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Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb

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Aims: A screening approach was developed to assess the potential of rhizobacterial strains to control *Verticillium* wilt caused by *Verticillium dahliae* Kleb.

Methods and Results: Sixty randomly chosen antagonistic bacterial strains originally isolated from rhizosphere of three different host plants of *V. dahliae* – strawberry, potato and oilseed rape – were evaluated for biocontrol and plant growth promotion by analysing *in vitro* antagonism towards *V. dahliae* and other plant pathogenic fungi, production of fungal cell wall-degrading enzymes and plant growth-promoting effects on strawberry seedlings. To test the plant growth-promoting effect, a microplate assay with strawberry seedlings was developed. Although the rhizobacterial strains were isolated from different plants they showed effects on the growth of strawberry seedlings. According to the *in vitro* biocontrol and plant growth-promoting activity, the three best candidates *Pseudomonas putida* B E2 (strawberry rhizosphere), *Ps. chlororaphis* K15 (potato rhizosphere) and *Serratia plymuthica* R12 (oilseed rape rhizosphere) were selected for greenhouse experiments to verify the *in vitro* screening results. Under greenhouse conditions the isolates selected according to this strategy were as effective, or more effective than commercial biocontrol agents and may therefore possibly be valuable as antagonists of *V. dahliae*.

Conclusions: In this study, the screening strategy resulted in a selection of three interesting biocontrol candidates against *Verticillium*: *Ps. putida* B E2 (strawberry rhizosphere), *Ps. chlororaphis* K15 (potato rhizosphere) and *Ser. plymuthica* R12 (oilseed rape rhizosphere).

Significance and Impact of the Study: A new combination of *in vitro* screening methods including a microplate assay with strawberry seedlings to test the plant growth promoting effect which allow to more efficiently select potential biological control agents was developed successfully.

INTRODUCTION

Verticillium wilt caused by the soilborne fungus *Verticillium dahliae* Kleb. is an important disease responsible for dramatic yield losses in many crops (Maas 1998; Tjamos 2000). *V. dahliae* has a broad host range which includes many important crops such as strawberry (*Fragaria* × *ananassa* Duch.), potato (*Solanum tuberosum* L.) and oilseed rape (*Brassica napus* L.). Since microsclerotia of *V. dahliae* that develop in the senescing tissues of the dead plant may persist in soil for several years, chemical control is nearly impossible (Maas 1998). Control of this disease is limited to heat treatment, or fumigation with broad-spectrum biocides such as methyl bromide (Fravel and Larkin 2000). In the coming years, methyl bromide will be phased out. This fact will lead to a further accumulation of microsclerotia in soil (Tjamos *et al.* 2000). An environmentally friendly alternative to protect roots against fungal pathogens is rhizobacteria-mediated biological control (Weller 1988; Emmert and Handelsman 1999). However, only a few biocontrol products are currently on the market (Whipps 1997) and it is

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necessary to find more efficient biocontrol bacteria. Previous results has been reported in controlling *Verticillium* wilt using antifungal rhizobacteria (Leben *et al*. 1987; Berg *et al*. 1994; Berg *et al*. 1999; Kurze *et al*. 2001). However, only a small number of rhizobacteria was evaluated in these studies.

The problem of developing an effective biological control of *V. dahliae* is the screening method used against the pathogen (Tjamos 2000). We have reported recently the successful selection of rhizobacteria antagonistic to *Verticillium* wilt (Berg *et al*. 2000). However, several studies showed discrepancies between the antagonistic effects under *in vitro* conditions and the corresponding *in situ* efficacy (Weller and Cook 1983; Reddy *et al*. 1993). Ideally, the candidate organisms should be screened on the plants rather than *in vitro* (Weller 1988). However, it is expensive and time consuming to conduct large-scale screening trials on whole plants. As a compromise, it is required to develop new *in vitro* screening methods which allow to more efficiently select potential antagonists for plant greenhouse and field experiments.

