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Screening of Bacterial Isolates Collected from Marine Bio-Films for Antifungal Activity against *Rhizoctonia solani*

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ABSTRACT

Rhizoctonia solani is one of the major damaging diseases of potato crops in Tunisia and worldwide. The use of fungicides and varieties with different levels of tolerance for the control of this disease is limited by the appearance of resistant fungal strains and with the non-availability of resistant varieties to *R. solani* attacks. The biological control of pathogens offers a promising approach in managing plant disease attacks. In this context, 30 bacteria isolates were isolated from marine bio-films from the Tunisian cost and their antifungal activity against *R. solani* was evaluated by the dual culture assay. Among the tested bacteria two showed an important antagonistic effect against this pathogen, which belong to the species *Bacillus subtillis* and *Bacillus cereus* according to their 16S rDNA gene sequence. The antagonistic activity of the two *Bacillus* species was also observed using their culture supernatant, demonstrating that the active substances are secreted in the medium. The maximum of activity was reached for both strains at 48 h of culture in LB liquid medium. The two antagonistic bacteria were cultured on LB, LB supplemented with glucose and sea water (LBGSW) and M2 supplemented with sea water (M2SW) for 48 and 60 h in order to optimize the production of active substances against three fungal pathogens *R. solani*, *Pythium ultimum* and *Aphanomyces euteiches*. The results showed that for the two *Bacillus* species maximal antifungal activity against *R. solani* was observed at 48 h culture on LB medium. The antifungal activity against *P. ultimum* was observed only with *B. cereus* after 48 h on M2SW. Maximum antifungal activity against *A. cochlioides* was observed with *B. subtilis* in culture on LBGSW at 60 h.

Keywords: antibiosis tests, *Bacillus* sp., black scurf of potato, Tunisian coast
Abbreviations: LB, Luria-Bertani liquid medium; **LBGSW**, LB supplemented with glucose and sea water**; PDA**, potato dextrose agar; **PDB,** potato dextrose broth

INTRODUCTION

Rhizoctonia solani causes pre- and post-emergence damping-off of potato and many other crops. The application of chemical fungicides has been the most and widely used to control *Rhizoctonia* diseases (Djébali and Belhassen 2010). Even-though chemical treatments showed promising results in controlling the diseases, phytotoxicity and chemical residues may pose a serious threat to the environment by soil pollution and determent effects for human health. Therefore, a continuous search for biocontrol agents from different microorganisms has been an ongoing process in order to find specific drugs to treat widespread diseases (Wang *et al*. 2009). The biological control of plant pathogens using antagonistic bacteria is a well known strategy for plant protection (Kloepper *et al*. 1999). Several bacteria species, specially belonging to the genus *Bacillus* were used for the biological control of plant diseases (Liu *et al.* 2009; Gajbhiye *et al* 2010). *Bacillus* bacteria are ubiquitous in soils, have high thermal tolerance and grow quickly in liquid culture. Furthermore, they form endospores that can be easily formulated readily into stable products (Wulff *et al*. 2002). *Bacillus* species are known to produce antifungal lipopeptides (Kim *et al*. 2004), bacteriocins or bacteriocin-like substances (Bizani and Brandelli 2002). Li and Yang (2005) reported that *Bacillus subtilis* produce protein against *Rhizoctonia* on potato dextrose agar plates. Yu *et al*. (2002) reported that *Bacillus amyloliquefaciens* produce iturin A that suppress *Rhizoctonia solani*.

In recent years, isolation of bacteria that produce bioactive secondary metabolites from marine origin was reported (Subramanian *et al*. 2008). A review of the literature found that nearly half of all marine natural products papers report bioactivity data for new compounds which can be used for the biocontrol of plant pathogens (Ongena and Jacques 2007). In this context, our work aimed to isolate and identify antagonistic bacteria species form marine bio-films for the biocontrol of black scurf of potato caused by *R. solani*.

