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Pseudomonads as Antagonists of Plant Pathogens in the Rhizosphere: Role of the Antibiotic 2,4-Diacetylphloroglucinol in the Suppression of Black Root Rot of Tobacco*

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Abstract

Pseudomonas fluorescens strain CHA0 is an effective biocontrol agent of diseases caused by soilborne plant pathogens. Strain CHA0 produces several secondary metabolites, notably cyanide, acetylpheoroglucinols and pyoluteorin. Cyanide plays an important role in the suppression of black root rot of tobacco, caused by *Thielaviopsis basicola*. A mutant, CHA625, has now been isolated, which does not produce 2,4-diacetylphloroglucinol. Strain CHA625 suppressed black root rot to a distinctly smaller extent than did wild-type CHA0 under gnotobiotic conditions. A cosmid obtained from a genomic library of strain CHA0 restored the ability of strain CHA625 to produce this metabolite and to suppress disease. Addition of synthetic 2,4-diacetylphloroglucinol to soil reduced disease severity in the absence of bacteria. These results suggest that the production of 2,4-diacetylphloroglucinol by *P. fluorescens* strain CHA0 is another important factor, in addition to cyanide, in the suppression of black root rot of tobacco.

Keywords: biocontrol, soilborne pathogens, *Pseudomonas*, antibiotics, phloroglucinol, cyanide

1. Introduction

Fluorescent pseudomonads are known to suppress plant diseases caused by soilborne pathogens and to promote plant growth in greenhouse and field experiments (Burr and Caesar, 1984; Bruehl, 1987; Davison, 1988; Défago and Haas, 1990; Digat and Gardan, 1987; Schippers, 1988; Schroth and Hancock, 1982; Suslow, 1982; Weller, 1988; Weller and Cook, 1986). Different hypotheses have been proposed to explain

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pathogen suppression: inhibition of the pathogen by siderophore-mediated competition for iron (Baker et al., 1986; Leong, 1986; Misaghi et al., 1982; Neilands and Leong, 1986; Schippers et al., 1987) or by production of antibiotic metabolites (Fravel, 1988; Weller, 1988). Besides these two major hypotheses, induction of resistance in the plant, aggressive root colonization, and stimulation of plant growth are discussed as possible mechanisms of disease suppression (Défago et al., 1990 Weller, 1988).

Pseudomonads produce a wide array of antibiotic metabolites (Leisinger and Margraff, 1979). Some of them, pyrroles, phenazines, and cyanide, have been shown to be of importance in biological control of soilborne pathogens (Howell and Stipanovic, 1979, 1980; Thomashow and Weller, 1988; Voisard et al., 1989). Antibiotic metabolites produced by pseudomonads exert in most cases an activity against a broad spectrum of bacteria and fungi (Défago and Haas, 1990; Kiprianova and Smirnov, 1981). Some of the antibiotics also show a pronounced phytotoxicity, e.g. phenazines (Toohey et al., 1965), pyoluteorin (Ohmori et al., 1978), and 2,4-diacetylphloroglucinol (Reddi et al., 1969).

Support for the hypothesis that antibiotics are involved in biological control has come from a correlation of pathogen inhibition *in vitro* and disease suppression *in vivo* (Ahl et al., 1986; Bencini et al., 1983; Garagulya et al., 1974; Gardner et al., 1984; Hasegawa et al., 1988; Lambert et al., 1987; Pidoplichko and Garagulya, 1974; Xu and Gross; 1986). In addition, Howell and Stipanovic (1979, 1980) have demonstrated that both the antibiotics produced by *P. fluorescens* strain Pf-5-pyoluteorin and pyrrolnitrin, are themselves sufficient to suppress damping-off of cotton. Direct evidence for the role of antibiotics in biocontrol has come from studies with chemically or genetically generated mutants that are defective in their antagonistic potential. However, in many of these studies the antibiotics involved in disease suppression were not identified (Colyer and Mount, 1984; Gutterson et al., 1986, 1988; Howie and Suslow, 1986, 1987; James and Gutterson, 1986; Kloepper and Schroth, 1981; Lam et al., 1987; Poplawsky and Ellingboe, 1989; Poplawsky et al., 1988). In a few recent studies the antibiotic metabolites produced by fluorescent pseudomonads were identified and mutants were obtained that are defective in the production of these specific compounds (Kraus and Loper, 1989; Pierson III and Thomashow, 1988; Thomashow and Weller, 1988; Voisard et al., 1989). By transposon mutagenesis and complementation tests two antibiotic metabolites, phenazine and cyanide, were proved to be responsible, in part, for the suppression of take-all of wheat and of black root rot of tobacco, respectively (Thomashow and Weller, 1988; Voisard et al., 1989).

