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Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties

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Abstract

Isolates of *Serratia* have been isolated from the rhizosphere of oilseed rape. The percentage of *Serratia* in this microenvironment was determined as 12.4% of the total antifungal bacteria. *Serratia liquefaciens*, *S. plymuthica* and *S. rubidaea* were found. All of the isolates showed an antifungal activity against different phytopathogenic fungi *in vitro* but the efficiency of strains was different. The antifungal mechanisms of 18 selected strains were investigated. Direct antifungal effect may be based on antibiosis (production of prodigiosin and pyrrolnitrin) and production of lytic enzymes (chitinases and β-1,3-glucanases). Potent siderophores were secreted by the strains to improve the availability of iron. No strain was able to produce cyanide. Most of the strains secrete the plant growth hormon indole-acetic-acid which can directly promote the growth of roots. The mechanisms were specific for each isolate.

Key words: *Serratia liquefaciens* – *Serratia plymuthica* – *Serratia rubidaea* – rhizobacteria of oilseed rape – antifungal mechanisms

Introduction

Plant growth promoting rhizobacteria (PGPR) are root-colonizing bacteria which exert a beneficial effect on plant development. The reported beneficial effect of PGPR includes plant growth promotion and reductions in the incidence of soilborne diseases (Kloepper *et al.* 1992).

Pseudomonads are, however, not the only plant-associated PGPR, *Enterobacteriaceae* are an important component of the beneficial rhizosphere flora too (Hedgens and Messens 1990). Species of the genus *Enterobacter*, *Erwinia*, and *Serratia* have been reported as antagonistic bacteria (Weller and Thomashow 1994). *Serratia* species were frequently found associated with plants (Grimmont and Grimmont 1992). Several *Serratia* isolates have proved to be antibacterial and sometimes good antifungal agents. This gave the possibility of use in the biological control of plant pathogens e.g. *Serratia liquefaciens* from carnation rhizosphere was used to protect root cuttings (Sneh *et al.* 1985) and *Serratia plymuthica* was found as a common rhizobacterium with antifungal effect to *Fusarium culmorum* and *Pythium* spec. (Alström and Gerhardson 1988). *Serratia marcescens* and *S. plymuthica* were described as endophytic bacteria in root and stem of sweet corn and cotton (McInroy and Kloepper 1994).

Bacterial antagonism responsible for the benefit of plants and biological control, may be due to the production of antibiotics, competition or parasitism (Chet *et al.* 1990). The relative importance of these mechanisms may differ considerably among strains. Antibiotics play a major role in disease suppression by some bacteria and often act in concert with other mechanisms such as competition and parasitism (Fravel 1988). Some strains of *Serratia (S. marcescens, S. plymuthica, S. rubideae)* produce a non-diffusible red pigment prodigiosin with antifungal activity. Another antibiotic pyrrolnitrin was found only in one strain of *Serratia marcescens* (Grimmont and Grimmont 1992). Competition for nutrients supplied by root and seed exsudates probably occurs in most interactions between bacteria and pathogens (Elad and Chet 1987; Weller 1988). Kloepper *et al.* (1980) were the first who demonstrate the importance of siderophore production as a mechanism of competition. Various siderophores were found in *Serratia marcescens*: ethylenediamine di-O-hydroxyphenylacetic acid, enterobactin and aerobactin (Grimmont and Grimmont

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1992). Parasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi. Microorganisms capable of lysing other organisms can serve as a powerful tool for biological control (Chet *et al.* 1990). Many rhizobacteria are classified as chitinolytic microorganisms, e.g. *Aeromonas caviae* (Inbar and Chet 1991), *Serratia marcescens* (Ordentlich *et al.* 1988) and *E. coli* cloned with the *chi*A gene from *Serratia marcescens* (Shapira *et al.* 1989). Subsequently, it was shown that the biocontrol of *S. marcescens* relied on the production and excretion of a chitinase. Induced systemic resistance, broadly defined as activation of latent defense mechanisms in plants prior to pathogenic attack, has been hypothesized in recent years to be an operable mechanisms in several rhizobacterial systems (Kloepper *et al.* 1992). It is also known, that *S. plymuthica* is able to induce systemic resistance to *Colletotrichum orbiculare* (Kloepper *et al.* 1992). Many strains of rhizobacteria influence plant growth direct by the production of phytohormons such as auxin derivates (Loper and Schroth 1986).

