically significant reduction or enhancement of fusaric acid production levels relative to the control treatment without 2,4-DAPG. However, exposure of the tolerant strains 91426 and Focub2 to 2,4-DAPG led to opposite responses. For strain 91426, addition of 2,4-DAPG resulted in a significant increase in fusaric acid production levels from 9 ± 0.04 to 42 ± 0.2 $\mu\text{g/g}$ of biomass dry weight, seven days postinoculation; in contrast, addition of 2,4-DAPG to cultures of strain Focub2 reduced fusaric acid production more than fourfold, from 414 ± 45 to 92 ± 1 $\mu\text{g/g}$ of biomass dry weight, at 7 days postinoculation (Fig. 7). Thus, the effect of 2,4-DAPG on fusaric acid production may be strain dependent.

Relationship between 2,4-DAPG tolerance and traits of *F. oxysporum*.

2,4-DAPG tolerance of the *F. oxysporum* strains tested in this study was not linked to their pathogenicity. The percentage of tolerant strains was 22 and 14.5% among the nonpathogenic and pathogenic strains analyzed, respectively. The pathogenic 2,4-DAPG-tolerant strains corresponded to eight different formae speciales and also had various geographic origins (Table 1). Similarly, tolerant strains were found within several IGS types, which were distributed all along the dendrogram, suggesting that 2,4-DAPG-tolerant strains of *F. oxysporum* are genetically distant (Fig. 1). There also was no evidence of any correlation between 2,4-DAPG sensitivity or tolerance and fusaric acid production levels. The 2,4-DAPG-tolerant strains R23, Fom38, Focub2, and 92T11 produced considerable amounts of fusaric acid (600, 464, 282, and 256 mg/g of biomass dry weight, respectively), whereas Fom1127, Foa2, and

Fig. 5. A, Chromatogram, recorded at 285 nm, of the methanol extract of the halo zone (zone C) of 2,4-diacetylphloroglucinol-tolerant *Fusarium oxysporum* R23. Peak 1, the antioxidant present in the methanol extraction solvent; peak 2, 2,4-DAPG concentration present in the plate; peak 3, monoacetylphloroglucinol; peak 4, fusaric acid; and peak 5, phloroglucinol **B**, Spectral characteristics analysis of peak 3 matches monoacetylphloroglucinol (MAPG) (see inset).

Table 2. Elemental composition and identification by mass spectrometry of the peaks detected in Figure 4^a

Peak number	Compound name	Elemental composition	Exact mass [M + H] ⁺		Accuracy Δppm
			Calculated	Measured	
2	2,4-DAPG	$\text{C}_{10}\text{H}_{10}\text{O}_5$	211.0606	211.0603	-1.7
	-H ₂ O	$\text{C}_{10}\text{H}_8\text{O}_4$	193.0501	193.0493	-4.1
3	MAPG	$\text{C}_8\text{H}_8\text{O}_4$	169.0501	169.0498	-1.7
	-H ₂ O	$\text{C}_8\text{H}_6\text{O}_3$	151.0400	151.0395	+3.2
5	Phloroglucinol	$\text{C}_6\text{H}_6\text{O}_3$	127.0395	127.0390	-3.9
4	Fusaric acid	$\text{C}_{10}\text{H}_{13}\text{NO}_2$	180.1025	180.1016	-4.7

^a Measured masses were determined by accurate mass liquid chromatography-mass spectrometry on a high-resolution time-of-flight mass spectrometer with lock mass correction. 2,4-DAPG = 2,4-diacetylphloroglucinol and MAPG = monoacetylphloroglucinol.

Foap2 produced no detectable amounts. The other 2,4-DAPG-tolerant *F. oxysporum* strains produced fusaric acid at concentrations ranging from 3.6 to 161 mg/g of biomass dry weight.