MATERIALS AND METHODS

Microbial materials

Bacteria strains were isolated from marine biofilms from northern Tunisian cost (Hammam-Chott). Bacteria strains were grown on Luria-Bertani solid medium (LB) at 30°C. The bacterial strains were stored at -80°C in LB liquid medium supplemented with 25% glycerol (v/v). The strain of *R. solani* (TN-RS3.2) was isolated form potato tuber-borne sclerotia and cultured on PDA medium. The fungi species *Pythium ultimum* and *Aphanomyces cochlioides* were kindly provided by Prof. Hartmut Laatsch from the Institute of Organic and Biomolecular Chemistry, University of Göttingen, Tammannstraβe 2, 37077 Göttingen, Germany.

In vitro antagonistic assay

1. The dual culture assay

The antagonistic activity of the bacteria isolates against *R. solani* TN-RS3.2 was evaluated the by dual culture assay on potato dextrose agar (PDA) medium. A one day old *R. solani* 9-mm agar

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disk containing grown mycelia was placed in the centre of Petri dishes. The bacteria isolates were streaked in both sides of the agar disks at 2 cm distance. The inhibition of the fungus mycelia was measured after 3 days of incubation at 25°C and 16 h photophase. The bacteria antifungal activity against *R. solani* was also tested in potato dextrose broth (PDB) liquid medium. The antagonistic effect in liquid medium was estimated by measuring the *R. solani* mycelia dry weight.

2. The supernatant activity test

The bacteria strains were grown in 100 ml of LB medium at 30°C with constant shaking at 150 rpm. Cells were harvested by centrifugation at 12000 × *g* for 15 min at 4°C. The resulted supernatant was filtered through 0.45 µm membranes and concentrated 10-fold by lyophilisation. Antifungal activity of the supernatant was carried out by the disc diffusion assay in Petri dishes containing PDA medium. After the mycelia colony of *R. solani* had developed, sterile filter discs (6-mm in diameter) were disposed 1 cm away from the rim of the mycelia colony. An aliquot (100 µl) of the supernatant was then added to the disc. Plates were incubated at 25°C until mycelia growth had formed inhibition crescents around discs.

Optimization of the conditions for maximum antifungal activity

1. Optimization of culture time

The bacteria strains were grown in the same conditions as previously described. The bacteria culture was stopped at 6, 12, 24 and 48 h and the supernatant was recovered by centrifugation at 12000 × *g* for 15 min at 4°C. The antifungal activity of the supernatant was carried out by the disc diffusion assay as described above.

2. Optimization of culture medium

The bacteria isolates were cultured in 1-litre Erlenmeyer flasks each containing 250 ml of LB medium, LB medium supplemented with glucose (5 g/l) and sea water (50%) and M2 (10 g malt extract, 4 g yeast extract, 4 g glucose, pH 7.8) medium supplemented with sea water (50%). The addition of sea water was done because these bacteria were isolated from marine biofilms. The flasks were shaken 150 rpm for 48 or 60 h at 27°C. The entire fermentation broth was freeze-dried and the residue extracted with ethyl acetate. The extracts were evaporated to dryness and then dissolved in dichloromethane/methanol (90/10) (concentration 50 mg/ml), in which the paper disks were dipped, dried under sterile conditions (flow box) and put on an agar plates containing the fungus agar disk. The plates were incubated at 27°C. The inhibition of the fungus mycelia was visually estimated at 72 h as follow: (-) no inhibition; (+) low inhibition; (++) moderate inhibition; (+++) strong inhibition.