Verticillium dahliae var. *longisporum* Stark, *Rhizoctonia solani* Kühn and *Sclerotinia sclerotiorum* (Lib.) De Bary are important pathogens causing diseases of oilseed rape. The increasing importance of oilseed rape for renewable biological raw material resulted in a marked expansion of the rape growing area in Europe. Over the years very short rotations have led to a build-up of Verticillium wilt caused by *V. dahliae* var. *longisporum* and Post emergence damping off and Brown girdling root rot caused by *R. solani* followed by yield losses. *Sclerotinia sclerotiorum* is not regarded as a typically soilborne fungus. Ascospores are the primary source of inoculum which infects plants but also infections of roots are known in oilseed rape (Zeise, pers. communication). The possibility of replacing chemical control of plant root diseases with a biological control agent (BCA) e.g. with seed bacterization containing antifungal rhizobacteria remains an exciting and challenging objective (O'Sullivan and O'Gara 1992).

The objective of our investigations was to demonstrate the importance of *Serratia* in the rhizosphere of oilseed rape and also as potential biological control agents of phytopathogenic fungi. Therefore it was necessary to characterize antifungal mechanisms and the efficiency of selected isolates *in vitro*.

Material and methods

Isolation of bacterial antagonists and determination of the colony forming units (cfu). Bacteria were isolated from roots of oilseed rape. The material was rinsed in sterile water and the suspension was plated in serial dilutions on nutrient agar (Gibco, Paisley, Scotland). Plates were incubated 5 days at 20 °C and bacterial colonies were counted to determine the amount of colonies (colony forming units = cfu). Colonies representative of different morphological types present were selected and purified. Selected isolates (12 h-culture, 30 °C) were stored in nutrient broth with 15% glycerol at –80 °C.

Bioassay for in vitro inhibition of V. dahliae, R. solani and S. sclerotiorum. The ability of the bacterial antagonists to produce antifungal substances against fungal pathogens was determined by the paired *in vitro* assay on Waksman agar [WA; containing: 5 g proteose-peptone (Merck, Darmstadt, FRG), 10 g glucose (Merck), 3 g meat extract (Chemex, München, FRG), 5 g NaCl (Merck), 20 g agar (Difco), 1 l distilled water, pH 6.8]. A suspension of hyphal fragments was plated on agar and bacteria were streaked as a broad band. Zones of inhibition were measured after 5 days of incubation at 20 °C. The isolates of *Verticillium dahliae* var. *longisporum, Sclerotinia sclerotiorum* and *Rhizoctonia solani,* which were isolated from oilseed rape in Rostock–Biestow (1989), were received from Dr. K. Zeise (University of Rostock).

Bacterial identification. For the identification of isolates, a standardized micromethod, employing the Analytical Profile Index strip which consists of a gallery of 20 biochemical tests separated into 7 groups was used (Bio Mérieux. Marcy-L'Etoile, France). API 20 E tests were used for identifying the isolates. Additionally characteristics described by Grimmont and Grimmont (1992) and Bergey's Manual of Systematic Bacteriology (Krieg *et al.* 1984).

Antibiotics. Prodigiosin: Bacteria were cultivated on Pepton-Glycerol-Agar [PGA; containing: 5 g Bacto-Pepton (Difco), 10 ml Glycerol, 20 g Bacto-Agar (Difco), 1 l distilled water] at 30 °C for 48 h. Cells extracted from petri dishes (containing 20 ml PGA) were harvested and dissolved in 25 ml Aceton for 30 min. The solution was centrifugated at 10.000 g for 10 min. The supernatant was then purified by gel filtration on sephadex LH-20 (Pharmacia, Uppsala, Sweden) using methanol-distilled water (1 : 1 vol/vol) as eluting solvent. The active fractions were concentrated under vacuum (50 °C) and then the dry residue was dissolved in 1 ml of ethanol. The characteristic spectrum of the red pigment was proved according to Süssmuth *et al.* 1987.

Pyrrolnitrin: Bacteria were cultivated on Nutrient Agar [NA (Gibco); containing additionally 2% glycerol] at 30 °C for 72 h. The purification of pyrrolnitrin was carried out according to Kraus and Loper (1992). The purified extract was tested in a bioautography assay (Berg *et al.* 1995).

