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Resistance to oxidation products of caffeic acid is important for efficient colonization of wheat seedlings by *Pseudomonas proteolytica* strain PSR114

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ABSTRACT

The interrelationships between plants and rhizosphere bacteria are strongly dependent on the quality and quantity of root exudates. The ability to colonize roots is crucial for pseudomonads to function as biological control agents of root- and soil-borne pathogenic microbes. The multiplication of rhizosphere bacteria is restricted in the presence of simple phenolic compounds, which are components of the resistance mechanisms of plants to pathogens. Caffeic acid is a phenolic compound, which is commonly found in wheat tissues. It is prone to oxidation into *o*-quinones, which are toxic to microorganisms. The aim of the present study was to determine whether the ability of microorganisms to resist caffeic acid and its oxidation products could play a role in the early colonization of wheat seedlings. Among the fluorescent pseudomonads that we have studied, strain PSR114 is one of the most efficient colonizers of wheat seedlings during the first 48 h after seed germination, and it is particularly resistant to products resulting from the spontaneous oxidation of caffeic acid. This strain was isolated from the rhizosphere of oilseed rape and identified as being closely related to *Pseudomonas proteolytica* through the analysis of 16S rRNA and *rpoB* gene sequences. At pH 7.0, this strain grew intensively in the presence of 1.50 mg mL^{-1} of caffeic acid. Its multiplication was partially reduced in the presence of oxidized caffeic acid at concentrations above 0.21 mg mL^{-1}, and completely inhibited at concentrations above 0.38 mg mL^{-1}. A Tn*5* transposon mutant of PSR114 had lower level of resistance to the oxidation products of caffeic acid, as well as reduced capacity to colonize wheat seedlings when compared to the wild type strain. This work demonstrates that resistance to oxidation products of caffeic acid can be important for successful bacterial colonization of wheat seedlings.

1. Introduction

The rhizosphere is considered to be a part of the soil ecosystem where plant roots, soil and soil microorganisms interact with each other (Lynch et al., 2002). Plant exudates have an effect on the activities of microbial populations. Moreover, they determine the associations of plant and soil-borne microorganisms (Singh et al., 2004). Root exudates consist of a mixture of organic acids, phenolic compounds, plant-derived siderophores, sugars, vitamins, amino acids, purines, molecules of CO_2, H_2, HCO_3^-, OH^-, H^+, enzymes and root border cells (Dakora and Phillips, 2002). Their availability and utilization is one of the most crucial factors for successful bacterial establishment in the rhizosphere and the effective colonization of roots is an essential step for their use in biological control of root- and soil-borne diseases (Chin-A-Woeng et al., 2000; Bloemberg and Lugtenberg, 2001). *Pseudomonas reactans* strains PSR2, PSR21 and PPS96, which were found to be very good colonizers of wheat seedlings during the first 48 h, distinguished themselves from the less efficient colonizers belonging to this species by their ability to utilize *p*-hydroxyphenylacetic acid, bromosuccinic acid, benzoic acid, methyl pyruvate, *N*-acetyl-D-glucosamine, D-trehalose and adonitol (Oksinska et al., 2011).

The growth of microorganisms in the rhizosphere can be inhibited by the presence of phenolic compounds, which besides being released from roots, are present in leaf leachates and in decomposing litter (Kobayashi et al., 1996; Dixon, 2001). Phenolics can serve as nutrient sources, but also as microbial chemoattractants, as microbial growth promoters, or as chelators of