DISCUSSION

Studies on interactions between antagonistic microorganisms and plant-pathogenic fungi mostly focus on a multitude of factors related to the behavior of the microbial antagonist (the antagonist perspective), i.e., how biocontrol agents affect pathogens; which mechanisms, metabolites, and genes are involved; and how antagonists behave and survive after introduction into a specific environment. In contrast, relatively few studies have focused on the 'pathogen perspective,' i.e., responses of plant pathogens to microbial antagonists (Duffy et al. 2003). The results of this study demonstrate that specific plant-pathogen populations harbor strains with adequate defense mechanisms against 2,4-DAPG, a broad-spectrum antibiotic produced by multiple strains of antagonistic *P. fluorescens*. Among a collection of 117 genotypically different strains of *F. oxysporum*, con-

Fig. 6. Fusaric acid production by *Fusarium oxysporum* strains, represented as the average amount (μg) per gram of dry-biomass mycelium. Potato dextrose broth was inoculated with agar plugs containing mycelium and incubated at 25°C for 7 days in a rotary shaker. The amount of fusaric acid was determined by high-performance liquid chromatography analysis. The mycelium was harvested by filtration, and subsequently freeze dried and weighed. Arrows indicate the 2,4-diacetylphloroglucinol-tolerant strains and the error bars represent the standard error of the mean of two experiments.

the sensitive *F. oxysporum* strains described in this study. Only one strain of *Pythium deliense* was tested; therefore, the question remains whether other *Pythium deliense* strains also are tolerant. In studies on the efficacy of biocontrol agents, often only one single strain of the target pathogen is tested. When considering the observed variations in sensitivity to antagonistic traits in this and other studies (Duffy et al. 2003), testing of only a few strains of a pathogen may well be inadequate for determining the value of a particular trait for the efficacy of biocontrol.

The results obtained in HPLC-PDA and LC-QTOF-MS analyses strongly indicated that deacetylation of 2,4-DAPG to MAPG and phloroglucinol is among the initial mechanisms by which tolerant *F. oxysporum* strains resist 2,4-DAPG. Previous studies by de Souza and associates (2003a) have clearly shown that the lower the number of acetyl groups the lower the toxicity of phloroglucinol derivatives. Although we were able to detect phloroglucinol, this compound itself probably is further metabolized by the process of ring fission as described previously for *F. solani* (Walker and Taylor 1983). We have not yet characterized the enzymes or the coding genes, which are responsible for the deacetylation process. Possible candidates may be deacetylases or acetyltransferases. The latter may be of particular interest because such an enzyme, trichothecene 3-*O*-acetyltransferase, already was described for trichothecene detoxification in *F. graminearum*, and probably is necessary for self protection (Kimura et al. 1998a). The coding gene, *Tri101*, is not located within the gene cluster necessary for the trichothecene synthesis; therefore, Kimura and associates (Kimura et al. 1998b) postulated that this gene has evolved independently and was acquired by horizontal gene transfer. A homologous gene was found in *F. sporotrichioides* (Kimura et al. 1998b).

Fig. 7. Fusaric acid production by *Fusarium oxysporum* strains Focub2 and 91426 in the presence (shaded bars) or absence (dotted bars) of 25 µg of 2,4-DAPG per ml. Fusaric acid production was measured over a period of seven days after initiation of mycelial growth and is represented as the average amount (µg) per gram dry-biomass mycelium. For each strain and treatment, 28 round flasks containing potato dextrose broth were inoculated with agar plugs containing mycelium and were incubated at 25°C in a rotary shaker. In the case of the antibiotic treatment, the medium was supplemented with 25 µg of 2,4-DAPG per ml. Every 24 h, two flasks were harvested and analyzed for fusaric acid content, 2,4-DAPG content (when applicable), and mycelium dry weight. In case the 2,4-DAPG concentration had decreased, 2,4-DAPG was added to the remaining flask to elevate the concentration to 25 µg/ml. The error bars represent the standard error of the mean (SEM). For each timepoint, different letters indicate a statistically significant difference ($P = 0.05$).

