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Determination of antibiotic activity on plasmids from fluorescent pseudomonads isolates CW2, WB15 and WB52 against pre-emergence damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* in cucumber

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ABSTRACT

Three fluorescent pseudomonads, isolates CW2, WB15 and WB52, were tested for their antagonistic activities against *Pythium ultimum* and *Rhizoctonia solani* isolates. *In vitro* experiments revealed pronounced inhibition zones on PDA agar medium against *P. ultimum* and *R. solani* by the isolates CW2 and WB15, respectively. *Escherichia coli* strains transformed with plasmids from the fluorescent pseudomonads isolates caused larger inhibition zones against both pathogens compared to the wild type isolates. In greenhouse experiments, the most effective isolate against *P. ultimum* was CW2 and isolate WB52 was the most effective against *R. solani.* The transformed *E. coli* strains proved to be significantly ($P < 0.05$) more effective in reducing pre-emergence damping-off in cucumber caused by *P. ultimum* than the wild type isolates, while their efficacy against damping-off incited by *R. solani* was similar to or less than that of the wild type fluorescent pseudomonads isolates. According to the results obtained by HPLC analysis, an isomer of 2,4-diacetylphloroglucinol (DAPG) was detected in culture filtrates of isolate CW2, while in the *E. coli* strain transformed with the CW2 plasmid, pyoluteorin and other phenazine derivatives were detected. In wild type isolates of WB15 or WB52 and in *E. coli* isolates transformed with plasmids from the fluorescent pseudomonads isolates, pyoluteorin and phenazine derivatives were detected. These results suggest that the expression of some genes might be blocked in the wild type isolates.

1. Introduction

Fluorescent pseudomonads represent an important group of rhizosphere bacteria that have promising antagonistic potentials for use in biological control of soil-borne fungal pathogens (De La Fuente et al., 2004; Andersen et al., 2003). Because of their catabolic versatility, their excellent root-colonizing abilities and their capacity to produce a wide range of antifungal metabolites, fluorescent pseudomonads have received particular attention (Walsh et al., 2001; O'Sullivan and O'Gara, 1992; Weller, 1988). *Pseudomonas* spp. are particularly suitable for applications as agricultural biocontrol agents since they: (1) can use compounds within root exudates as nutrients (Lugtenberg et al., 1999); (2) are abundantly present in natural soils, in particular on plant root systems, which is indicative for their adaptative potential (Chin-A-Woeng et al., 2003); (3) have a high growth rate relative to many other rhizosphere bacteria; (4) possess diverse mechanisms by which they can exert inhibitory activity towards phytopathogens and thereby mediate crop protection (Chin-A-Woeng et al., 2003; Lugtenberg et al., 1999); (5) can easily be grown *in vitro*; (6) subsequently be reintroduced into the rhizosphere by seed bacterization (Chin-A-Woeng et al., 2003); and (7) represent suitable organisms for mutation and modifications using molecular biological tools (Haas and Keel, 2003). In addition, certain non-pathogenic pseudomonads are capable of inducing a systemic resistance in plants against pathogens known as induced systemic resistance (ISR) (Chin-A-Woeng et al., 2003; Van Loon et al., 1998).

In recent years intensive work has been done on bacteria and their use as biocontrol agents against different plant diseases. The molecular aspects of pathogenicity and the mechanisms of action of such agents were studied (Bolwerk et al., 2003; Bloemberg and Lugtenberg, 2001; Lugtenberg et al., 2002; Whipps, 2001). The objective of this work was to investigate the antagonistic potential of different pseudomonades against *Pythium ultimum* Trow and *Rhizoctonia solani* Kuhn pre-emergence damping-off and check whether genes encoding for the antifungal activity can be located on plasmids of different fluorescent pseudomonad isolates.

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2. Materials and methods

2.1. Cultivation and maintenance of antagonistic Pseudomonas fluorescens and fungal isolates

The antagonistic *P. fluorescens* isolates CW2, WB15 and WB52 as well as the pathogen isolates *P. ultimum* and *R. solani* isolate BBA were obtained from the culture collection of the Institute of Phytomedicine, University of Hohenheim, Germany. Stock cultures of the bacterial isolates were prepared by growing the bacteria in 125-ml Erlenmeyer flasks containing 25 ml King's B (King et al., 1954) liquid medium on a rotary shaker (150 rpm) for 20 h at 28 °C. Aliquots of 800 µl of each bacterial isolate were transferred into Eppendorf tubes containing 200 µl of sterile glycerol. The cultures were stored after mixing at −80 °C. The fungal isolates were grown on potato dextrose agar medium (PDA) at 22 °C for 4 days. Stock cultures were stored at 4 °C and subcultured routinely every 2 weeks.

2.2. In vitro antagonistic tests

The antagonistic activity of the bacteria against *P. ultimum* or *R. solani* was determined using the dual culture technique (Fakhouri and Buchenauer 2003). Each bacterial strain was streaked on the center of a PDA plate and incubated for 24 h at 28 °C. Then two disks of *P. ultimum* or *R. solani* grown for 4 days on PDA medium were placed about 3 cm apart from the bacterial streak. Cultures were then incubated at 22 °C for 48 h. Control experiments were done by using sterile distilled water instead of bacteria. The effect of each bacterial strain was determined by measuring the inhibition zone of mycelial growth. The rating scale was: –, no inhibition zone and often *P. ultimum* or *R. solani* were growing over the bacterial streak; +, weak inhibition, the growth of *P. ultimum* or *R. solani* was stopped at the bacterial-streak line; ++, moderate inhibition with 1–5 mm inhibition zone; +++, strong inhibition with inhibition zone between 5 and 10 mm and ++++, very strong inhibition with inhibition zone > 10 mm (Bardin et al., 2003).