The objective of our study was to develop a screening method to evaluate rhizobacteria originally isolated from three different host plants of *V. dahliae* – strawberry, potato and oilseed rape – to see whether their potential to control *Verticillium* wilt on strawberry was affected by the host plant from which the antagonist was obtained. Therefore, different screening methods were applied. First, the traditional dual culture assay was used to characterize the ability of antagonistic isolates to antagonize *in vitro* pathogenic fungi. Secondly, antifungal mechanisms were evaluated by analysing the production of fungal cell wall-degrading enzymes. Lysis is a very efficient antifungal mode of action of rhizobacterial strains (Chet *et al*. 1990). In addition, a plant growth-promotion assay in microplates was developed and applied. For this, strawberry seedlings were treated with rhizobacterial suspension and the development of seedlings was compared to a non-treated control. Based on the three different test systems an assessment scale was proposed. The results of the *in vitro* tests were verified by greenhouse tests. The study was carried out using an improved screening strategy to find new potential biocontrol agents to *Verticillium* wilt.

MATERIALS AND METHODS

Rhizobacterial strains

Bacteria were isolated from three different crop plants, potatoes cv. Element, oilseed rape cv. Licosmos and strawberries cv. Elsanta grown in a randomized block design with six replicates per crop plant located at the site of the Federal Biological Research Centre for Agriculture and Forestry in Braunschweig, Germany (Smalla *et al*. 2001).

Screening for antifungal *in vitro* activity

Randomly chosen strains isolated from the rhizosphere of potatoes, oilseed rape and strawberries were screened for their antifungal activity. Bacterial isolates were tested in a dual culture with pathogenic fungi on Waksman agar containing 5 g proteose–peptone (Merck, Darmstadt, Germany), 10 g glucose (Merck), 3 g meat extract (Chemex, München, Germany), 5 g NaCl (Merck), 20 g agar (Difco, Detroit, USA) and distilled water (to 1 l), pH 6·8. Zones of inhibition were measured after 5 days' incubation at 20°C according to Berg (1996). All strains were tested in three independent replicates. Fungi used in this bioassay include (1) *V. dahliae* KLEB. V16, (2) *Rhizoctonia solani* KÜHN and (3) *Sclerotinia sclerotiorum* LIB. The fungal strain *R. solani* DSMZ 63010 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). All other pathogenic fungi were obtained from the strain collection of the University of Rostock, Department of Microbiology. These fungi were routinely grown on Sabouraud medium (Gibco, Paisley, UK) and stored in broth containing 15% glycerol at −70°C.

Identification of bacterial antagonists

All antagonists were identified based on analysis of fatty acid methyl-esters (FAMEs) of total cellular fatty acids by gas chromatography using the MDI system (Microbial Identification System, Inc., Newark, USA).

Screening for the production of fungal cell wall-degrading activity

Protease activity (casein degradation) was determined from clearing zones in skimmed milk agar according to Nielsen and Sørensen (1997). Colonies were screened for chitinolytic activity by plating on chitin-agar plates (CA) containing: nutrient broth (Sifin, Berlin, Germany) 1·62 g, NaCl (0·5 g), M9 salts (6 g), chitin 2 g, 0·1 mM $CaCl_2$, 1 mM $MgSO_4$ and 3 nM Thiamin-HCl (all from Sigma, Deisenhofen, Germany), Bacto-Agar (Difco) 15 g and distilled water (1 l). Clearance halos indicating the enzymatic degradation were measured after 5 days of incubation at 30°C. β-1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin (Fluka, Buchs, Switzerland). The standard assay (1 ml) contained 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 for 1 h at 50°C. Total reducing sugars were assayed by a colorimetric method and expressed as glucose equivalents.

Phytochamber assay for effect on plant growth

Seeds of strawberry cv. Rügen Selecta (Erfurter Samenzucht, Erfurt, Germany) were pregerminated in moist chambers at 30°C for six days. Three standard 24-well microplates (Roth, Karlsruhe, Germany) were filled with 1 ml water agar containing 20 g agar (Difco) made up to 1 l with distilled water (pH 6·8). One pregerminated seed followed by 10 μl bacterial suspension (nutrient broth 2 [Sifin], grown for 18 h) was added to each well. Rhizobacterial isolates were evaluated at 10^5 colony-forming units (cfu) ml^{-1} and compared with a control of 10 μl of distilled water. Five weeks after incubation (16 h artificial light, 22°C) in a chamber (Percival Scientific, Boone, USA) first leaves and radial roots were counted, the lengths of stem and root were measured, and the development of roothairs was assessed to determine effects of bacterial treatment on plant growth. The strain was tested in six replicates and the experiment was repeated three times.