Identification of bacterial strains

A 1500 bp 16S rRNA gene fragment was amplified using the primers fd1 (5'-AGA GTTTGATCCTGGCTCAG-3') and rd1 (5'-AAGCTTAAGGAGGTGATCCAGCC-3') according to Weisburg (1991). The PCR amplification products were purified from agarose gels using the Promega PCR purification kit (Wizard SV gel and PCR clean-up systems). Nearly a full-length 16S rRNA gene was sequenced by Macrogen, Korea. Sequences were assembled by the CAP program available on the Infobiogen server (http://bioinfo.hku.hk/services/menuserv.html) and checked manually. The FASTA program was used to search for sequence similarities in DNA databases. Nucleotide sequences were aligned using ClustalW software (Thompson *et al*. 1994).

Statistics

Each experiment was carried out in triplicate and standard errors are indicated in figures. Data were compared on the basis of standard deviation of the mean values. Mean values were compared using Duncan's multiple range test at $P < 0.05$ in STATISTICA software version 5.1H (StatSoft, France).

RESULTS

Screening and identification of antagonistic bacteria

Among the 30 bacteria isolates tested, two were found to be good antagonists against *Rhizoctonia solani* on PDA medium (**Fig. 1**). The antagonistic effect of these bacteria was clearly observed by the complete inhibition of *R. solani* mycelia growth. According to the 16S rDNA gene sequence, these two bacteria were identified as *Bacillus subtilis* and *Bacillus cereus.* Gene sequences were deposited in GenBank under accession numbers bankit1208200 FJ908706 for *B. subtilis* and bankit1208201 FJ908707 for *B. cereus*. *B. subtilis* showed a superior antagonistic effect against *R. solani* than *B. cereus*. In fact, by measuring the mycelia dry weight of *R. solani* in co-culturing with two bacteria separately in PDB liquid medium, the results showed 15% and 31.25% of growth inhibition with *B. cereus* and *B. sublitis*, respectively (**Fig. 2**). In addition, the antagonistic activity of *B. subtilis* was persistent even after 1 month of the co-culture with *R. solani*, however it was not the case with *B. cereus* (data not shown).

Optimizing conditions for maximum antifungal activity

1. Optimization of culture time

The antagonistic activity of the two *Bacillus* species was observed using their culture supernatant. The two bacteria (*B. subtilis* and *B. cereus*) strains were cultured in LB liquid medium for 6, 12, 24 and 48 h and the relative supernatant was tested for antifungal activity against *R. solani* by the disc diffusion assay. The results showed that the antagonistic activity of the two bacteria was detected as soon as 6 h (the end of log phase) and the maximum was reached at 48 h (the end of the stationary phase) (**Fig. 3**).

Table 1 Optimization of the culture medium for two antagonistic bacteria for maximum antifungal activity against three phytopathogens.

Antagonistic bacteria	Culture period	Medium	*Rhizoctonia solani**	*Pythium ultimum*	*Aphanomyces cochlioides*
Bacillus cereus	48 hours	LB	+++	-	++
		LB + Glucose + 50% sea water	++	-	-
		M2 + 50% sea water	-	++	-
	60 hours	LB	++	-	-
		LB + Glucose + 50% sea water	+	-	++
		M2 + 50% sea water	-	++	-
Bacillus subtilis	48 hours	LB	+++	-	++
		LB + Glucose + 50% sea water	+	-	-
		M2 + 50% sea water	-	-	-
	60 hours	LB	++	-	++
		LB + Glucose + 50% sea water	+++	-	+++
		M2 + 50% sea water	-	-	-

*(-) no inhibition; (+) low inhibition; (++) moderate inhibition; (+++) strong inhibition.