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poorly soluble mineral nutrients (Dakora and Phillips, 2002). For example, iron deficiency provokes the release of caffeic acid by tomato plants (Olsen et al., 1981). The presence of simple phenolic acids is universal in plant tissues and in soils (Blum et al., 1991). Such compounds as *p*-hydroxybenzoic, vanillic, syringic, caffeic, chlorogenic, 3,4-dimethoxycinnamic, *cis*-ferulic, *trans*-ferulic, *trans*-*p*-coumaric, *cis*-*p*-coumaric, sinapic, protocatechuic, salicylic and gentisic acids were found in wheat tissues or root exudates (Vancura, 1964; Onyeneho and Hettiarachchy, 1992; Weidner et al., 1999; Wu et al., 2000, 2001; Mpofu et al., 2006; Verma et al., 2009). Some of these phenols can inhibit the growth of several species of bacteria (Fernandez et al., 1996; Campos et al., 2003; Maddox et al., 2010). Yao et al. (1995) found that production of chlorogenic acid (an ester of caffeic acid and quinic acid) in potato tubers increased during 48–72 h after wounding, and that these tubers were less susceptible to *Phytophthora infestans*. Cutinase activity of *Monilinia fructicola* was inhibited in the presence of chlorogenic acid and caffeic acid, compounds which are abundant in epidermal cells (Agrios, 2005). Four phenolic compounds: L-tyrosine, D,L-phenylalanine, caffeic acid and hydroquinone were present in wheat tissues at higher levels than normal in resistant plants during the early stages of infection with *Neovossia indica* (Gogoi et al., 2001). The exposure of plants to pathogens, their toxins or enzymes results in the release of signal transduction molecules, superoxide, hydroxyl radical, hydrogen peroxide, lipoxygenase, loss of compartmentalization and the oxidation of phenolic compounds (Iwamura et al., 1996; Kobayashi et al., 1996; Agrios, 2005).

Caffeic acid is one of the most common secondary metabolites of plants. It was found in the outer bran layer of wheat seeds (Mpofu et al., 2006; Verma et al., 2009), in wheat caryopses (Weidner et al., 1999), in rice root exudates (Seal et al., 2004) and in exudates of germinating seeds of groundnuts during the first 48 h of growth (Reddy et al., 1977). This compound and its derivatives are prone to autoxidation by oxygen and by biological oxidants due to their *o*-diphenol feature, as well as to enzymatic oxidation by polyphenol oxidases and peroxidases to *o*-quinones, which are more toxic to microorganisms (Bassil et al., 2005). Quinones can undergo attacks by nucleophilic substrates, such as amino acids, proteins, oxidation of lower redox potential molecules, or condensation with other quinones (Kroll and Rawel, 2001; Pati et al., 2006). The composition of caffeic acid ions in aqueous solutions is a function of pH (Cilliers and Singleton, 1989). At pH 7.0, almost 100% of the caffeic acid was found in the form of caffeic-e$^-$ ions, and less than 6% of the species was in the form of caffeic-2e$^-$. At pH 8.5, the concentration of caffeic-e$^-$ ions only comprised 58% and caffeic-2e$^-$ ion had increased to 42% of the total caffeic acid ions (Giacomelli et al., 2002). The caffeic-2e$^-$ ion is characterized by its high nucleophilicity and it is responsible for the instability of *o*-quinones, which formed as result of its oxidation at pH values exceeding 7.4.

In a previous study plant-associated pseudomonads had been classified as belonging to three different nutrient utilization groups, named α, β1 and β2 (Oksinska et al., 2011). The strains of the β1 group, despite having similar nutrient utilization profiles, differed among themselves in their effectiveness to colonize 48-h-old wheat seedlings. These bacteria were unable to utilize *o*-coumaric acid, caffeic acid and *trans*-cinnamic acid as sole carbon and energy sources (Oksinska et al., 2011). Among these compounds, caffeic acid could play a significant role in the defense system of the wheat rhizosphere (Kobayashi et al., 1996; Agrios, 2005). Considering these observations, the present study was conducted to test whether phenolic compounds can inhibit the growth of *Pseudomonas* spp. strains. The second aim was to test if the ability of the best colonizers of wheat seedlings to grow in the presence of these phenolic acids can be a determinant for effective multiplication in the rhizosphere of 48-h-old wheat seedlings.