Biological control and growth promotion in the greenhouse

Soil artificially infested by *V. dahliae* was used to evaluate the potential of biological control of selected rhizobacteria. Sterilized soil mixed with humus (3 : 1 v/v) was infested with 15% of substrate (1 v vermiculite and 0·1 v Czapek–Dox agar [Difco] grown with the pathogenic fungus *V. dahliae* [6 weeks, 18°C]). Polypropylene boxes (0·7 l) were filled with soil and *Verticillium* inoculum (4%) and planted with strawberry frigo plants cv. Elsanta (Janssen, Kalkar, Germany). Prior to planting the roots were dipped in a suspension of rhizobacteria (2×10^9 cfu ml^{-1}) for 15 min. The non-treated control plants were dipped in tap water and planted in infested soil. A negative control using pathogen-free soil was included in each test. Ten replicates of each treatment were performed in a completely randomized block design. All treatment combinations were repeated three times. The experiments were conducted under greenhouse conditions (18 h light, sodium lamps, 100 mE m^{-2} s^{-1} 25 ± 1°C) over a 10-week period. After inoculation, disease incidence based on a 0–2 scale with 0 = no disease, 1 = infected plant showing wilting symptoms and 2 = dead plants was recorded and the buds and blossoms were counted. The fruits were weighed to evaluate the impact of the treatment on yield.

At the end of the 10-week trial, plant roots with adhering soil taken from five plants per treatment were aseptically sampled to sterile Stomacher bags. Five g of each sample was extracted in a laboratory blender (BagMixer, Interscience, St. Nom, France) with sterile NaCl solution (8·5 g l^{-1}). Solutions were serially diluted and plated on nutrient agar 2 (Sifin) containing 100 g ml^{-1} rifampicin (Fluka for isolation of rifampicin-resistant mutants of rhizobacteria). After a five-day incubation at 20°C cfu were determined. The total culturable bacterial populations in the rhizosphere were determined using the same procedure on nutrient agar 2.

Statistical analysis

Data on the percentages of disease incidence and yield were analysed for significance using *U*-test 'Mann–Whitney' ($P \leq 0{\cdot}05$) by Statistical Product and Service Solutions for Windows, Rel. 9. 0. 1 (SSPS Inc., Chicago, USA).

RESULTS

Antifungal activity and production of lytic enzymes

Table 1 shows the results of *in vitro* tests of antagonism toward the plant pathogens *V. dahliae*, *S. sclerotiorum* (ascomycetes with a chitin–glucan-containing cell wall) and *R. solani* (basidiomycete with a chitin–glucan-containing cell wall) using the dual culture technique on agar plates. Generally, the fungi grew as well as the rhizobacterial isolates on Waksman agar. Inhibition was clearly discerned by limited growth or by the complete absence of fungal mycelium in the inhibition zone surrounding a bacterial colony. All of the 60 isolates showed antifungal activity against *V. dahliae*, the model pathogen for the screening procedure, but their activity showed a high variability (Table 1). Two additional fungi were used to find out if isolates have a broad antifungal activity or if the antifungal activity is specific for *V. dahliae*. Nearly half of the isolates (= 31 isolates) were antagonistic towards *R. solani*. Isolates obtained from the oilseed rape rhizosphere showed a higher antifungal potential against *R. solani* than isolates from potato and strawberry. Only 17 strains (= 28%) were active against the plant pathogenic fungus *S. sclerotiorum*. The majority of them originated from oilseed rape. The percentage of isolates with an antifungal activity against *V. dahliae* was higher for strawberry (65% = 13 isolates) than for potato (50% = 10 isolates) and oilseed rape (20% = four isolates). Twenty-eight isolates were active against all of the fungi tested.

Production of fungal cell wall-degrading enzymes was analysed because this is an important mechanism of fungal inhibition. Protease production was shown for most strains (Table 1). Four of 60 (7%) rhizosphere strains showed fungal cell wall-degrading enzyme β-1·3-glucanase activity. Chitinolytic activity was detected for nine (15%) strains. Most of them were oilseed rape isolates. Altogether, isolates from the oilseed rape rhizosphere possessed a higher lytic potential than isolates from strawberry and potato rhizosphere.