Different factors, e.g. adsorption by clay minerals or nutrient supply, can affect the production, behavior, and activity of antibiotics in soil (Gottlieb, 1976; Howell and Stipanovic, 1979, 1980; Stotzky, 1986; Williams, 1982; Williams and Vickers, 1986). Up to now, only in the case of phenazines has it been demonstrated that antibiotics are produced in the rhizosphere by fluorescent pseudomonads (Thomashow et al., 1990)

otherwise there is not direct proof of antibiotic production in natural rhizosphere soil (Williams, 1982; Williams and Vickers, 1986).

P. fluorescens (Trevisan) Migula strain CHA0 was isolated from a soil near Payerne (Switzerland) which is naturally suppressive to tobacco black root rot caused by *Thielaviopsis basicola* (Gasser and Défago, 1981; Stutz et al., 1986). Suppression of this disease by strain CHA0 occurs in iron-rich natural and artificial soils containing vermiculitic clay minerals (Keel et al., 1989; Stutz et al., 1986, 1989). Furthermore strain CHA0 protects wheat from *Gaeumannomyces graminis* var. *tritici* in greenhouse and field experiments (Défago et al., 1987, 1990) and suppresses diseases caused by *Rhizoctonia solani, Fusarium oxysporum* and *Aphanomyces euteiches* (Défago et al., 1990, Keel, 1989). Strain CHA0 produces a siderophore of the pyoverdine type and several antibiotic metabolites that may contribute to disease suppression (Ahl et al., 1986). Five of the metabolites produced were identified as hydrogen cyanide (HCN) (Ahl et al., 1986; Voisard et al., 1989), 2,4-diacetylphloroglucinol, monoacetylphloroglucinol, pyoluteorin, and salicylic acid (Défago et al., 1990). By means of genetically engineered derivatives of *P. fluorescens* strain CHA0, cyanide has been shown to be an important factor in the suppression of black root rot of tobacco (Voisard et al., 1989). Iron is necessary for cyanogenesis by *P. fluorescens*. Vermiculitic clay minerals provide conditions of iron sufficiency for cyanogenesis and, in parallel, for efficient disease suppression by strain CHA0 (Keel et al., 1989). A pyoverdine-negative Tn5 mutant of strain CHA0 had normal suppressive properties, indicating that siderophore-mediated iron competition is not a suppressive mechanism in the systems tested (Défago et al., 1990, Keel et al., 1989).

The purpose of this study was to determine if the production of 2,4-diacetylphloroglucinol by *P. fluorescens* strain CHA0 is an important factor in the suppression of black root rot of tobacco.

2. Materials and Methods

Microorganisms and culture conditions

Thielaviopsis basicola strain ETH D 127, *Fusarium oxysporum* f. sp. *lycopersici* strain FOL 15 (obtained from C. Alabouvette, I.N.R.A., Dijon, FR) and *Pseudomonas fluorescens* strain CHA0 (Stutz et al., 1986) were cultivated on malt agar, unless otherwise specified.

Production of acetylphloroglucinols and pyoluteorin

Ten-day-old bacterial cultures were extracted with 80% aqueous acetone according to Howell and Stipanovic (1979). The extracts were chromatographed on TLC plates (Silica gel 60 F_{254}, Merck) with toluene-acetone (4:1, v/v) and sprayed with 1% (w/v) vanilline in sulphuric acid (95–97%). For HPLC an aliquot of the extracts was

analyzed by a Hewlett Packard 1090 Liquid Chromatograph equipped with a diode-array-detector, using a column (4 × 250 mm) packed with Nucleosil 120-5-C8 (Macherey-Nagel). The samples were eluted with an increasing gradient of methanol from 15% (v/v) (in 0.05% ethanolamine, acidified with 85% *o*-phosphoric acid to pH 2.8) to 100%, with a flow rate of 1 ml min^{-1}. Specific components were detected by UV-absorption at 270 nm for 2,4-diacetylphloroglucinol, at 286 nm for monoacetylphloroglucinol (2,4,6-trihydroxyacetophenone) and at 312 nm for pyoluteorin. The retention times of authentic samples of 2,4-diacetylphloroglucinol (synthesized according to Campbell and Coppinger, 1951), monoacetylphloroglucinol (Fluka) and pyoluteorin (synthesized according to Birch et al., 1964 and Cue et al., 1981) were 21.3, 11.7, and 16.9 min, respectively.