2. Materials and methods

2.1. Bacteria

The fluorescent pseudomonads investigated in the present study originated from the rhizosphere of oilseed rape (*Brassica napus* L. cv. Jantar) and wheat (*Triticum aestivum* L. cv. Jawa) and were isolated on Gould's agar medium (Gould et al., 1985). These strains were previously described in Oksinska et al. (2011). *Escherichia coli* strain DH5α, which harbors plasmid pRL765 was used to generate random Tn*5* transposon insertions in the genome of *Pseudomonas* sp. strain PSR114, and these were subsequently screened for loss of resistance to the oxidation products of caffeic acid. *E. coli* DH5α was received from the Plant Pathology and Biocontrol Unit, at the Swedish University of Agricultural Sciences (Uppsala, Sweden). All bacteria were stored at −70 °C in medium (pH 7.0) containing 1000 mL distilled water, 10 g bacto tryptone, 5 g yeast extract (Difco Laboratories, Detroit, MI, USA), 0.5 g NaCl, 6.3 g K_2HPO_4, 1.8 g KH_2PO_4, 0.45 g sodium citrate, 0.09 g $MgSO_4 \times 7H_2O$, 0.9 g $(NH_4)_2SO_4$ and 50.6 mL glycerol (Sigma Aldrich, Inc., St. Louis, MO, USA).

2.2. Growth of Pseudomonas spp. strains in the presence of phenolic acids

The ability of pseudomonads to multiply in the presence of phenolic acids was tested on solid King's medium B (KB) (King et al., 1954) enriched with one of the following compounds: *o*-coumaric acid, caffeic acid and *trans*-cinnamic acid (Sigma Aldrich, Inc.). Phenolic acids were dissolved in 1 M solution of NaOH, sterilized by filtration (pore size 0.22 μm, Millipore, Carrigwohill, Co. Cork, Ireland), and introduced into KB medium (pH 7.0) to final concentrations ranging from 0.0 (control) to 1.0 mg mL^{-1}.

A 24-h-old culture of bacteria grown in KB medium was centrifuged and suspended in 0.1 M solution of $MgSO_4 \times 7H_2O$. Five microliter aliquots of this suspension (approximately 1×10^8 colony forming units (CFU) mL^{-1}) were placed on the surfaces of KB agar plates at 0 h and at 48 h after the medium had been supplemented with *o*-coumaric acid, caffeic acid and *trans*-cinnamic acid. During this time period, different concentrations of oxidation products should have been formed (Cilliers and Singleton, 1989). Bacterial growth was visually estimated after 48 h of incubation at 28 °C, on KB medium supplemented with each phenolic acid to be tested. KB medium without any phenolics was used as a positive control, to observe the uninhibited bacterial growth. Minimal inhibitory concentration (MIC) was determined for each phenolic compound, which suppressed the bacterial growth. Significant differences among the means of three replicates were revealed through Duncan's multiple range test at 95% level of significance (ANOVA/MANOVA, Statistica Version 5, 97 Edition, StatSoft, Inc., Tulsa, OK, USA).

2.3. Identification of Pseudomonas sp. strain PSR114

Strain PSR114 was characterized by its particularly high level of resistance to phenolic compounds and was therefore selected for further research. It was identified by analysis of its 16S rRNA and *rpoB* gene sequences. Total bacterial DNA was isolated by using the Bactozol™ kit (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the protocol of the manufacturer. The 16S rRNA gene was amplificated by PCR in the following conditions: initial denaturation for 2.5 min at 98 °C, denaturation for 1 min at 93 °C, annealing for 45 s at 55 °C, elongation for 1.5 min at 72 °C and final extension for 10 min at 72 °C. The primers 27f (5′-AGAGTTTGATCCTGGCTCAG) and 1541r (5′-AAGGAGGTGATCCAGCCGCA) (Lane, 1991) were used. The reaction