The strategies for coping with antibiotics from biocontrol agents appear to be as diverse and based, in part, on similar mechanisms as described for resistance to other toxins, i.e., enzymatic degradation, efflux mechanisms, and changing the target site of the toxin. Next to our example of degradation of 2,4-DAPG by different *F. oxysporum* strains, *Botrytis cinerea* also can degrade 2,4-DAPG by means of laccase activity (A. Schouten, G. van den Berg, and J. M. Raaijmakers, unpublished data). Two tolerant *F. oxysporum* strains, 91015 and 91B50, did not show an apparent degradation of 2,4-DAPG. Although these strains were not further studied in detail, their tolerance to 2,4-DAPG may be caused, in part, by an efflux mechanism as demonstrated previously for *B. cinerea* (Schoonbeek et al. 2002).

To obtain additional information on the defense capabilities of both sensitive and tolerant *F. oxysporum* strains, fusaric acid production levels also were determined. Fusaric acid can directly affect 2,4-DAPG biosynthesis by repressing *phlA* expression (Notz et al. 2002). In the present study, a significant variation in fusaric acid production levels was observed for *F. oxysporum*. These results confirm and extend results obtained by Bacon and associates (1996), who analyzed 78 strains belonging to different *Fusarium* spp. The results in this study showed that there is a significant percentage of *F. oxysporum* strains that does not produce detectable levels of fusaric acid under the tested conditions. Other strains were capable of producing relatively high amounts of fusaric acid. These levels were not correlated with formae speciales, genetic background, and, in particular, 2,4-DAPG tolerance. The different tolerant strains produced various amounts of fusaric acid. For three of those, Fom1127, Foa2, and Foap2, no fusaric acid production was detected, whereas four strains, R23, Fom38, Focub2, and 92T11, produced relatively high amounts. Thus, plant-pathogenic strains Fom38 and Focub2 would be particularly difficult to control by 2,4-DAPG-producing biocontrol agents. Furthermore, one may postulate that the presence of sublethal concentrations of 2,4-DAPG may trigger fusaric acid production in *F. oxysporum* strains to repress 2,4-DAPG synthesis in *Pseudomonas fluorescens* to prevent further accumulation of 2,4-DAPG in their habitat to toxic concentrations. The results of this study further showed that the effect of 2,4-DAPG on fusaric acid production in *F. oxysporum* is strain dependent. Interestingly, fusaric-acid-mediated repression of 2,4-DAPG synthesis in *Pseudomonas* spp. also was shown to be strain dependent: fusaric acid blocked 2,4-DAPG biosynthesis in *P. fluorescens* CHA0 but not in strain Q2-87 (Duffy et al. 2004). In this context, Duffy and associates (2004) suggested that sensitivity of 2,4-DAPG-producing *Pseudomonas* strains to fusaric acid partly explains the variation in biocontrol efficacy. Given that 2,4-DAPG production in *Pseudomonas* spp. may vary depending on abiotic or biotic conditions, it would be interesting to determine whether there are differences with respect to fusaric acid production between the sensitive and tolerant *F. oxysporum* isolates when exposed to different 2,4-DAPG concentrations. The results of the present study, showing considerable variation in sensitivity of *F. oxysporum* strains to 2,4-DAPG and the ability of multiple strains to degrade 2,4-DAPG, provide a new addition to the multitude of biotic factors that determine the efficacy of biological control with antagonistic *Pseudomonas* strains.

The result on fusaric acid production capabilities may lead to an additional conclusion, which is not within the scope of this article but nevertheless is noteworthy. Because there were enormous variations in fusaric acid production levels within all the formae speciales, one may postulate that fusaric acid is not a virulence factor, as suggested before (Bacon et al. 1996; Bryden et al. 2001). Several pathogenic strains, which did not produce detectable amounts of fusaric acid under the tested conditions,

are known to be virulent and produce symptoms on their host plant. Conversely, the saprophytic strain Fo47, a strain which can colonize plants without showing any symptoms and which frequently is used in the biocontrol of pathogenic *F. oxysporum* strains (de Souza et al. 2003a; Duijff et al. 1998; Fravel et al. 2003), has high fusaric acid production capability. Similar results were reported for *F. moniliforme* (Bacon et al. 1996). Therefore, the role of fusaric acid in pathogenicity remains unresolved. In this respect, Duffy and associates (2004) speculated that the broad-spectrum antimicrobial activity of fusaric acid and other mycotoxins may be of potential importance for survival of the producing strains in crops and crop residues.