Transposon mutagenesis and characterization of mutants

Transposon mutagenesis using pLG221 and characterization of Tn5-mutants were carried out according to the methods described earlier (Voisard et al., 1988, 1989). In a screening for 2,4-diacetylphloroglucinol-negative mutants, inhibition of *F. oxysporum* f. sp. *lycopersici* (which was found to be very sensitive to 2,4-diacetylphloroglucinol) by strain CHA0 and its derivatives *in vitro* was assayed on malt agar plates. Siderophore production of the mutants was assessed by growing them on succinate minimal medium (Meyer and Abdallah, 1978), with or without 100 μg/ml EDDHA (ethylenediaminedi[*o*-hydroxyphenylacetic acid]). Hydrogen cyanide (HCN) production was measured according to Voisard et al., (1989).

Black root rot suppression in the gnotobiotic system

The gnotobiotic system consists of pure vermiculite clay (expanded with 30% H_2O_2), quartz sand, and modified Knop nutrient solution and contains a sterile-grown tobacco plant (5 wk old), *P. fluorescens* (10^7 cfu per cm^3 soil) and *T. basicola* (10^4 endoconidia per cm^3 soil) in a flask plugged with cotton wool (Keel et al., 1989). The plants were grown for 3 wk, washed, weighed and assessed for disease severity and bacterial root colonization (Keel et al., 1989; Stutz et al., 1986). In some experiments, 2,4-diacetylphloroglucinol was added to soil before planting. *T. basicola* was reisolated according to Keel et al., (1989).

Toxicity of 2,4-diacetylphloroglucinol

Synthetic 2,4-diacetylphloroglucinol was dissolved in EtOH and added to the media at concentrations of 0-5120 μM. All media including controls without the antibiotic contained 0.1% (v/v) EtOH.

(a) Germination of tobacco: surface-sterilized seeds (Keel et al., 1989) were spread on 0.85% water agar (Difco). Incubation was in a growth chamber containing 70%

relative humidity at 22 C for 16 hr with light (80 μmol m^{-2} sec^{-1}) and an 8-hr dark period at 15 C. After 7 days, the percentage of germinated seeds was evaluated. (b) Growth of tobacco: after 1 wk on 1.7% water agar (Difco), seedlings with similar root length were transferred to Petri dishes containing modified Knop nutrient solution (Keel et al., 1989) solidified with 0.8% agar. The plants were grown in the growth chamber for 3 wk, washed and weighed.

(a) Germination of *T. basicola*: a suspension of *T. basicola* endoconidia was prepared (Keel et al., 1989) and immediately spread on malt agar. The malt agar plates were incubated at 24 C in the dark for 3 h and assessed for the percentage of germinated endoconidia. (b) Growth of *T. basicola*: agar plugs (diameter 6 mm) of 3 wk old cultures of *T. basicola* were transferred to malt agar and grown at 27 C in the dark for 7 days.

Statistics

Each experiment was repeated at different times; means of three experiments are presented. Each mean was compared with all other means by the Student *t*-test (multiple *t*-test).

3. Results

Isolation and characterization of a 2,4-diacetylphloroglucinol-negative mutant

After Tn5 mutagenesis of *P. fluorescens* strain CHA0 a mutant, strain CHA625, was isolated that was unable to produce detectable amounts of 2,4-diacetylphloroglucinol (less than 0.1 μM) on malt agar. Strain CHA625 is prototrophic, produces siderophores of the pyoverdin type, hydrogen cyanide, and pyoluteorin, and gives wild-type growth rates in batch culture. A cosmid (pME3101) obtained from a genomic bank of strain CHA0 restored the capacity of strain CHA625 to produce 5 μM 2,4-diacetylphloroglucinol on malt agar and to inhibit *F. oxysporum* f. sp. *lycopersici*. Strain CH625/pME3101 did not differ from wild-type CHA0 in the production of this antibiotic and in the inhibition of the pathogen.