2.3. Plasmid extraction, transformation of Escherichia coli cells and screening for 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn) and pyoluteorin (Plt) biosynthetic loci

Midi plasmid DNA preparations and transformation of *E. coli* DH10B cells preparation using the calcium chloride technique were done according to the protocols of Sambrook and Russell (2003). The antifungal activity of recovered transformed *E. coli* cells was tested against *P. ultimum* and *R. solani* using a dual culture technique as mentioned above.

Oligonucleotide primers listed in Table 1 were obtained from MWG-Biotech AG (Germany). PCR amplification was carried out in 25 µl reaction mixtures as described by Mavrodi et al. (2001). Each reaction mixture consisted of 1X *Taq* DNA polymerase buffer, 200 µM each of dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 1.5 mM $MgCl_2$ and 2.0 units of Ampli*Taq* DNA polymerase. Amplifications were performed with an Eppendorf Mastercycler. The PCR programs were performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C (for DAPG and Prn primers) and 55 °C (for Plt primers) for 1 min and 72 °C for 2 min. The programs were followed with a final extension cycle at 72 °C for 5 min. Ten microliter aliquots of PCR products were separated on 1.5% agarose gel in 1 × TBE buffer (Tris base 27 g, boric acid 13.75 g and 0.5 M EDTA 10 ml (pH 8.0)) at 80 V for 1 h. After staining with ethidium bromide, gels were visualized under UV transilluminator (Raajimakers et al., 1997).

2.4. Antibiotics extraction, isolation and detection by HPLC

The fluorescent pseudomonads isolates CW2, WB15, WB52 and CHA0 (control), and the transformed and non transformed *E. coli* isolates were grown in 50 ml KB medium for 3 days at 28 °C on a rotary shaker at 150 rpm. The fluorescent pseudomonads isolate CHA0, which is well known for its ability to secrete 2,4-diacetylphloroglucinol and pyoluteorin (Schnider-Keel et al., 2000), was used as a control. Cultures were centrifuged at 10,000*g* for 10 min and extracted twice with ethyl acetate. Extracts were dried with sodium sulphate and the solvent was evaporated by a rotary evaporator. Extracts were dissolved in acetonitril: water (1:1 v:v) solutions and further purified using preparative HPLC. Separation of the compounds was done at room temperature at a flow rate of 6 ml min^{-1} using phosphate buffer (1 mM, pH 3.0): acetonitril (ACN) gradient. Fractions of each extract were collected and analyzed using HPLC (Merk-Hitachi®), coupled to a waters 991 Photoperiod Array Detector (DAD) and a Merck-Hitachi (4000A) autosampler. Analytical separation was carried out using a Hydro-RP-Column (10 µm, 250 × 4.6 mm) at 35 °C at a flow rate of 0.7 ml min^{-1} using phosphate buffer/ACN gradient. For each bacterial isolate there were three replicates. The experiment was repeated twice.

2.5. Efficacy of bacteria to control damping-off caused by P. ultimum and R. solani in cucumber

Cucumber (*Cucumis sativus* L.) cv. 'Delikatess' was used as a model plant because of its importance in vegetable production. Experiments were conducted in nine cm dia pots filled to two thirds with potting soil (Humosoil®: sand mixture 2:1, v:v) as mentioned in Vogt and Buchenauer (1997). Three disks of *P. ultimum* and *R. solani* were placed on the surface of 'infested pots' and covered with additional soil. The control pots were not infested. All pots were watered daily and incubated for 3 days in greenhouse

Table 1
Oligonucleotide primers used for the detection of antibiotic coding genes on plasmids of fluorescent pseudomonads isolates CW2, WB15 and WB52.

Primer	Sequence (5′→3′)	Target[a]	Position[b]	T_m[c] (°C)
Phl2a	GAG GAC GTC GAA GAC CAC CA	PhlD	1915	73
Phl2b	ACC GCA GCA TCG TGT ATG AG	PhlD	2660	72
PltBf	CGG AGC ATG GAC CCC CAG	PltB	8160–8178	64
PltBr	GTG CCC GAT ATT GGT CTT GAC	PltB	8927–9857 (complement)*	63.8
PrnCf	CCA CAA GCC CGG CCA GG	PrnC	3478–3497	66.9
PrnCr	GAG AAG AGC GGG TCG ATG	PrnC	4175–4197 (complement)*	62.9

[a] phlD encodes a protein of 349 amino acids that is homologous to chalcone synthase from plants (Raajimakers et al., 1997). PltB encodes a protein similar to type I polyketide synthase in *P. fluorescens* Pf-5 (Nowak-Thompson et al., 1999). PrnC encodes a halogenase catalyzing chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin in *P. fluorescens* BL915 (Kirner et al., 1998).
[b] position of the primers in the database sequence.
[c] T_m melting temperature.
* Reverse primer.