Handelsman and Stabb (1996) suggested that most biocontrol agents suppress disease via more than one mechanism, and resistance to multiple antagonistic traits should occur only at a very low frequency. Second, antagonistic microorganisms are thought to operate in microsites on the plant surface where only a fraction of the pathogen population is exposed during a short period of its life cycle. Additionally, Sevéno and associates (2002) suggested that, in case of antibiosis, only minute amounts of the compounds are produced by the biocontrol agent as opposed to the inundative application of chemical pesticides. These conditions are, among others, expected to exert a relatively low selection pressure for the buildup of resistance in pathogen populations to biocontrol. Overall, our results emphasize again that this view is based merely on the initially limited amount of experimental data and that plant pathogens probably already have developed ample and effective defense strategies against biocontrol agents (Duffy et al. 2003). Consequently, as found for any other control measure, one may postulate that the incidence of insensitive plant pathogens will increase with the increasing use of biocontrol agents. Preliminary results of recent experiments performed in collaboration with P. A. H. M. Bakker and M. Viebahn (Utrecht University, The Netherlands) suggest that this is indeed happening. In a field trial, in which the survival of genetically modified *P. fluorescens* strains was monitored during successive wheat cultivation for several years, introduction of a transgenic *Pseudomonas* strain producing 2,4-DAPG caused transient shifts in indigenous *Fusarium* populations toward strains that efficiently degrade 2,4-DAPG. Thus, it becomes increasingly evident that biocontrol strategies have to anticipate the fact that plant pathogen populations are not static but, rather, dynamic entities, capable of rapidly adapting to and coping with adverse conditions.

MATERIALS AND METHODS

Fusarium oxysporum strains, growth, and storage conditions.

In all, 117 strains of *F. oxysporum* were analyzed (Table 1). The collection included 76 pathogenic strains representative of 13 different formae speciales, and 41 strains representative of the genetic diversity found within soil borne populations of different soils (Edel et al. 1997, 2001). The 29 strains with the first two digits 91 or 92 were nonpathogenic on flax, melon, and tomato (Steinberg et al. 1997). The other soil borne strains were isolated from samples collected from cultivated fields with various vegetable crops, including melon and tomato, in which no *Fusarium* diseases were observed. Thus, the soil borne strains included in this study were considered to be saprophytic strains.

F. oxysporum strains were grown at 25°C on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, England) plates by inoculation with conidia from frozen (–80°C) stock solutions. To preserve *F. oxysporum* strains, conidia were collected from the sporulating cultures 7 to 14 days after inoculation by scraping

and using 2 ml of potato dextrose broth. This suspension was mixed with an equal volume of 50% (vol/vol) sterilized glycerol, dispensed in 1 ml aliquots, and stored at –80°C.

Genetic characterization of the plant-pathogenic *F. oxysporum* strains.

A subsample of 32 pathogenic strains was characterized by restriction fragment length polymorphism (RFLP) analysis of the ribosomal IGS as described previously (Edel et al. 1995, 1997). A fragment of the IGS was amplified by polymerase chain reaction with oligonucleotide primers PNfo (5'-CCCGCCTGGCTGCGTCCGACTC-3') and PN22 (5'-CAAGCATATGACTACTGGC-3') and digested with seven restriction enzymes: *AluI*, *HaeIII*, *HinfI*, *MspI*, *RsaI*, *ScrFI*, and *XhoI*. Each strain was assigned to an IGS type defined by the combination of the restriction patterns obtained with the seven enzymes. In addition, the IGS types corresponding to 40 non-pathogenic strains were determined previously (Edel et al. 1997, 2001). The pairwise restriction site differences between IGS types were represented as a dendrogram with the computer program NTSYS (numerical taxonomy and multivariate analysis system, version 2.0, State University of New York, Stony Brook, U.S.A.).