Siderophore production. To analyze the ability of selected bacterial isolates to produce siderophores we used the

plate assay according to Schwyn and Neilands (1987) and varied by Heupel (1988). The ternary complex chrome azurol S/iron(III)/hexacyltrimethyl-ammonium bromide (Sigma) served as an indicator.

Lytic enzymes. Chitinase: Bacterial colonies were screened for chitinase production and excretion by planting on nutrient agar (Merck) containing 5% colloidal chitin (Sigma, Deisenhofen, FRG). The clear halos indicating of the enzymatic degradation were measured after 14 days of incubation at 20 °C.

Glucanase: β-1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin (Fluka) according to Daugrois *et al.* (1990). The standard assay (1 ml) contains the enzyme extract, 2.5 mg laminarin, 100 mM acetate buffer pH 5.2. The laminarin substrate was dissolved in acetate buffer by heating at 60 °C before use. The reaction mixture was incubated 1 h at 50 °C. Total reducing sugars were assayed by colorimetric method and expressed as glucose equivalents.

Cyanide. The direct proof was made with "Aquaquant 14417-Testsystem" (Merck) with culture broth of the isolates.

Indole-acetic acid (IAA): The standard method by Gordon and Weber (1991) in which IAA present in culture filtrate (3 ml) was reacted with Salkowski reagent (2 ml) to yield a pink coloured product after 30 min incubation, was used. The contents of IAA in the Medium was qualitatively measured on a spectrophotometer at 530 nm.

Results

Occurrence of Serratia in the rhizosphere of oilseed rape

Over 5000 bacterial isolates were screened for antifungal properties against *Verticillium dahliae* in further investigations (Berg and Ballin 1994; Berg 1995). The origin of the investigated isolates are shown in Tab. 1. 146 of the active isolates were determined, 18 isolates belong to the genus *Serratia*. These are 12.4% of the total antifungal bacterial populations.

Identification of the isolates

Isolates were identified by using API-system, the schemes outlined in Bergey's Manual of Systematic Bacteriology (Krieg *et al.* 1984) compared with informations from (Grimmont and Grimmont 1992). Results of organism identification are presented in Tab. 2. All isolates were aerobic, Gram-negative, catalase-positive, motile rods with petrichous flagella. The isolates C2, C3 and C27 produced the visible red pigment prodigiosin. Production of prodigiosin is typical for *S. plymuthica, S. rubidaea* and *S. marcescens*. The strains requiring additional tests comprised *S. plymuthica* and *S. rubidaea* because of the low identification indices in part in API system (Tab. 2). After additional testing the majority of the beneficial bacteria belonged to *Serratia plymuthica*. The isolate C27 was identified as *Serratia rubidaea*, therefore we proved the assimilation of D-arabinose (+), hydrolysis of chitin (+), growth at 4 °C (+), 37 °C (+),

Table 1. Origin of the root samples

No.	No. of isolate	origin	Locality[1]	date[2]
1	GP1	rhizosphere of *Brassica napus*	Rostock	6/93
2	GP2	rhizosphere of *Brassica napus*	Rostock	6/93
3	C2	rhizosphere of *Brassica napus*	Pölchow	4/94
4	C3	rhizosphere of *Brassica napus*	Pölchow	4/94
5	C27	rhizosphere of *Brassica napus*	Pölchow	6/94
6	C38	rhizosphere of *Brassica napus*	Pölchow	6/94
7	C44	rhizosphere of *Brassica napus*	Pölchow	6/94
8	C45	rhizosphere of *Brassica napus*	Pölchow	6/94
9	C46	rhizosphere of *Brassica napus*	Pölchow	6/94
10	C47	rhizosphere of *Brassica napus*	Pölchow	6/94
11	C48	rhizosphere of *Brassica napus*	Pölchow	6/94
12	C50	rhizosphere of *Brassica napus*	Pölchow	6/94
13	C52	rhizosphere of *Brassica napus*	Pölchow	6/94
14	C53	rhizosphere of *Brassica napus*	Pölchow	6/94
15	C54	rhizosphere of *Brassica napus*	Pölchow	6/94
16	C63	rhizosphere of *Brassica napus*	Pölchow	6/94
17	P184	rhizosphere of *Brassica napus*	Biestow	11/94
18	P485	rhizosphere of *Brassica napus*	Biestow	2/95

[1] Locality (town and M.-W. = Mecklenburg-Western Pomerania)
[2] Date (Month and year) of the sample