Table 1 *In vitro* activity against plant pathogenic fungi and production of lytic enzymes by bacterial strains isolated from strawberry, potato and oilseed rape

Strain no.	Species	Antagonism toward†			Production of lytic enzymes‡			Plant growth promoting effects on§					
		V. d.	R. s.	S. s.	Glucanases	Proteases	Chitinases	Length stem	Length root	Number leaves	Number roots	Root hairs	Assessment¶
E1	*Ps. syringae*	+	+	–	–	+	–	–	+	–	+ +	–	4
E2	*Ps. putida* B	+ + +	–	–	–	+	–	+ +	+ +	–	+ +	–	9
E3	*Ps. putida* B	+ + +	+	–	–	+	–	–	–	–	–	–	6
E4	*Ps. putida* B	+	–	+	–	+	–	–	–	–	–	–	4
E5	*Ps. putida* B	+	+	–	–	+	–	–	–	+ + +	–	+ + +	8
E6	*Ps. putida* B	+	–	–	–	+	–	+	+ +	–	+ + +	–	7
E7	*Ps. putida* B	+ +	–	–	–	+	–	–	–	–	–	–	4
E8	*Ps. putida* B	+ +	–	–	–	+	–	+	+ +	–	+ +	–	9
E9	*Ps. putida* B	+ +	+	–	–	+	–	–	–	–	–	–	5
E10	*Ps. fluorescens*	+ +	+	–	–	+	–	–	–	–	–	–	5
E11	*Ps. putida* B	+	+	–	–	+	–	–	–	–	–	–	4
E12	*Comamonas acidovorans*	+ +	–	–	–	+	–	+ +	+ +	–	–	–	5
E13	*Ps. putida* B	+ +	–	–	–	+	–	–	+ +	–	–	–	6
E14	*Bacillus megaterium*	+	–	–	–	+	–	–	–	–	–	–	3
E15	*Ps. putida* B	+	–	–	–	+	–	–	–	–	+ +	–	3
E16	*Ps. putida* B	+ +	–	–	–	+	–	–	–	–	–	–	4
E17	*Ps. putida* A	+ + +	–	–	–	+	–	–	–	–	–	–	5
E18	*Ps. putida* B	+ + +	–	–	–	+	–	–	–	–	–	–	3
E19	*Ps. syringae*	+ + +	–	–	–	+	–	–	–	–	+ +	–	7
E20	*Ps. syringae*	+	–	–	–	+	–	–	–	–	–	–	1
P1	*Ps. putida* B	+ + +	–	–	–	–	–	–	–	–	–	–	2
P2	*Janthinobac. lividum**	+	–	–	–	+	–	–	–	–	–	–	3
P3	*Kluyvera cryorescens*	+	–	–	–	+	+	–	–	–	–	–	2
P4	*Ps. putida* B	+	+	+	–	+	–	–	–	–	–	+ + +	6
P5	*Cytophyga johnsonae*	+	–	–	+	–	–	+ +	+ +	–	+ +	–	7
P6	*P. putida* A	+	+ +	–	–	+	–	–	–	–	–	–	5
P7	*Comamonas acidovorans*	+	+ + +	–	–	+	–	–	–	+ +	–	–	5
P8	*S. maltophilia*	+	–	–	–	–	–	+ +	+ + +	–	–	–	6
P9	*Ps. putida* B	+	–	–	–	+	–	+ +	+ +	–	–	–	7