2,4-DAPG sensitivity assay.

Plates containing one-fifth-strength potato dextrose (Oxoid), supplemented with 1.5 mM McIlvaine's citric acid-phosphate buffer (Sober 1970) set at pH 6.0, and 1.5 % (wt/vol) agar (Oxoid) were supplemented with HPLC-purified 2,4-DAPG (de Souza et al. 2003a) at 0 (control), 25, 50, 100, 200, or 400 µg/ml, and inoculated with a plug (5 mm in diameter) of mycelium. To properly dissolve 2,4-DAPG, 100× concentrated stock solutions were prepared in ethanol and added to liquid agar medium (50°C) immediately prior to pouring. An equal amount of ethanol was added to the control. After incubation for 5 days at 25°C, the radial growth was measured. For each 2,4-DAPG concentration, two replicates were used. The experiment was performed twice.

Fusaric acid production by *F. oxysporum* strains.

To determine the fusaric acid production capabilities of the *F. oxysporum* strains, the method described by Notz and associates (2002) was used with modifications. Liquid Czapek Dox (Oxoid) medium, supplemented with 1.5 mM McIlvaine's citric acid-phosphate buffer (pH 6.0), was prepared according to the manufacturer's instructions and dispensed in 100-ml aliquots in flat-bottom flasks. After sterilization, the medium was inoculated with three PDA plugs containing mycelium and incubated for 7 days at 24°C in a rotary shaker at 180 rpm. The mycelium was harvested by filtration over Miracloth (Calbiochem, San Diego, CA, U.S.A.) and the fungal biomass was determined after freeze-drying. The total volume of the culture filtrate was measured and the fusaric acid concentration was calculated by measuring the area under the fusaric acid peak detected at 270 nm by the array detector after HPLC. Different concentrations of purified fusaric acid (Sigma-Aldrich, St. Louis) were used to calculate the relation between peak area and concentration.

The effect of 2,4-DAPG on the fusaric acid accumulation was determined by inoculating 28 flat-bottom flasks containing 100 ml of Czapek Dox medium, supplemented with 1.5 mM McIlvaine's citric acid-phosphate buffer set at pH 6.0, with mycelium from a *F. oxysporum* strain. 2,4-DAPG was added to 14 of these cultures to a final concentration of 25 µg/ml at the time of inoculation. For 7 days, four flasks were sampled every 24 h, two with and two without 2,4-DAPG. The mycelium was harvested by filtration over Miracloth (Calbio-

chem) and the fungal biomass was determined after freeze-drying. The total volume of the culture filtrate was measured and both the average 2,4-DAPG (when applicable) and the fusaric acid concentrations were calculated by measuring the area under the 2,4-DAPG and fusaric acid peaks, respectively, both detected at 270 nm by the array detector after HPLC. When necessary, 2,4-DAPG was added to the remaining 2,4-DAPG-containing cultures in order to maintain the antibiotic concentration at 25 µg/ml throughout the incubation period. Differences in fusaric acid production levels by *Fusarium* isolates exposed or not to 2,4-DAPG were analyzed statistically by a *t* test (*P* = 0.05).

HPLC-photodiode array analysis.

2,4-DAPG was quantified by HPLC analysis on a Waters HPLC system using a Symmetry C18 reversed phase column (150 by 3.9 mm, 5 µm), preceded by a Symmetry C18 reversed-phase guard column (20 by 3.9 mm, 5 µm), which were connected to a 600S system controller, 616 pump unit, 996 photodiode array detector, and 717S autosampler.