Table 2. Identification of representative rape root associated *Serratia* with antifungal activity

Strain	API profile[1]	% id.[2]	Species	growth and acid production					
				Xylitol	Rhamnose	Sorbitol	Arabitol	4 °C	8.5% NaCl
GP1*	1-0-0-7-5-6-3	96.5	*S. plymuthica*	–	–	–	–	+	–
GP2*	1-0-0-7-5-6-3	96.5	*S. plymuthica*	–	–	–	–	+	–
C2	1-0-0-6-3-2-3	57.5	*S. plymuthica*	–	–	–	–	+	–
C3	1-2-0-7-3-2-3	37.6	*S. plymuthica*	–	–	–	–	+	–
C27	5-0-0-6-1-2-3	99.3	*S. rubidaea*	–	–	–	–	+	+
C38	1-2-0-7-5-6-3	74,3	*S. plymuthica*	–	–	+	–	+	–
C44	1-2-0-7-5-6-3	74.3	*S. plymuthica*	–	–	+	–	+	–
C45	1-2-0-6-7-6-3	67.6	*S. plymuthica*	–	–	+	–	+	–
C46	1-2-0-6-3-6-3	46.4	*S. plymuthica*	–	–	–	–	+	–
C47	1-0-0-7-5-6-3	96.5	*S. plymuthica*	–	–	+	–	+	–
C48	1-0-0-6-3-2-3	57.5	*S. plymuthica*	–	–	–	–	+	–
C50	1-0-0-7-7-6-3	97.1	*S. plymuthica*	–	–	+	–	+	–
C52	1-0-0-5-7-6-3	84.3	*S. plymuthica*	–	–	+	–	+	–
C53	1-2-0-6-3-2-3	39.9	*S. plymuthica*	–	–	–	–	+	–
C54	1-2-0-7-3-2-3	37.6	*S. plymuthica*	–	–	–	–	+	–
C63	5-1-0-6-7-2-3	96	*S. liquefaciens*	–	+	+	–	+	–
P184	1-2-0-6-1-2-3	39.1	*S. plymuthica*	–	–	–	–	+	–
P485	1-2-0-7-7-6-3	78.7	*S. plymuthica*	–	–	+	–	–	–

[1] API 20 E: Identification of *Enterobacteriaceae* and other Gram-negative rods

[2] Quality of the identification selected on the value of the percentage of identification

* These isolates are available at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig under the following numbers: *Serratia plymuthica* Isolat 18 = DSM 8571, Isolat 19 = DSM 8572.

Table 3. Efficiency of isolates of *Serratia* against different phytopathogenic fungi *in vitro*

No. strain	*Verticillium dahliae*	*Rhizoctonia solani*	*Sclerotinia sclerotiorum*
GP1*	++	+++	+++
GP2*	++	++	++
C2	+++	+++	++
C3	+++	+++	++
C27	+++	+++	++
C38	+++	+++	++
C44	+++	+++	++
C45	+++	+++	++
C46	+++	+++	+++
C47	+++	+++	++
C48	+++	+++	++
C50	+++	+++	+++
C52	+++	+++	++
C53	+++	+++	++
C54	+++	+++	–
C63	+	++	–
P184	+++	++	–
P485	+++	+++	+++

Bioassay: dual culture assay +++ represent > 10 mm wide zone of inhibition; ++ represents 5–10 mm wide zone of inhibition, + represents 0–5 mm wide zone of inhibition

40 °C (–), growth on 8.5% NaCl (+) and the odour (musty). Alltogether three different species were determined: *Serratia liquefaciens* (Grimes and Hennerty 1931) Bascom *et al.* 1971, *Serratia plymuthica* (Lehmann and Neumann 1896) Breed *et al.* 1948 and *Serratia rubidaea* (Stapp 1940) Ewing *et al.* 1973.

Antifungal effects

The isolates were screened for their ability to suppress *Verticillium dahliae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* in an *in vitro* bioassay (Tab. 3). The efficiency of the isolates was different. Most isolates showed an inhibition against all of the pathogens. The activity against *Sclerotinia sclerotiorum* was lower in general.

We have tried to prove the involvement of antifungal mechanisms by the antagonistic bacteria. We found only 3 strains that produced prodigiosin (Tab. 4). It was a surprise, that so many isolates produced pyrrolnitrin, which was never found before in these three species of *Serratia*. Only 3 strains (isolate GP1, GP2, P184) showed an activity of β-1.3-glucanase. For half of the isolates chitinolytic activity could be detected (Tab. 4). Most of the isolates of *Serratia* (72%) produced siderophores. No isolate produced cyanide.