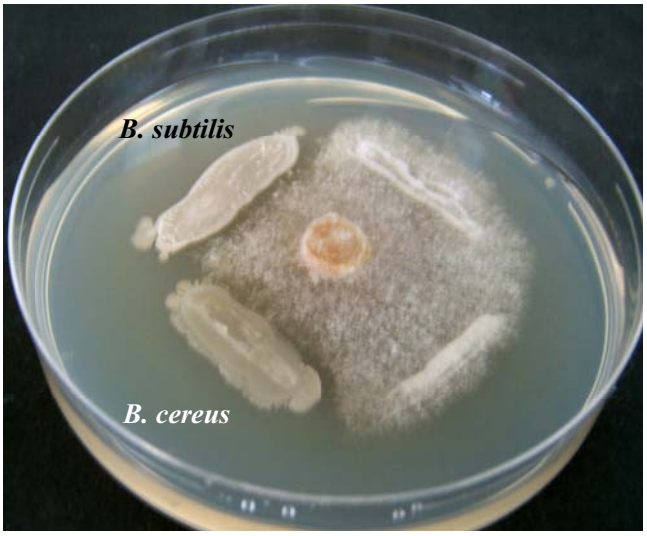

Fig. 1 Antifungal activity on direct antagonist of bacteria strain isolated against *Rhizoctonia solani.*

2. Optimization of culture medium

The two antagonistic bacteria were cultured on LB medium, LB supplemented with glucose and sea water (LBGSW) and M2 medium supplemented with sea water (M2SW) for 48 and 60 h in order to optimize the production of active substances against three fungal pathogens: *R. solani*, *Pythium ultimum* and *Aphanomyces euteiches*. The results showed that for the two *Bacillus* species the maximal antifungal activity against *R. solani* was observed at 48 h culture on LB medium. The antifungal activity against *P. ultimum* was observed only with *B. cereus* since 48 h on M2SW. The maximum antifungal activity against *A. cochlioides* was observed with *B. subtilis* in culture on LBGSW at 60 h (**Table 1**).

DISCUSSION

We focused our attention to screen the potential bacteria species from marine biofilms for their biological control against *Rhizoctonia solani*. An *in vitro* assay was employed for the screening of antagonistic bacteria against *R. solani* by the dual culture assay on PDA medium. Among the 30 bacterial isolates, two were found to be highly active against *R. solani*, whereas the others showed no activity. In

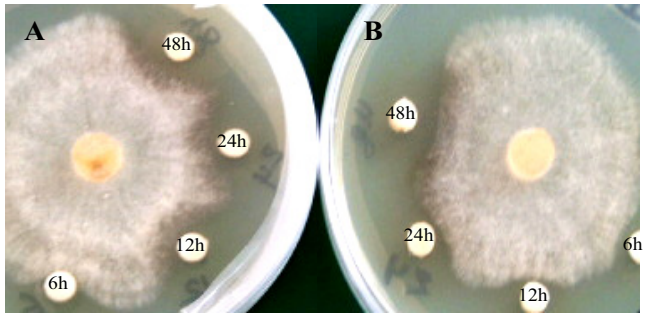

Fig. 3 The antagonistic activity of the culture supernatant of two bacteria at different time of culture (6, 12, 24 and 48 h) in LB liquid medium against *Rhizoctonia solani* by the disc diffusion assay. (A) *Bacillus subtilis*; **(B)** *Bacillus cereus*.

the basis of 16S rDNA this two bacteria were identified as *Bacillus subtilis* and *Bacillus cereus.* According to Ivanova *et al*. (1999), *B. subtilis* is the most abundant bacterium associated with marine sponges, ascidians, soft corals, and they were present in sea water as well. The two bacteria cultures showed antagonistic effect against *R. solani* in the supernatant, proving that the active compounds are secreted in the culture medium. The bacteria *B. subtilis* and *B. cereus* are known to secrete secondary metabolites which inhibit the growth of fungal mycelia (Montealegre *et al*. 2003; Lee *et al*. 2008). In our study, we found that the maximum of activity was clearly in the end of the stationary phase. In concordance with this result, several works showed that the synthesis of antimicrobial compounds by *Bacillus* species generally starts at the end of the exponential phase and reaches its maximum level during the stationary phase (Bizani and Brandelli 2002; Tabbene *et al*. 2009). Our *Bacillus subtilis* strain showed a strong and a persistent inhibitory effect on the growth of *R. solani* mycelia, event after one month of direct antagonism and by using the supernatant, implying that the antifungal metabolites are very higher active and stable compounds. In fact, *B. subtilis* strains are described to produce several higher antifungal metabolites such as iturine (Alippi and Monaco 1994). *B. subtilis* induce a change in mycelia colour of *R. solani* in front of the bacteria cells, which is the result of vacuolization and deformation of hyphae (data not shown). The colour and the aspect of *R. solani* inhibited hyphae may be due to the effect of antibiotics secreted by *B. subtilis* with fungicidal action. The optimization of the production of the antifungal compounds against *R. solani* in different media,

