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The Potential of *Serratia marcescens*: An Indigenous Strain Isolated from Date Palm Compost as Biocontrol Agent of *Rhizoctonia solani* on Potato

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Abstract

Rhizoctonia solani, associated with stem canker and black scurf diseases of potato, was one of the most destructive pathogens in Tunisia and elsewhere. A bacterial strain was isolated from date palm compost, identified as *Serratia marcescens* using amplification and sequencing of the 16SrRNA in combination with biochemical characterization. The antifungal properties against *R. solani* AG3 strain were assessed on potato. Mycelial growth inhibition of the pathogen was evaluated after 6 days of incubation at 28°C in the presence of the bacterium and its cell-free culture filtrates. The application of bacterial suspension adjusted to 10^8CFU/ml as pre-planting treatment of potato seeds tubers cv. Nicola reduced the incidence and severity of diseases under greenhouses conditions. In pot experiments, no stem canker was detected and the percentage of progeny tubers showing symptoms of black scurf was significantly reduced With *S. marcescencs* treatment (36.47%) as compared to the controls. Results of this study suggest also that *S. marcescencs* was an effective biocontrol agent against black scurf and stem canker of potato since severity was reduced up to 49.31% and 83.16% respectively. Therefore, the bacterium could be considered as promising alternative to chemical products.

Keywords: *R. solani*; *S. marcescens*; Cell free culture filtrate; Disease incidence; Disease severity; Plant growth

Introduction

The widespread soil-borne pathogen *Rhizoctonia solani* is the causal agent of Rhizoctonia disease complex in potato (*Solanum tuberosum* L.) resulting in two different appearances of the disease, namely stem canker and black scurf which lead to tuber yield reductions and losses in tuber quality [1].

Stem canker consists of brown and black sunken stem lesions which involve the reduction of nutrient in the whole plant as a result losses of yield, whereas black scurf is revealed by the formation of brown or black sclerotia on tuber surfaces, which may affect their quality and marketability [2-4].

R. solani perpetuates in soil and tubers in the form of sclerotia. Seed-borne inoculum of *R. solani* can be a significant factor in the development of disease [5,6].

Control measures through cultural practices and chemical fungicides are used but they are not completely effective and Rhizoctonia disease remains a persistent problem. In addition chemical treatments are becoming increasingly difficult due to concerns about health and environmental hazards.

Biological control of Rhizoctonia diseases using microorganisms or their secretions has been demonstrated in some cases and offers an attractive alternative to overcome the disease and may provide effective and sustainable management [4,7-9].

Effectiveness of bacteria isolated from composts as potential biocontrol against plant diseases has been extensively described in many papers [10-13].

Several bacterial strains have been identified as biocontrol agents with antifungal activity against a large spectrum of plant diseases in natural environments and may be used to replace chemical control.

Serratia species, usually found in diverse natural environments, have been found to reduce disease severity of various foliar and soil-borne diseases [14-18]. Among *Serratia* species, *S. marcescens* was reported as an important bacterium that has the ability to induce systemic resistance to various pathogens and to exhibit antifungal activity against several soil-borne fungi.

The objective of this research was to evaluate the potential of using a strain of *S. marcescens* isolated from date palm compost as biological control agent to reduce the impact of stem canker and black scurf caused by *R. solani* in potato. The efficacy of *S. marcescens* to control *R. solani* was tested using *in vitro* plate agar assay and under greenhouses conditions in artificially infested peat-sand growth media. The effect of the antagonist isolate on plant growth was also investigated.

Materials and Methods

Source of the antagonistic bacterium

The bacterium was isolated from date palm waste compost obtained by aerobic windrow composting. The composting crushed dry date palms wastes material was mixed with sheep manure at the ratio of 2:3 and 1:3 (v/v), respectively and piled in a 3.90 m long by 2.70 m wide by 1 m high windrow on a concrete floor with an open air. The moisture content of the compost was maintained at approximately 60% by sprinkling with water. The compost from the pile was used as

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the source of microorganisms. At the end of the process, the compost had the following major physico-chemical characteristics: pH 6.3, 1% nitrogen, 91.8% organic matter, 78.3% carbon and 37.3 C/N.