P10	*Ps. putida* A	+	+ + +	+ +	–	+	–	–	–	–	–	–	8
P11	*Ps. putida* B	+ +	–	–	–	+	–	–	–	–	–	+ + +	7
P12	*Ps. putida* B	+ +	+	–	–	+	–	–	–	–	+ +	–	7
P13	*Ps. chlororaphis*	+	–	–	–	+	–	+	+ +	–	+ + +	–	9
P14	*Ps. putida* B	+ + +	+ + +	+	–	+	–	–	–	–	–	–	9
P15	*Ps. chlororaphis*	+ + +	+ + +	+	–	+	–	–	–	+ + +	–	+ + +	15
P16	*Ps. chlororaphis*	+ +	+	–	–	+	–	–	–	–	–	+ + +	6
P17	*Ps. putida* B	+ +	–	–	–	+	–	–	–	–	+ + +	–	7
P18	*Ps. fluorescens* B	+	–	–	–	+	–	–	–	–	–	+ + +	4
P19	*Ps. marginalis*	+ +	+	–	–	+	–	+	+ +	–	–	–	6
P20	*Ps. putida* B	+ +	+	–	–	+	–	+ +	+ +	–	–	–	9
R1	*Serratia odourifera*	+ + +	+ +	+ + +	–	+	–	–	–	–	–	+ + +	11
R2	*Ps. marginalis*	+ + +	+ +	–	–	+	–	+	–	–	–	–	8
R3	*Proteus vulgaris*	+ +	+ + +	+	–	+	+	–	–	–	+ + +	–	10
R4	*Micrococcus kristinae*	+ + +	+ + +	–	–	+	–	–	–	–	–	–	8
R5	*Ps. putida* A	+ + +	+ +	+	–	–	–	–	–	–	+ + +	–	10
R6	*Serratia fonticola*	+ + +	+ +	+ + +	–	+	+	–	–	–	–	–	9
R7	*Pantoaea agglomarans*	+ + +	+ +	+ + +	+	–	+	–	–	–	–	–	9
R8	*Chryseobacterium balustinum*	+	–	–	–	+	–	–	–	–	–	–	1
R9	*Burkholderia cepacia*	+	+	+ +	–	+	–	–	–	–	–	–	4
R10	*Enterobacter intermedius*	+ +	+ + +	+	–	+	+	+	–	–	+ +	–	12
R11	*Serratia grimesii*	+ +	+ + +	+	+	+	+	–	–	–	–	–	9
R12	*Pantoaea agglomarans*	+ +	+ + +	+	+	+	+	+ + +	+ + +	–	+ +	–	18
R13	*Ps. putida* B	+ + +	–	–	–	+	–	–	+	–	–	–	6
R14	*Ps. chlororaphis*	+ + +	–	–	–	+	–	–	–	+ + +	–	–	8
R15	*Serratia grimesii*	+ + +	+ + +	+ + +	–	+	+	–	–	–	–	+ + +	13
R16	*Acidovorans avenae*	+	–	–	–	–	–	–	–	+ + +	–	–	3
R17	*Ps. corrugata*	+	+	+ +	–	+	–	–	–	–	–	–	4
R18	*Ps. syringae*	+ + +	+ +	–	–	+	–	–	–	–	–	–	7
R19	*Xenorhabdus nematophilus*	+ + +	+	+	–	+	+	–	–	–	–	–	9
R20	*P. chlororaphis*	+ + +	–	+ + +	–	+	–	–	–	–	+ +	–	8

**Janthinobac.* = *Janthinobacterium.* †Antagonism toward V. d. = *Verticillium dahliae*, R. s. = *Rhizoctonia solani*, S.s. = *Sclerotinia sclerotiorum*, dual culture assay: + represents 0–5 mm wide zone, + + represents 5–10 mm wide zone, + + + represents > 10 mm wide zone of inhibition. ‡Plant growth promotion on strawberry seedlings: length stem: + represents 10–12 mm, + + represents 12–14 mm, + + + represents > 14 mm; length root: + represents 7–8 mm, + + represents 8–10 mm, + + + represents > 10 mm; number leafs: + represents 2·4–2·6, + + represents 2·6–2·8, + + + represents > 2·8; number roots: + represents 6·3–7, + + represents 7–8, + + + represents > 8 mm; root hairs + represents 1·4–1·6, + + represents 1·6–1·8, + + + represents > 1·8 mm. §β-1,3-glucanase, protease and chitinase activity: plate assay (+ represents hydrolysis, – represents no hydrolysis). ¶Assessment on the basis of a developed scale (Fig. 1).

The selected bacterial antagonists were identified by gas chromatography-fatty acid methyl ester (GC-FAME) analysis. Altogether, 25 different bacterial species were identified (Table 1).

Plant growth promotion test in a phytochamber

A bioassay to evaluate the plant growth promoting effect by rhizobacterial strains was developed. Therefore, strawberry seeds treated or untreated with rhizobacteria were germinated in microplates in a phytochamber. The effect of rhizobacteria on the growth of seedlings was monitored by measuring stem and root length, and by counting leaves, roots and root hairs (Table 1). Most isolates (58% = 25 isolates) induced statistically significant plant growth-promoting effects compared with the non-treated control. The main influence was seen on the number of roots; 15 isolates (= 25%) were able to enhance this parameter to a statistically significant extent. Thirteen isolates (= 22%) enhance the length of stems, 14 isolates (= 23%) the length of roots. The majority of isolates which enhance the length of the stem simultaneously enhance the length of the root. The highest effect was found for the *Serratia plymuthica* R12 isolate which enhances the length of the stem by 30% (14·89 mm/10·04 mm non-treated control) and of the root by 41% (11·8 mm/7·1 mm non-treated control). Only five isolates were able to enhance the number of leaves. For eight isolates a positive influence on the development of root hairs was found.