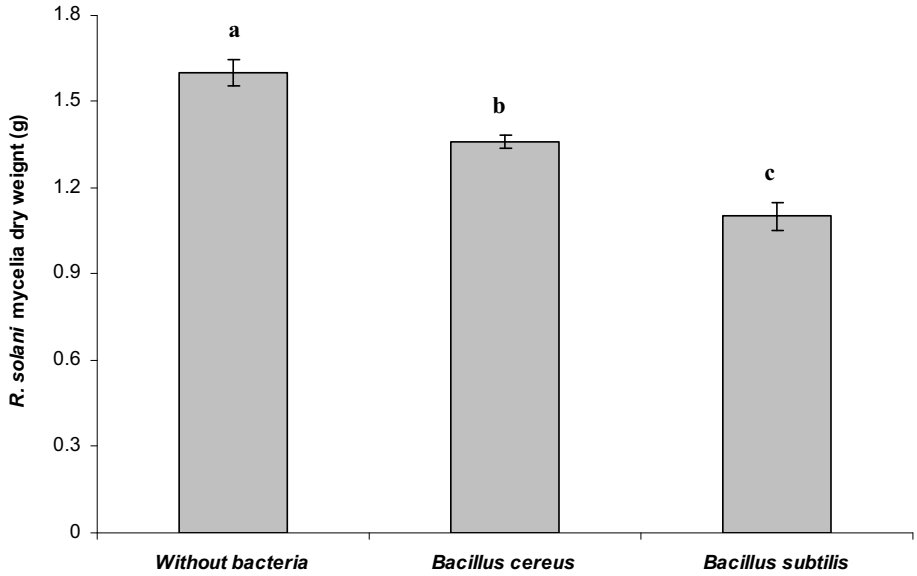

Fig. 2 *Rhizoctonia solani* mycelia growth cultured in potato dextrose broth medium and in presence with *Bacillus cereus* and *Bacillus subtilis*.

showed that the composition of the culture medium for the antagonistic bacteria influence the amount and the specificity of inhibition for the tested fungal pathogen. Variation in culture medium composition was previously shown to affect the production and/or secretion of the antifungal activity (Naclerio *et al*. 1993; Motta and Brindelli 2003).

Bacillus species are well-known as beneficial bacteria for the biocontrol of plant diseases. Indeed, *Bacillus*-based products represent about half of the commercially available biocontrol agents (Fravel 2005). *Bacillus* species synthesize numerous antimicrobial compounds with well-established activity *in vitro*. But only few of these metabolites have been studied for their production in natural field conditions in a way to demonstrate their implication in the biocontrol potential of the producing strains (Ongena *et al*. 2009). However, some studies have linked an *in vitro* antimicrobial activity with a direct antagonism of the phytopathogens but not with other ways of biocontrol so far. Haggag (2008) show that *Bacillus brevis* and *Bacillus polymyxa* produce gramicidin S and polymyxin B peptide antibiotics that strongly inhibited *in vitro Botrytis cinerea* germination, growth and extra-cellular enzyme production but also exhibited under natural field conditions high activity against the *Botrytis* grey mould on strawberry. In this context, we will test the selected bacteria for the biological control of *R. solani* in greenhouse conditions. In addition, we will identify the biochemical nature of the antifungal compounds produced by our *B. subtilis* strain.

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