Role of 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco

The role of 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco by *P. fluorescens* strain CHA0 and its derivatives was investigated under gnotobiotic conditions. In the presence of *T. basicola* the final weight of plants was drastically reduced (Fig. 1A), compared with that of uninoculated controls, and a large proportion of the root surface was covered with chlamydospores (Fig. 1B). The wild-type CHA0 provided good protection against symptoms induced by *T. basicola* (Fig. 1A and 1B): plant weights were increased five times and the percentage of the

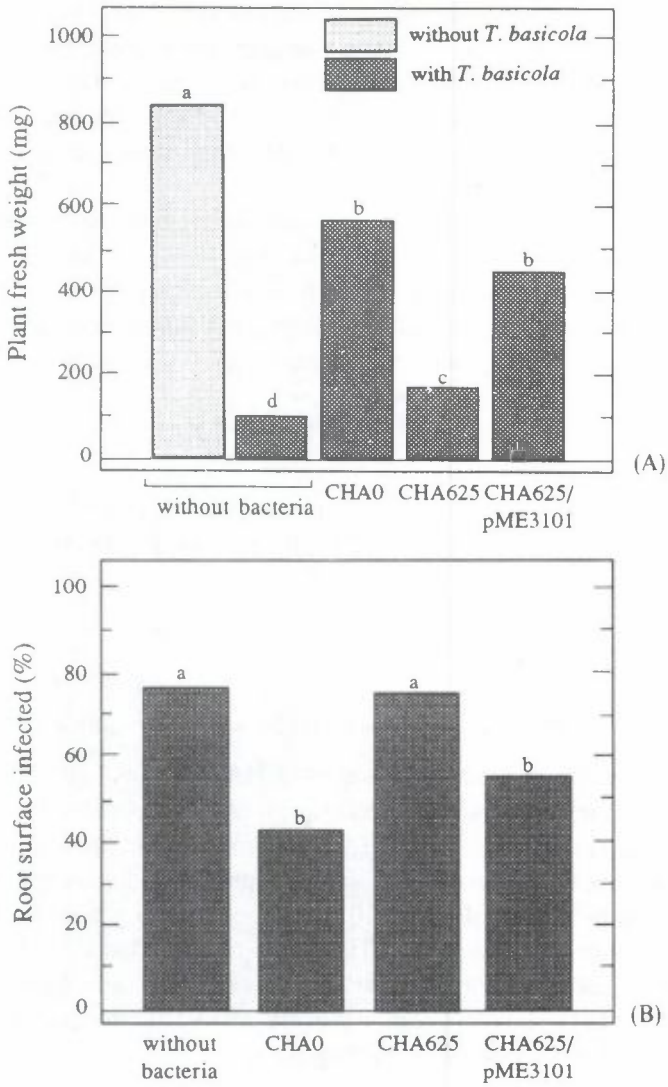

Figure 1. Influence of 2,4-diacetylphloroglucinol on the suppression of tobacco black root rot, caused by *Thielaviopsis basicola*, by addition of *Pseudomonas fluorescens* strain CHA0 and its derivatives: CHA625 is a 2,4-diacetylphoroglucinol-negative mutant of wild-type CHA0; CHA625/pME3101 is a transconjugant of CHA625 which was restored in its 2,4-diacetylphloroglucinol production. Disease suppression is evaluated in terms of total plant fresh weight (A) and of root surface infected (B). Bars with the same letter are not significantly different at $P = 0.05$ according to the multiple t-test. Each value is the mean of three experiments, with ten replicates per experiment and one flask per replicate. Root surface infected was measured as the percentage of root surface darkened by the presence of chlamydospores of *T. basicola* and transformed to arc sines (Keel et al., 1989; Stutz et al., 1986).

root surface covered with chlamydospores was reduced to half. The 2,4-diacetylphloroglucinol-negative mutant CHA625 afforded a significantly lower degree of protection. The infected root surface was not reduced (Fig. 1B) and plant weights were more than three times lower than weights of plants protected by strain CHA0 (Fig. 1A). In strain CHA625 carrying the recombinant cosmid pME3101 the suppressive capacity was restored (Fig. 1A and 1B), in terms of both plant weight and disease severity. In the absence of the pathogen, the bacteria did not significantly influence the final plant weight. All bacterial strains colonized the roots to the same extent (0.3 to 4.3×10^9 cfu per g fresh weight at the end of the experiment). After incubation for four weeks in the gnotobiotic system, 70–80% of the bacteria still contained pME3101.