Isolation and purification of the antagonistic bacterium

The bacterium was isolated on Trypto-Casein Soja agar medium (TSA) from compost suspension obtained by adding 10 grams of compost sample to 90 ml sterile distilled water. After being shaked at room temperature on a rotating shaker for 1 h at 120 rpm, the mixture was then filtered through four layers of sterile gauze. The suspension was serially diluted (10^{-1} to 10^{-7}) and spread onto TSA plates then incubated at 28°C for 48 h and the subsequent purified bacterium was stored at 4°C on the same medium until future use.

Valuation of the antifungal activity of the isolated bacterium against *Rhizoctonia solani in vitro*

Plate antagonism assay: *In vitro* PDA plate pairing assay was used, as described by Bezert et al. [19], to determine the effect of *S. marcences* on *R. solani* mycelial growth. Two mycelial plugs of actively growing *R. solani* were placed 5 cm apart on PDA in a 90 mm Petri dish. A streak of the test bacterium was placed between the plugs (2.5 cm from each plug). Control plates consisted of the mycelial plugs without bacterium. Three replicate plates were prepared and then incubated at 28 °C. Inhibition of fungal growth was noted after 5-7 days. The experiment was repeated to confirm initial results.

Effect of cell free culture filtrates of *S. marcescens*

After 24-h growth on a TSA medium at 28°C, the bacterium was re-inoculated into a liquid medium (Trypto-Casein Soja broth medium:TSB) for 72 h. Crude extracts were obtained by centrifuging broth culture at 8000 rpm at +4°C for 20 min. The extracts were further filter-sterilized (0.22 µm filter) prior to use. Antifungal potential of cell-free extracts of *S. marscens* was detected using the agar-well diffusion test and the pouring test.

The effectiveness of the bacterium crude culture filtrate by agar diffusion was tested by placing 100 µL aliquots of the cell free extract into a 6 mm well cut in PDA plate equidistant from the 6 mm plug of *R. solani* [20,21].

In the pouring method, filter-sterilized extracts were mixed in PDA (cooled at 45°C) based on the ratio of 1:4. Once the agar has solidified, a 6 mm mycelial plug of *R. solani* was placed centrally on the agar [22].

Plates were incubated for 7 days at 28°C. Control plates were prepared and incubated similarly, substituting the extracts with sterile distilled water. The diameter of *R. solani* inoculated on medium with extracts was calculated against the diameter of the pathogen on agar with sterile distilled water and the percentage of inhibition of the growth diameter was determined. Three plates were used per experiment and the whole experiment was repeated twice.

Bacterial identification: 16S rDNA gene sequencing

The genomic DNA of antagonist was extracted using the innuPREP DNA/RNA Mini Kit (Analytik Jena, Biometra) according to manufacturer instructions. The 16S rDNA primers used are universal bacterial primers: 27F and 1492R. PCR reaction was performed in a total reaction volume of 25 µL containing 5 µl of buffer (5x), 2.5 µl of dNTP (2 mM), 1.5 µl of $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (5 U/µl), 2.5 µl of each primer (6 µM), 5.75 µl of ultra-pure water and 5 µl of genomic DNA templates (10 ng). The amplification program, performed in an OpticonII (Biorad) Thermocycler, included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. Amplification was terminated by a final extension step of 7 min at 72°C. PCR products, checked on agarose gel, were subsequently purified and sequenced by private company (Biotools, Tunisia) using Forward and Reverse PCR primers of 16S r-DNA. Finally, the BLAST program of the National Center for Biotechnology Information was used to analyze the obtained sequences (http://www. blast.ncbi.nlm.nih.gov/)

Biocontrol activity of the antagonistic bacterium in greenhouse trials

Inocula preparation and pot bioassays: The efficacy of the selected bacterium was evaluated for its control of Rhizoctonia stem canker and black scurf on potato. Pathogen inoculum was prepared by scraped off mycelium and sclerotia of *R. solani* from 10 day-old cultures and then, homogenized with sterile distilled water in a blender for 5 min. The pathogen preparation was mixed with autoclaved peat-sand mixture (one Petri dish per 100 ml distilled water per pot). Non-infested mixture served as a control [23].