From the plates containing 2,4-DAPG at 400 µg/ml, 0.1 to 1 g of agar was extracted with two volumes (wt/vol) of methanol, supplemented with 0.75% (wt/vol) butylated hydroxytoluene (BHT; Sigma-Aldrich), by overnight incubation. From the methanol fraction, 10 to 50 µl was injected onto the column, which had been equilibrated with 10% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid (TFA) (solvent A), and eluted at a flow rate of 0.5 ml/min using an isocratic flow of 100% solvent A for 2 min, a linear gradient from 100% solvent A to 100% solvent B (90% [vol/vol] acetonitrile and 0.1% [vol/vol] TFA) in 23 min and an isocratic flow of 100% solvent B for 5 min. Under these conditions, the 2,4-DAPG standard eluted at a retention time of 25.7 min with an absorption maximum at 270 nm.

The simultaneous detection and quantification of 2,4-DAPG and fusaric acid in liquid cultures was done on the same Waters HPLC system as mentioned above, but lacking the guard column. A culture filtrate sample was centrifuged at 14,000 × *g* for 10 min to remove fungal debris. An equal volume of methanol was added to the supernatant. Of this mixture, 10 to 50 µl was injected onto the reverse phase column, which was equilibrated with 25% (vol/vol) methanol and 0.03% (vol/vol) TFA (solvent C), and eluted by a flow rate of 1 ml/min using an isocratic flow of 100% solvent C for 2 min, a linear gradient from 100% solvent C to 100% solvent D (methanol and 0.3% [vol/vol] TFA) in 13 min, and an isocratic flow of 100% solvent D for 5 min. Under these conditions, fusaric acid (Sigma-Aldrich) and 2,4-DAPG standards were detected at 270 nm (optimal wavelength) and at retention times of 7.9 and 16.2 min, respectively.

LC-MS analysis.

To identify 2,4-DAPG derivatives, samples were subjected to accurate-mass LC-MS on a high-resolution time-of-flight mass spectrometer with lock mass correction (Wolff et al. 2001). Samples were injected and separated using a Waters Alliance 2795 HT system equipped with a Luna C18-reversed phase column (150 by 2.1 mm, 3 µm; Phenomenex, Torrance, CA, U.S.A.) at 40°C and a gradient from 5 to 50% (vol/vol) acetonitrile acidified with 0.1% (vol/vol) formic acid at a flow rate of 0.2 ml/min. Eluting compounds were detected online at 210 to 600 nm by a 2996 photodiode array detector followed by a Q-TOF Ultima MS with ESI-source. The following settings were applied during the LC-MS runs: desolvation temperature of 300°C with a nitrogen gas flow of 500 liters/h, capillary spray at 3 kV, source temperature of 120°C, cone at 35 eV with nitrogen gas flow at 50 liters/h, collision energy at 5 eV. Positive ions in the

100 to 1,000 *m/z* range were detected using a scan time of 0.9 s and an interscan delay of 0.1 s. The MS was calibrated with 0.5% (vol/vol) phosphoric acid in 50% (vol/vol) acetonitrile and leucine enkephalin was used as a lock mass. Masslynx software version 4.0 was used to control all instruments and to calculate accurate masses.

ACKNOWLEDGMENTS

The work presented here was financed in part by the Royal Netherlands Academy of Arts and Sciences. Additional financial support was obtained by an NWO-Van Gogh grant (no. VGP 83-192).