The capacity of 2,4-diacetylphloroglucinol to suppress disease was also tested by the addition of pure, synthetic substance to plants grown in the absence of bacteria. When 40 μg 2,4-diacetylphloroglucinol was added per g moist soil infested with *T. basicola*, the percentage of root surface covered with chlamydospores was reduced drastically, whereas the final plant weight was not improved (Table 1). The survival of *T. basicola* in soil supplemented with the antibiotic was not reduced (Table 1). In contrast, 2,4-diacetylphloroglucinol reduced plant and root weight in absence of *T. basicola* (Table 1). Roots were stunted and deformed, and showed increased root hair formation.

Toxicity of 2,4-diacetylphloroglucinol

2,4-Diacetylphloroglucinol was more toxic to the germination of tobacco seeds than to the germination of endoconidia of *T. basicola* (Fig. 2); the germination of tobacco

Table 1. Influence of 2,4-diacetylphloroglucinol on the growth of tobacco[a] and on the suppression of *Thielaviopsis basicola*-induced black root rot under gnotobiotic conditions.

Addition of 2,4-diacetylphloroglucinol (Phl)[b] and D127[c]	Plant fresh weight[d] (mg)	Root fresh weight[d] (mg)	Root surface infected[d] (%)	Propagules of *T. basicola* per g dry soil[d]
none	870a	250a	0a	0a
Phl	526b	222a	0a	0a
D127	126c	20b	67b	2.2×10^3 b
Phl + D127	124c	19b	2a	0.8×10^3 b

[a] Plants were grown for 3 wk under gnotobiotic conditions in an artificial soil containing vermiculite as clay mineral.

[b] 40 μg of 2,4-diacetylphloroglucinol dissolved in 1 ml of EtOH were added per g moist soil before planting.

[c] D127: 10^4 endoconidia of *T. basicola* strain D127 were added per g soil 7 days before planting.

[d] Means within columns followed by the same letter are not significantly different at $P = 0.05$ according to the multiple *t*-test. Each value is the mean of three experiments, with ten replicates per experiment and one flask per replicate. Root surface infected was measured as the percentage of root surface darkened by the presence of chlamydospores of *T. basicola* and transformed to arc sines (Keel et al., 1989; Stutz et al., 1986).

was completely inhibited at a concentration of 160 μM, whereas 1280 μM were necessary for the same effect on the endoconidia. The growth of the plant and of the fungal pathogen was completely inhibited at a concentration of 640 μM of the antibiotic (Fig. 3).

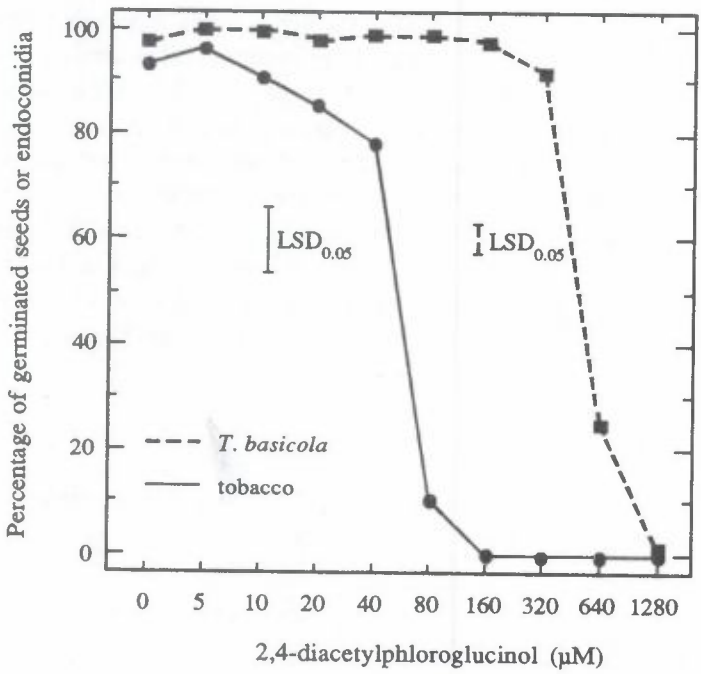

Figure 2. Influence of increasing concentrations of 2,4-diacetylphloroglucinol on the germination of tobacco seeds and endoconidia of *Thielaviopsis basicola*. Each value is the mean of three experiments, with three replicates per experiment. $LSD_{0.05}$ = least significant difference at $P = 0.05$.