Potato (*Solanum tuberosum* L.) seed tubers (cv. Nicola, Class A) were superficially disinfected with a solution of sodium hypochlorite (diluted at 2%) for 20 min and then rinsed with sterile distilled water and air dried. Seed treatment was performed using a suspension of *S. marcescens* produced by culturing the bacterium in trypto-caseine soja broth (TSB) on a rotary shaker (120 rpm, 28°C). Tuber seeds were immersed for 30 min in the bacterial cell suspension obtained from a 48 h culture broth and adjusted to 10^8 cfu/ml as determined by dilution plating and 560 nm optic density analysis. Seeds treated with water were used as controls. Seeds were planted in 5 liters plastic pot and grown in the greenhouse (18–25°C, 16 h light) for eight weeks and watered every 3 days.

Effect of *S. marcescens* on plant yield and on suppression of stem canker and black scurf

Plant yield and biocontrol potential were assessed on plants after harvest. Dry aerial parts and tuber fresh weights were determined. Stems and tubercles were washed with tap water, dried in air and examined for Rhizoctonia disease incidence and severity. Disease severity was assessed using the collar necrosis index (CNI) and the index of progeny tubers (IPT) infection by *R. solani* sclerotia according to the scales used by Daami-Remadi et al. [24] and the Official French scale disease on tubers [25].

Incidence of stem canker was calculated as the percentage of plants with a severity rating of 2 or more and the incidence of black scurf was calculated as the percentage of tubers with a severity rating of 1 or more according to the method of Brewer and Larkin [4].

Statistical analysis

The plant bioassay was designed as a randomized complete block with five replicates. Each replicate consisted of one pot containing one potato seed pieces. This study was conducted in two separate experiments. The agar assay was analyzed as a paired comparison between bacterium and the pathogen-only. Statistical analysis was performed with the STATISTICA software version 5 and comparisons of group means were accomplished with the Newman-Keuls test at $P = 0.05$

Results

Antifungal activities of *S. marcescens* and its cell free culture filtrates on mycelium growth of *R. solani*

In vitro antagonism assays (Figure 1) showed that the compost strain of *S. marcescens* is an antagonist to *R. solani*. The bacterium exhibited an antagonistic activity through antibiosis. After 6 days, growth of *R. solani* at 28°C was reduced to approximately 30% compared with that in the absence of bacterium.

The cell free culture filtrates collected from liquid culture show an inhibition in both test agar –well diffusion and pouring tests, relative to the *R. solani* control (Figure 2). Pouring test was found to be more efficient than agar-well diffusion with reductions of respectively 65.6% ± 0.21 and 21.8% ± 0.24.

Identity of the antagonist strain

Biochemical tests achieved using the API20E indicate that the bacterium strain belonged to the genus *Serratia*. After sequencing 16S r-DNA PCR product with Forward and Reverse primers, NCBI BLAST results showed that the sequence has ≥99% DNA identity with *S. marcescens* 16S rDNA gene. On the basis of BLAST result, it was found that the strain isolated from compost date palms was *S. marcescens*.

Suppression of stem canker and black scurf after *S. marcescens* treatment

In the *R. solani*-potato pathosystem, the experiment was ended after 8 weeks under greenhouse conditions. *S. marcescens* isolate was screened for suppression of stem canker and black scurf (Table 1). The bacterium applied as a seed treatment in the peat-sand infested with *R. solani* provided significant suppression (P=0.05) relative to the pathogen check without bacterium. Black scurf incidence was reduced by 36.47% compared with 100% of progeny tubers with sclerotia in the absence of bacterium treatment. All treated plants have healthy stems.

Effect of *S. marcescens* on stem canker and black scurf severity

Disease severity assessment was determined by using 0-5 disease severity scale. Disease severity over the experimental bioassay was significantly low as compared to the infested controls. The results showed that the application of the strain *S. marcescens* reduced significantly the severity of symptoms of black scurf (Figure 3) and stem canker. Treatments with the bacterium strain led to reductions in the disease index of 49.31% and 83.16%, respectively for black scurf and stem canker compared to the non-inoculated pathogen controls (Figures 4 and 5).

Figure 1: Radial growth inhibition of *Rhizoctonia solani* co-cultured with *Serratia marcescens* strain at 28°C for 6 days.