Concluding assessment of *in vitro* screening

All of the tested parameters were used to evaluate the rhizobacterial strains. The strategy and the assessment which was used to characterize and evaluate rhizobacteria is shown in Fig. 1. For antifungal activity, it was possible to obtain three points for high activity against each pathogen (totalling nine points). Production of lytic enzymes (chitinases, glucanases and proteinases) was evaluated with one point (totalling three points). Main importance is yielded for the plant growth promoting effect, three points were possible for each tested parameter (totalling 15 points). Based on this scale, points for each isolate were established (Table 1). From the strawberry isolates, *Pseudomonas putida* B E2 was the most efficient isolate (nine points). From the potato rhizosphere, *Ps. chlororaphis* P15 was the best isolate (15 points). Isolate R12, identified as *S. plymuthica*, was the most effective antagonist from oilseed rape rhizosphere (18 points). For all antagonists, there was no correlation between the antifungal and plant growth promoting activity. As a conclusion, isolates

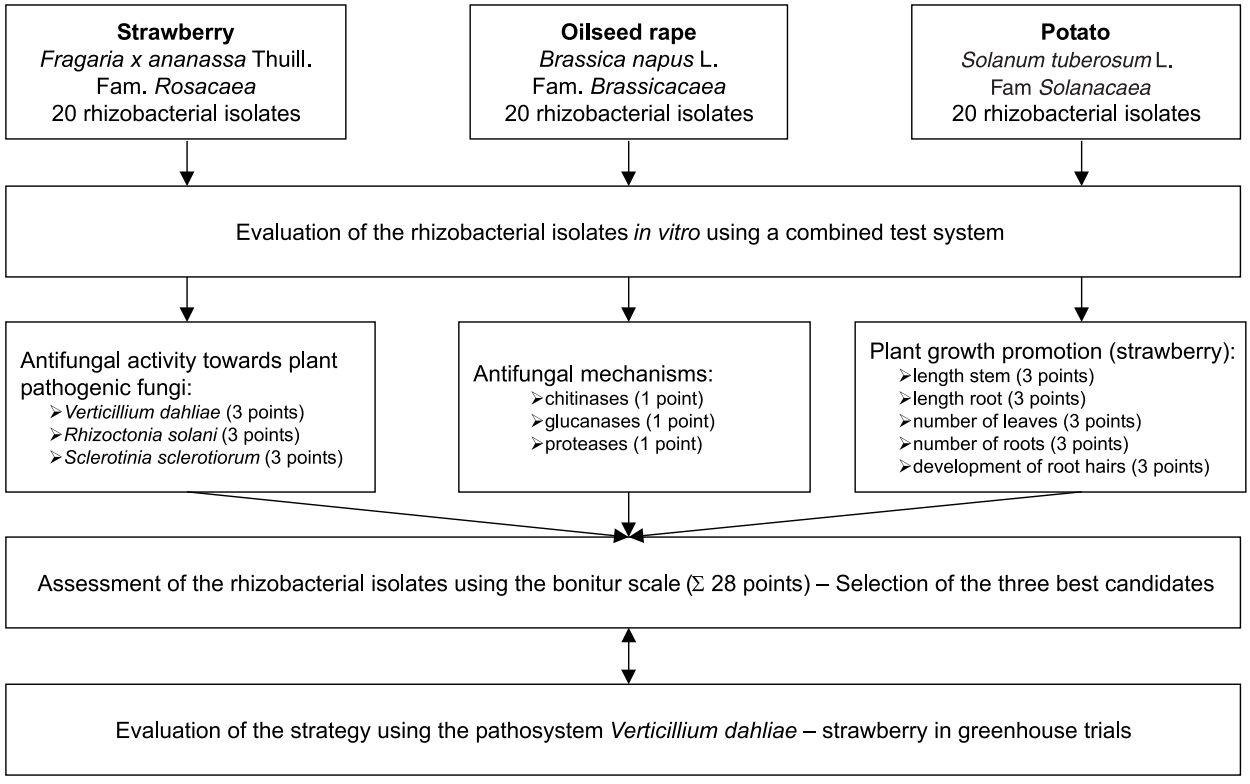

Fig. 1 Scheme for the screening and assessment strategy

from the rhizosphere of oilseed rape showed the highest antifungal activity and the lowest plant growth-promoting effects. The highest plant growth-promoting effect was found for the isolates from the potato rhizosphere.