LITERATURE CITED

- Alexander, N. J., McCormick, S. P., and Hohn, T. M. 1999. TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: Gene isolation and expression in yeast. *Mol. Gen. Genet.* 261:977-84.
- Bacon, C. W., Porter, J. K., Norred, W. P., and Leslie, J. F. 1996. Production of fusaric acid by *Fusarium* species. *Appl. Environ. Microbiol.* 62:4039-4043.
- Bryden, W. L., Logrieco, A., Abbas, H. K., Porter, J. K., Vesonder, R. F., Richard, J. L., and Cole, R. J. 2001. Other significant *Fusarium* mycotoxins. In: *Fusarium*: Paul E. Nelson Memorial Symposium. B. A. Sumner, J. F. Leslie, D. Backhouse, W. L. Bryden, and L. W. Burgess, eds. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Chelkowski, J. 1989. *Fusarium*: Mycotoxins, taxonomy and pathogenicity. In: *Topics in Secondary Metabolism*, vol. 2. Elsevier, Amsterdam.
- Chin-A-Woeng, T. F. C., Bloemberg, G. V., and Lugtenberg, B. J. J. 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol.* 157:503-523.
- de Souza, J. T., Arnould, C., Deulvot, C., Lemanceau, P., Gianinazzi-Pearson, V., and Raaijmakers, J. M. 2003a. Effect of 2,4-diacetylphloroglucinol on *Pythium*: Cellular responses and variation in sensitivity among propagules and species. *Phytopathology* 93:966-975.
- de Souza, J. T., de Boer, M., de Waard, P., van Beek, T. A., and Raaijmakers, J. M. 2003b. Biochemical, genetic, and zoosporecidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. *Appl. Environ. Microbiol.* 69:7161-7172.
- de Souza, J. T., Weller, D. M., and Raaijmakers, J. M. 2003c. Frequency, diversity, and activity of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. in Dutch take-all decline soils. *Phytopathology* 93:54-63.
- De Waard, M. A. 1997. Significance of ABC transporters in fungicide sensitivity and resistance. *Pestic. Sci.* 51:271-275.
- Duffy, B., Keel, C., and Defago, G. 2004. Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. *Appl. Environ. Microbiol.* 70:1836-1842.
- Duffy, B., Schouten, A., and Raaijmakers, J. M. 2003. Pathogen self-defense: Mechanisms to counteract microbial antagonism. *Annu. Rev. Phytopathol.* 41:501-538.
- Duffy, B. K., and Defago, G. 1997. Zinc improves biocontrol of *Fusarium* crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. *Phytopathology* 87:1250-1257.
- Duijff, B. J., Pouhair, D., Olivain, C., Alabouvette, C., and Lemanceau, P. 1998. Implication of systemic induced resistance in the suppression of *Fusarium* wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. *Eur. J. Plant Pathol.* 104:903-910.
- Edel, V., Steinberg, C., Avelange, I., Laguerre, G., and Alabouvette, C. 1995. Comparison of 3 molecular methods for the characterization of *Fusarium oxysporum* strains. *Phytopathology* 85:579-585.
- Edel, V., Steinberg, C., Gautheron, N., and Alabouvette, C. 1997. Populations of nonpathogenic *Fusarium oxysporum* associated with roots of four plant species compared to soilborne populations. *Phytopathology* 87:693-697.
- Edel, V., Steinberg, C., Gautheron, N., Recorbet, G., and Alabouvette, C. 2001. Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 36:61-71.
- Fleissner, A., Sopalla, C., and Weltring, K. M. 2002. An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. *Mol. Plant-Microbe Interact.* 15:102-108.
- Fravel, D., Olivain, C., and Alabouvette, C. 2003. *Fusarium oxysporum* and its biocontrol. *New Phytol.* 157:493-502.