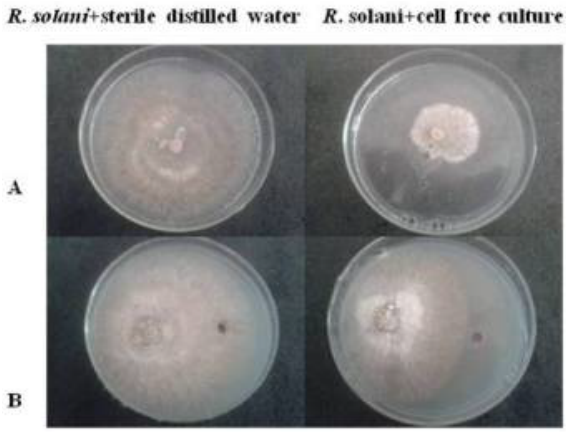

Figure 2: Radial growth inhibition of *Rhizoctonia solani* co-cultured with *Serratia marcescens* cell free culture filtrates at 28°C for 6 days in pouring test (A) and agar-well diffusion test (B).

Treatment	Black scurf		Stem canker	
	DI%	Reduction in DI (%)	DI%	Reduction in DI (%)
Uninfested controls	0 b	-	0 c	-
Infested controls	100 a	-	82.14 d	-
S. marcscens	63.53a	36.47	0c	100

Table 1: Effect of *S. marcescens* treatment on Rhizoctonia stem canker and black scurf incidence (DI) noted on potato plants eight weeks post-planting in substrate artificially infested with *R. solani*. Means followed by the same letter are not significantly different from each other at P=0.05 based on Newman-keuls test.

Figure 3: Severity of black scurf observed, eight weeks post-planting, on potato cv. 'Nicola' plants inoculated with *Rhizoctonia solani* and treated *Serratia marcescens* as compared to the controls. (A): Non inoculated with *R. solani* and non-treated; (B): Inoculated with *R. solani* and non treated; (C): Inoculated with *R. solani* and treated with *Serratia marcescens*.

Growth-promoting effects

The tested strain *S. marcescens* did not have a good growth-promoting effect on potato. None of the growth parameters, such as tuber dry weight and dry aerial part weight, was significantly (P=0.05) increased as compared to non-inoculated controls (Table 2).

Discussion

Rhizoctonia disease, caused by *R. solani*, is one of the soil-borne diseases in potato plants. The fungus does not sporulate; thus, the sclerotia are the survival and dissemination units. Sclerotia in soil are very hard to be suppressed and the control using either fungicides or resistant cultivars was unsuccessful up to date.

In our experiment, *S. marcescens,* an isolated strain from compost

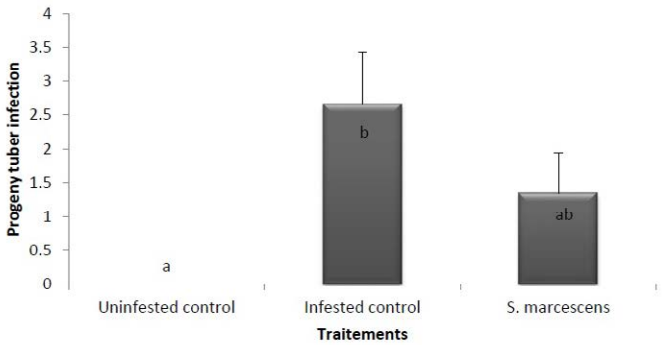

Figure 4: Progeny tuber infection, noted eight weeks post-planting, on potato plants cv. 'Nicola' treated with *S. marcescens* as compared with controls. Means followed by same letter are not significantly different from each other at *P*=0.05 based on Newman-keuls test.

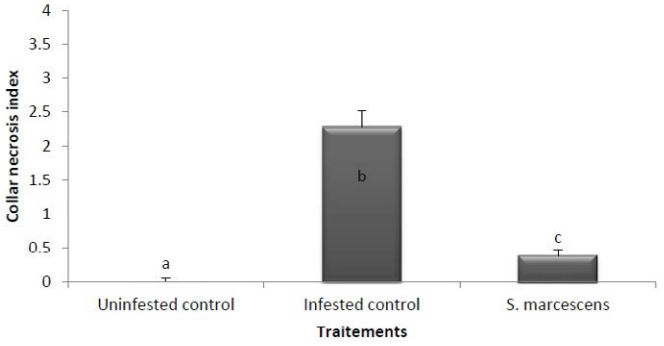

Figure 5: Collar necrosis index, noted eight weeks post-planting, on potato plants cv. 'Nicola' treated with *S. marcescens* as compared with controls. Means followed by same letter are not significantly different from each other at *P*=0.05 based on Newman-keuls test.