Table 4. Antifungal mechanisms of the isolates of *Serratia*

No.	Antibiosis		Siderophores	Glucanase	Chitinase	HCN
	Prodigiosin	Pyrrolnitrin				
GP1*	–	+	+++	+	+	
GP2*	–	+	+++	+	+	–
C2	+	+	++	–	–	–
C3	+	+	++	–	–	–
C27	+	+	++	–	–	–
C38	–	+	+++	–	+	–
C44	–	–	+++	–	+	–
C45	–	–	+++	–	–	–
C46	–	+	–	–	+	–
C47	–	–	++	–	+	–
C48	–	–	–	–	+	–
C50	–	+	–	–	+	–
C52	–	–	+++	–	–	–
C53	–	+	+	–	–	–
C54	–	+	+	–	–	–
C63	–	+	–	–	+	–
P184	–	+	–	+	–	–
P485	–	+	+++	–	–	–

Antibiosis: proof with extraction
Siderophores according to Schwyn & Neilands 1987 (+++ represents 20 mm wide orange zone, ++ represents 5–20 mm wide orange zone, + represents 5–3 mm orange zone)
Chitinase-Activity: plate assay (+ represents hydrolysis, – represents no hydrolysis)
β-1,3-Glucanase-Activity: according to Daugrois *et al.* 1990 (+++ >10 IE/ml, ++ 10–1 IE/ml, + 0.1–1 IE/ml)
Production of Cyanide: Merck. Schnelltest 14417: – represents <0,001 mg/l

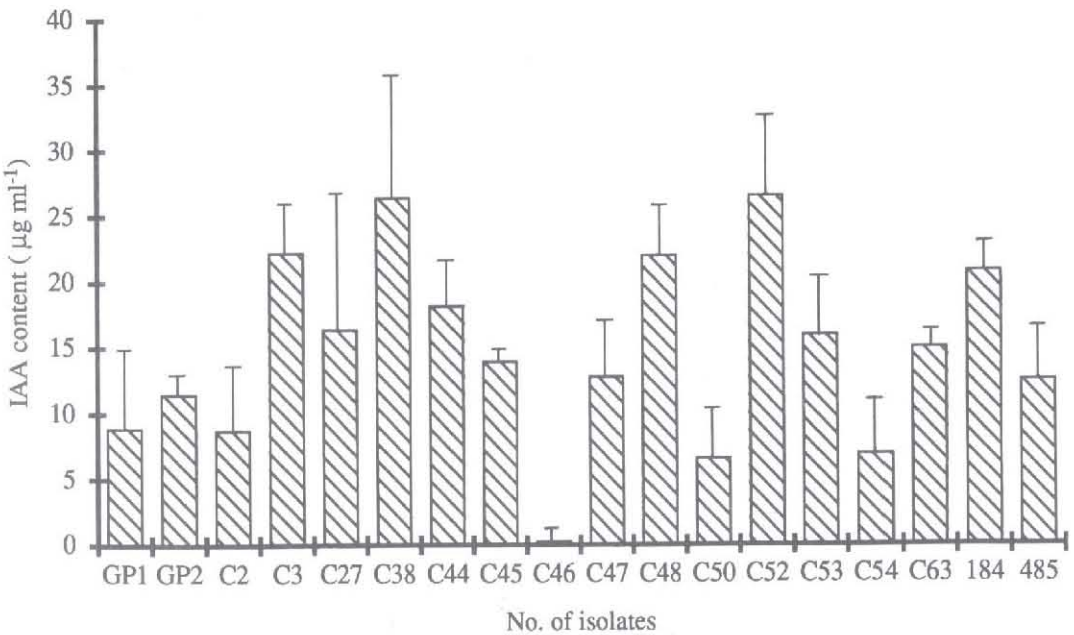


Fig. 1. Production of indole-3-acetic-acid (IAA) by strains of *Serratia*
Colorimetric IAA assay: culture filtrate (3 ml) reacted with Salkowski reagent (2 ml) to yield a pink-coloured product after 30 min incubation, which was quantitatively measured at 530 nm (growth medium contained in g l^{-1}: glucose 5,0; yeast extract 0.025; L-TRP 0.204)

Production of Indole-acetic acid

IAA-production is often an indication for plant growth promoting activity of rhizobacteria. We investigated the production of IAA *in vitro,* when grown in the presence of a suitable precursor such as L-tryptophan. All isolates produce IAA in various amounts (Fig. 1). Concentrations of IAA were comparable to results of other authors (Loper and Schroth 1986; Roy and Basu 1992).