- Gupta, A. K., Baran, R., and Summerbell, R. C. 2000. *Fusarium* infections of the skin. *Curr. Opin. Infect. Dis.* 13:121-128.
- Handelsman, J., and Stabb, E. V. 1996. Biocontrol of soilborne plant pathogens. *Plant Cell* 8:1855-1869.
- Joffe, A. Z. 1986. *Fusarium* Species: Their Biology and Toxicology. Wiley, New York.
- Kamei, Y., and Isnansetyo, A. 2003. Lysis of methicillin-resistant *Staphylococcus aureus* by 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. AMSN isolated from a marine alga. *Int. J. Antimicrob. Agents* 21:71-4.
- Keel, C., Weller, D. M., Natsch, A., Dfago, G., Cook, R. J., and Thomashow, L. S. 1996. Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl. Environ. Microbiol.* 62:552-563.
- Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., and Yamaguchi, I. 1998a. Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of *Tri101*. *J. Biol. Chem.* 273:1654-61.
- Kimura, M., Matsumoto, G., Shingu, Y., Yoneyama, K., and Yamaguchi, I. 1998b. The mystery of the trichothecene 3-O-acetyltransferase gene. Analysis of the region around *Tri101* and characterization of its homologue from *Fusarium sporotrichioides*. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 435:163-8.
- Lagopodi, A. L., Ram, A. F. J., Lamers, G. E. M., Punt, P. J., Van den Hondel, Camj, Lugtenberg, B. J. J., and Bloembergen, G. V. 2002. Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Mol. Plant-Microbe Interact.* 15:172-179.
- Lorie, G., Edel-Hermann, V., Gautheron, N., and Alabouvette, C. 2004. Genetic diversity of pathogenic and nonpathogenic populations of *Fusarium oxysporum* isolated from carnation fields in Argentina. *Phytopathology* 94:661-668.
- Mazzola, M., Fujimoto, D. K., Thomashow, L. S., and Cook, R. J. 1995. Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp. and effect on biological control of take-all of wheat. *Appl. Environ. Microbiol.* 61:2554-2559.
- Morrissey, J. P., and Osbourn, A. E. 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* 63:708-24.
- Nielsen, T. H., Sorensen, D., Tobiasen, C., Andersen, J. B., Christophersen, C., Givskov, M., and Sorensen, J. 2002. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. *Appl. Environ. Microbiol.* 68:3416-3423.
- Notz, R., Maurhofer, M., Dubach, H., Haas, D., and Dfago, G. 2002. Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of wheat. *Appl. Environ. Microbiol.* 68:2229-2235.
- Raaijmakers, J. M., Vlami, M., and de Souza, J. T. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* 81:537-547.
- Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot Bonnefoy, C., Reimann, C., Notz, R., Dfago, G., Haas, D., and Keel, C. 2000. Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J. Bacteriol.* 182:1215-1225.
- Schoonbeek, H. J., Raaijmakers, J. M., and De Waard, M. A. 2002. Fungal ABC transporters and microbial interactions in natural environments. *Mol. Plant-Microbe Interact.* 15:1165-1172.
- Sveno, N. A., Kallifidas, D., Smalla, K., van, Elsas J. D., Collard, J. M., Karagouni, A. D., and Wellington, E. M. H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. *Rev. Med. Microbiol.* 13:15-27.
- Sober, H. A., ed. 1970. *Handbook of Biochemistry*. 2nd ed. The Chemical Rubber Co., Cleveland.
- Steffens, J. J., Pell, E. J., and Tien, M. 1996. Mechanisms of fungicide resistance in phytopathogenic fungi. *Curr. Opin. Biotechnol.* 7:348-55.
- Steinberg, C., Edel, V., Gautheron, N., Abadie, C., Vallaey, T., and Alabouvette, C. 1997. Phenotypic characterization of natural populations of *Fusarium oxysporum* in relation to genotypic characterization. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 24:73-85.
- Summerbell, R. C., and Schroers, H. J. 2002. Analysis of phylogenetic relationship of *Cylindrocarpon lichenicola* and *Acremonium falciforme* to the *Fusarium solani* species complex and a review of similarities in the spectrum of opportunistic infections caused by these fungi. *J. Clin. Microbiol.* 40:2866-2875.
- Thomashow, L. S., and Weller, D. M. 1996. Current concepts in the use of introduced bacteria: Mechanisms and antifungal metabolites. In: *Plant-Microbe Interactions*. G. Stacey and N. T. Keen, eds. Chapman & Hall, New York.
- VanEtten, H., Temporini, E., and Wasmann, C. 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: Why is it not required by all pathogens? *Physiol. Mol. Plant Pathol.* 59:83-93.
- Walker, J. R. L., and Taylor, B. G. 1983. Metabolism of phloroglucinol by *Fusarium solani*. *Arch. Microbiol.* 134:123-126.
- Wolff, J.-C., Eckers, C., Sage, A. B., Giles, K., and Bateman, R. 2001. Accurate mass liquid chromatography/mass spectrometry on quadrupole orthogonal acceleration time-of-flight mass analyzers using switching between separate sample and reference sprays. 2. Applications using the dual-electrospray ion source. *Anal. Chem.* 73:2605-2612.