4. Discussion

Until now, there has been circumstantial evidence for bacterial 2,4-diacetylphloroglucinol production as a mechanism in the suppression of soilborne plant pathogens. A *Pseudomonas aurantiaca* strain, which produced 2,4-diacetylphloroglucinol, inhibited *Fusarium oxysporum in vitro* and protected wheat from the attack by the pathogen (Garagulya et al., 1974; Pidoplichko and Garagulya, 1974). From the results reported

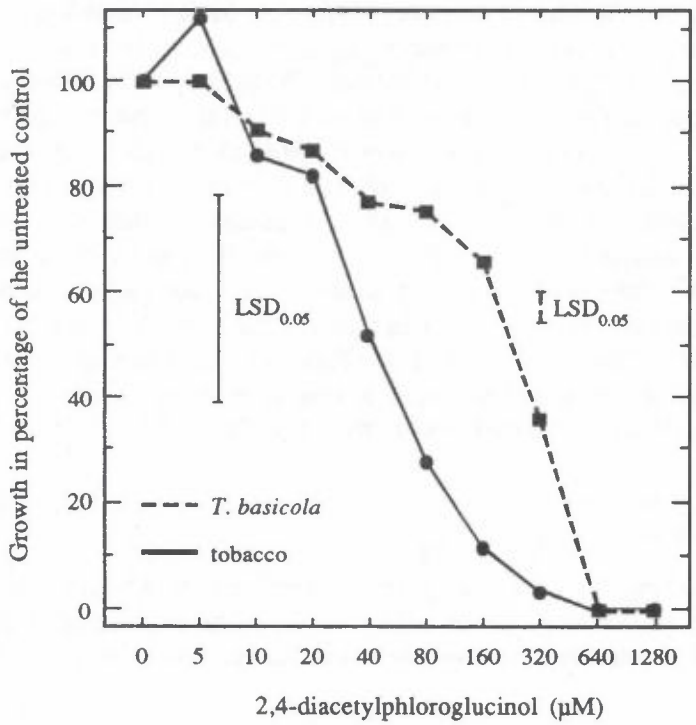

Figure 3. Influence of increasing concentrations of 2,4-diacetylphloroglucinol on the growth of tobacco plants (plant fresh weight) and of *Thielaviopsis basicola* (radial mycelial growth). Each value is the mean of three experiments, with three replicates per experiment. $LSD_{0.05}$ = least significant difference at $P = 0.05$.

here it appears that, in addition to cyanide (Keel et al., 1989; Voisard et al., 1989), 2,4-diacetylphloroglucinol production is another factor involved in the suppression of tobacco black root rot by *P. fluorescens*. Strain CHA625, a 2,4-diacetylphloroglucinol-negative mutant, was impaired in plant protection. 2,4-Diacetylphloroglucinol production and plant protection were both restored when CHA625 carried the cosmid pME3101. However more genetic work will be necessary to characterize the genes required for 2,4-diacetylphloroglucinol synthesis and to prove the role of this compound in disease suppression. In further experiments we obtained evidence that 2,4-diacetylphloroglucinol may also be involved in the suppression of take-all, caused by *G. graminis* var. *tritici*, by strain CHA0, and that it can be detected in the rhizosphere of wheat grown under gnotobiotic conditions (Keel et al., 1990). A number of fluorescent *Pseudomonas* strains produce 2,4-diacetylphloroglucinol (Aizenman et al., 1975; Broadbent et al., 1976; Garagulya et al., 1974; Kirprianova et al., 1985; Kiprianova

and Smirnov, 1981; Reddi and Borovkov, 1970). The bacterio- and fungitoxic activity of this antibiotic has been described by different authors (Broadbent et al., 1976; Garagulya et al., 1987; Reddi and Borovkov, 1970; Smirnov et al., 1989; Strunz et al., 1978) and its phytotoxicity has been compared with the herbicidal activity of 2,4-D (Kataryan and Torgashova, 1976; Reddi et al., 1969). Our results confirmed the broad toxic activity of 2,4-diacetylphloroglucinol. This substance was more toxic to tobacco than to *T. basicola*. Therefore, 2,4-diacetyl phloroglucinol production by strain CHA0 might directly antagonize *T. basicola* on the root, or alternatively, the antibiotic could also act on the plant and induce plant defence mechanisms against the pathogen. It is known that some herbicides can induce resistance in the plant (Altmann and Campbell, 1977; Cohen et al., 1986). It is noteworthy that phloroglucinol derivatives with antifungal activity have been found in certain plant species and may play a role as a biochemical defence mechanism against fungi (Tomás-Lorente et al., 1989).

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