Effects of selected rhizobacteria on disease incidence and strawberry growth and yield under greenhouse conditions

The biocontrol activity of the three best candidates was compared with commercial biological control agents and a non-treated control. Plants treated with antagonistic rhizobacteria showed a reduced number of wilted and necrotic plants when grown in soil artificially inoculated with *V. dahliae* (Fig. 2). Ten weeks after the treatment, the average reduction of *Verticillium* wilt by *Ps. putida* E2 was 11% compared with the non-treated control. Plants treated with *Ps. chlororaphis* P15 and planted in soils infested by *V. dahliae* reduced the disease by 6%. In contrast, *Ser. plymuthica* R12 treatment significantly enhanced the disease incidence. In these trials, *Ps. putida* E2 showed a better effect than the commercial biocontrol agents *Streptomyces albidoflavus* 1W1 and *Strep. rimosus* 7W1 (Rhizovit).

In all trials yield of strawberries was increased by treatment with antagonistic rhizobacteria (Fig. 3). Yield was increased by 104% in plants treated with *Ps. putida* E2, and in plants treated with *Ps. chlororaphis* P15 yield was increased by 113%. The lowest yield enhancement was found for *Ser. plymuthica* R12 treatment (72%). Surprisingly, only one parameter was statistically enhanced by each treatment, either the number or the length of stolons. The highest enhancement was found for *Ser. plymuthica* R12, with a fivefold enhancement of the number of stolons.

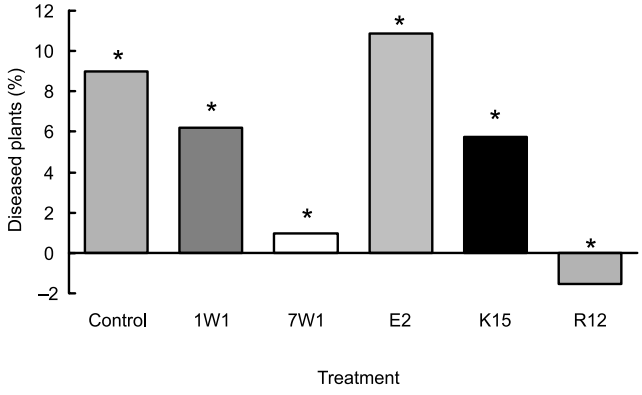

Fig. 2 Effects of rhizobacteria treatment on *Verticillium* wilt in two independent greenhouse trials using soil artificially infested by *V. dahliae* in comparison to the untreated non-bacterized control. Rhizobacteria (*Streptomyces albidoflavus* 1W1, *Strep. rimosus* 7W1 (both biocontrol agents = control), *Ps. putida* B E2 *Rif*^{r}, *Ps. chlororaphis* K15 *Rif*^{r}, *Ser. plymuthica* R12 *Rif*^{r}) were added at a concentration of 2×10^9 cfu ml^{-1} using root dipping for 15 min. After 10 weeks, the disease was monitored. Statistically significant differences ($P \leq 0{\cdot}05$) were analysed by Mann–Whitney and indicated by asterisks

After 10 weeks, the population of rhizobacteria and the total bacterial populations in the strawberry rhizosphere were determined. Strains colonized the treated roots of strawberry at population densities between $5{\cdot}4 \times 10^4$ (*Ser. plymuthica* R12), $7{\cdot}9 \times 10^4$ (*Ps. putida* B E2) and $4{\cdot}6 \times 10^5$ (*Ps. chlororaphis* K15) cfu g^{-1} root. Rifampicin-resistant mutants were not detected in the nontreated control rhizospheres. The total bacterial populations in the rhizosphere ranged from $2{\cdot}8\ 10^8$ to $1{\cdot}0\ 10^9$ cfu g^{-1} root and were not different among treatments.

DISCUSSION

Antagonistic root-associated bacteria are an important functional group of beneficial bacteria responsible for the control of soilborne pathogens (Weller 1988; Sørensen 1997). In order to find new biocontrol agents antagonistic to the soilborne fungus *V. dahliae*, 60 rhizobacterial isolates were evaluated using a combination of different screening steps.