Treatment	Tuber fresh weight (g)	Dry aerial part weight (g)
Uninfested controls	159.4 ± 11.6	9.8 ± 1.5
Infested controls	126.1 ± 8.8	8.3 ± 0.3
S. marcscens	142.1 ± 7	9.6 ± 0.4

Table 2: Progeny tubers fresh weight and dry aerial part weight ± SE, noted eight weeks post-planting, on potato plants cv. 'Nicola' treated with *S. marcescens* compared with controls.

date palm and sheep manure was isolated, identified, characterized and used to control rhizoctonia disease. Fungal growth and sclerotial germination of *R. solani* AG3 strain were inhibited in the presence of the bacterium and its cell free culture filtrates *in vitro*. These results suggested that *S. marcescens* could be used as an effective biocontrol agent against *R. solani*. Our results indicate that under greenhouse conditions, the bacterium significantly improved suppression of incidence and severity of the disease. The highest control efficiency was observed on stem canker than on black scurf. In accordance with our results, many studies have proved the successful use of *S. marcescens* as biocontrol agent. Villar de Queiroz and Soares de Melo [26] reported that the biological control of *Phytophthora parasitica* by a strain of *S. marcescens* R-35, isolated from citrus rhizosphere in greenhouse trials, suppressed more than 50% of the disease. In addition, Jaiganesh et al. [18] found that out of the six-bio protectants testes, *S. marcescens* was very effective against *Pyricularia oryzae* under *in vitro* conditions and when applied in rice field with a concentration of 2.5 kg/ha, the bacterium achieved the maximum disease control. In the rice sheath blight caused by *R. solani* AG1, Someya et al. [27] have used *S. marcescens* as effective and persistent biological control.

The occurrence of an inhibition zone by the bacterium isolate is believed to be the result of antibiosis through which the antagonist suppresses the disease. Many researches confirm our findings. Indeed, Kamensky et al. [28] suggested that the antifungal action of the *Serratia* may be based on antibiosis, as well as the production of siderophores and fungal cell-wall degrading enzymes-chitinases. This broad-spectrum activity is likely due, in part, to inhibitory metabolites produced by these organisms. Inhibitory metabolites produced by isolates of *Serratia* include mainly cell wall and cell-membrane degrading enzymes such us chitinases.

Someya et al. [14] tested the antifungal activities against *R. solani* sclerotia by *S. marcescens* strain culture filtrate. They demonstrate that suppression germination of *R. solani* sclerotia was attributed to chitinolytic enzymes and antifungal low-molecular-weight compounds present in filtrates of *S. marcescens*. The same result was found by Akutsu et al. [15]. Those authors showed the potential of *S. marcescens,* a strain isolated from tomato phylloplane, to inhibit several species of the genus *Botrytis* and suggest, via culture filtrate of the bacterium, that the inhibitory effect of *S. marcescens* was correlated with its chitinolytic activity.

Many of the previous studies that characterized the role of bacterial chitinases in biocontrol focused on *S. marcescens* in which the ability to produce this cell wall degrading enzyme of the fungus is considered crucial for antifungal activity [29-33].

Another approach was suggested by Dipanwita et al. [34] which involve the production of antibiotics such as the red pigment prodigiosin and pyrrolnitrin, besides chitinases and siderophores as main protagonist of the biological control by *Serratia* species.

Despite the potential role in reducing *Rhizoctonia* disease, the obtained *S. marcescens* strain has no significant effect on plant growth. This result is in contrast with those obtained by Villar de Queiroz and Soares de Melo [26] for which the strain *S. marcescens* R-35, can promote the growth of citrus.

According to our research work, the compost strain *S. marcescens* could be used as a potential biological control agent against *R. solani* AG3. Further evaluation of *serratia* chitinase for control of *R. solani* AG3 will be needed to valorize the chitinolytic ability in development of successful biocontrol strategy.

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