Discussion

Members of the genus *Serratia* belonged to the potential biocontrol bacteria (Hemming 1990). In the rhizosphere of oilseed rape we found 3 different species of this genus: *Serratia liquefaciens, S. plymuthica* and *S. rubidaea. S. liquefaciens* and *S. plymuthica* are typical rhizobacteria, which were isolated from the rhizosphere of other plants before. *S. rubidaea* has not been reported as a rhizosphere competent bacterium, but sometimes it was found with antagonistic activity e.g. an isolate from sclerotia of *Phymatotrichum omnivorum* (Zuberer *et al.* 1988). The species has been repeatedly isolated from coconuts (Grimmont and Grimmont 1992). In soil or in the rhizosphere *Serratia* might play a role in the biological cycle of metals by mineralizing organic iron and dissolving gold and copper (Grimmont and Grimmont 1992).

All of the investigated isolates showed an antifungal activity in bioassay. They inhibited growth of the phytopathogenic fungi *Verticillium dahliae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* and were evaluated as potential biocontrol agents. Antagonistic microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens (Weller 1988). The use of bacterial antagonists in biological control of soilborne pathogens sometimes gives good results (Marois *et al.* 1987; Berg *et al.* 1994).

The antifungal mechanisms of rhizosphere bacteria include production of antibiotics (antibiosis), production of siderophores (competition), production of lytic enzymes like chitinase and β-1.3-glucanase and production of cyanide (induction of resistance in the host plant). We have investigated the production of siderophores, enzymes, cyanide and the antibiotics prodigiosin and pyrrolnitrin. It is necessary to prove the mechanisms for each isolate, because the production and especially the quantity of secondary metabolites are often specific for a strain. This could be confirmed for our isolates too (Tab. 4). Six different combinations of antifungal mechanisms were found in the eighteen isolates. The combinations of antibiotics, siderophores and lytic enzymes were found for three isolates (GP1, GP2, GP38). Most of the isolates possess two antifungal mechanisms antibiotics combined with siderophores (C2, C3, C27, C53, C54, P485) or antibiotics combined with lytic enzymes (C46, C50, C63, P184). This support the thesis of Fravel (1988), that antibiosis is involved in most antagonism and is often combined with other mechanisms. The cooperation of siderophores and lytic enzymes were found in 2 isolates (C44, C47) and only one mechanism was found in C45, C52 (siderophores) and C48 (lytic enzymes). An efficient biocontrol agent may affect pathogens by a combination of mechanisms (Chet *et al.* 1990). Two different antibiotics were isolated: prodigiosin and pyrrolnitrin. Only three strains produced prodigiosin. Pigmented *S. marcescens* biotypes were rarely isolated from plants (Grimmont and Grimmont 1992). Strains producing the red pigment prodigiosin seem to be toxic to protozoa (Groscop and Brent 1964) and this may be an ecological advantage in water and soil (Grimmont and Grimmont 1992) and in the rhizosphere too. The role of pyrrolnitrin in antagonism of *Serratia* was not established before.

All of the strains were able to produce auxins, this can support their beneficial function of rhizobacteria on roots. The auxins stimulate the growth of roots especially of root hairs. This supports the health of plants in an indirect way. Generally, bacteria isolated from the rhizosphere and rhizoplane of various plants are more active in producing auxins than those from root free soil (Sawar and Kremer 1995).

Before it is possible to introduce bacteria in the rhizosphere it is necessary to investigate the ecology of this habitat. And there is another remark to be made: many of the antagonistic bacteria are facultative pathogenic and sometimes associated with opportunistic infections. At present *Serratia marcescens* is the only known nosocomial species of *Serratia. S. liquefaciens* and *S. rubidaea* are occasionally isolated from clinical specimens, but their pathogenic role is not established. Nevertheless, there is a potential risk for humans and that's why they were presented as facultative pathogenic bacteria (Anonymous 1990). It is an important target to develop alternatives to chemical control or as appropriate control measures for a pathogen which cannot be suppressed.

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