As a result of the first screening, isolates were found to produce detectable inhibition zones against *Verticillium* and other fungi on agar. The production of clear inhibition zones in dual culture screens is due to the production of either antibiotics, toxic metabolites or siderophores as mechanisms for biological control (Swadling and Jeffries 1996). The antagonism of selected isolates towards other fungal pathogens was assessed because under field conditions synergistic interactions of pathogens occurred (Scholte and Jacob 1989). Biological control agents which can control more than one pathogen are extremely interesting. In our study, only a small number of selective *Verticillium* antagonists was found and the majority was also active against other fungal pathogens.

The second parameter of the screening approach has an advantage for the selection of potential biocontrol agents over the first *in vitro* screen because it also selects modes of action involving lysis of fungal cell walls by hydrolytic enzymes. The ability of antagonistic bacteria to produce proteolytic enzymes was distributed widely between our rhizosphere isolates, but only a low percentage of glucanolytic and chitinolytic isolates were found. The composition of antagonistic mechanisms was specific for each isolate.

Many root-associated bacteria have a direct positive influence on plant growth and can stimulate plant health indirectly (Höflich *et al.* 1994). In our study, we could not detect a plant specific effect using the test system. Isolates from oilseed rape and potato rhizosphere were as effective or more effective in plant growth-promotion than isolates from

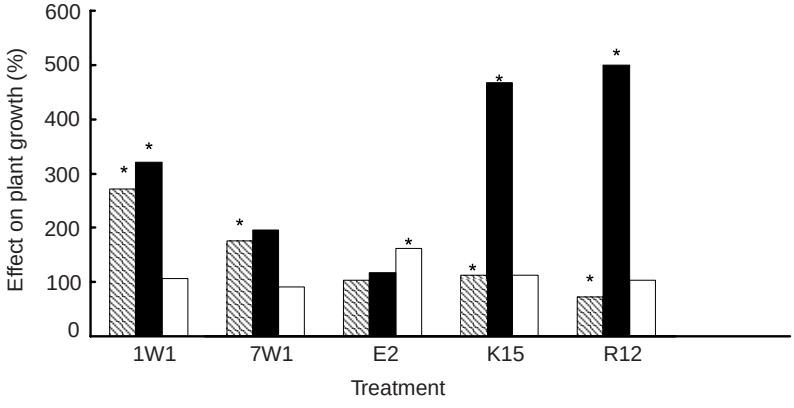

Fig. 3 Effect of rhizobacteria on the yield and on the development of stolons (number and length) of strawberry plants in greenhouse trials using soil naturally infested by *V. dahliae* compared with an untreated control. Rhizobacteria (*Streptomyces albidoflavus* 1W1, *Strep. rimosus* 7W1, *Ps. putida* B E2 *Rif*^{r}, *Ps. chlororaphis* K15 *Rif*^{r}, *Ser. plymuthica* R12 *Rif*^{r}) were added at a concentration of 2×10^{9} cfu ml^{-1} using root dipping for 15 min. Each treatment was replicated three times, 10 plants per pot. After 10 weeks, the plants were monitored. Statistically significant differences ($P \leq 0{\cdot}05$) were analysed by Mann–Whitney and indicated by asterisks

the strawberry rhizosphere. The newly developed plant growth-promotion assay in microplates is an easier *in planta* test than a whole plant system in terms of time, plant material and growth facilities. The microplate assay allowed many repetitions and screening of a large number of bacterial isolates.

Greenhouse trials were used to evaluate our test system. All the selected rhizobacteria showed plant-promoting effects compared to the non-treated controls and to the commercial biocontrol agents used as biological controls. Surprisingly, the effect was either seen in the production of fruits or the development of runner plants. The same effect was found by Taube-Baab *et al*. (1996) after treatment of strawberry plants with mycorrhiza fungi. This may be an interesting fact with potentially practical consequences. Usually, strawberries are cultivated either to produce strawberries or to produce runner plants. A requirement for an efficient biological control agent is the ability to survive and to become established in the rhizosphere (Lugtenberg and Dekkers 1999). The rhizosphere competence of the selected rhizobacteria was demonstrated by reisolation of the rifampicin-resistant mutant from the rhizosphere for a period of 10 weeks under greenhouse conditions. In this study, the screening resulted in a selection of three interesting biocontrol candidates against *Verticillium*: *Ps. putida* B E2 (strawberry rhizosphere), *Ps. chlororaphis* K15 (potato rhizosphere) and *Ser. plymuthica* R12 (oilseed rape rhizosphere).

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