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Full Length Research Paper

Evaluation of antifungal activity from *Bacillus strains* against *Rhizoctonia solani*

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In this study, 30 bacterial strains isolated from marine biofilms were screened for their antifungal activity against *Rhizoctonia solani* by dual culture assay. Two bacterial strains, *Bacillus subtilis* and *Bacillus cereus,* showed a clear antagonism against *R. solani* on potato dextrose agar (PDA) medium. The antagonistic activity of *B. subtilis* against this pathogen persisted after one month of co-culture. An *in vitro* antagonistic assay using potato tuber slices was set up allowing both the screening of bacteria for their biocontrol proprieties and for their rotting effect on plant tissues. Potato tuber slices treated with *B. cereus* showed a heavy tissues rotting, probably due to the secretion of several enzymes. Whereas, *B. subtilis* did not show any tissues rotting on treated potato tuber slices. In addition, the potato tuber slices treated with *B. subtilis* and subsequently inoculated with *R. solani* agar culture showed a reduced fungal infection in comparison to the control tuber slices inoculated with the pathogen only. The crude extract of *B. subtilis* strain culture in Luria-Bertani (LB) medium at 48 h of incubation showed a high antifungal activity against *R. solani* growth and no cytotoxic effect on Brine shrimp larvae.

Key words: Antagonism, *Bacillus* spp., biological control, black scurf of potato, marine biofilms.

INTRODUCTION

In Tunisia, potato is an important crop which accounts for 16% of the cultivated area (Azzouz, 1996). However, yield of this crop is reduced due to diseases such as *Rhizoctonia solani* which causes pre- and post-emergence damping-off of potato (El Bakali and Martin, 2006). In a recent study done by Djébali and Belhassen (2010), it was found that the commonly cultivated potato varieties in Tunisia are susceptible to *R. solani* attack. The use of chemical fungicides reduced the infection level (Djébali and Belhassen, 2010), but chronic treatment with these fungicides may lead to the emergence of resistant fungal strains. In addition, the use of these chemical products is costly for farmers, human health and environment (Vurro and Gressel, 2006). Thus, searching for biocontrol agents against fungal pathogens constitute a valuable alternative to chemical pesticides (Ongena et al., 2009) which could allow the finding of natural drugs for the treatment of several diseases.

Antagonistic bacteria, specially belonging to the *Bacillus* genus are among the most used biological agents to fight against many plant pathogens (El-hamshary and Khattab, 2008) including *Rhizoctonia* (Mizumoto et al., 2007). *Bacillus* strains are well known for their ability in producing bioactive natural peptides

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which are potentially active against bacteria, fungi and enveloped viruses (Bizani and Brandelli, 2002; Li and Yang, 2005; Jing et al., 2009; Ongena et al., 2009). In addition, bacteria from the *Bacillus* genus are able to colonize endophytic plants (Mahaffee and Kloepper, 1997) and to produce metabolites that affect bacterial and fungal growth (Stein, 2005).

In the present work an attempt was made to identify antagonistic bacteria from marine biofilms against *R. Solani.* In addition, the antifungal and the cytotoxicity activities of the crude extracts of the antagonistic bacterial cultures were performed.

MATERIALS AND METHODS

Isolation and culture conditions of *Rhizoctonia solani*

For the isolation of *R. solani*, sclerotia grown on the skin of infected potato tubes were secluded and immersed in 95% ethanol for 30 s and then washed several times with sterile distilled water. Disinfected sclerotia were cultured on potato dextrose agar (PDA, Pronadisa) medium at 25°C and 16 h photophase until the mycelia started to grow. The isolate RS3.2 was purified by the hyphal tip method which consist in cutting the hyphal tip of a growing mycelium from sclerotia using a sterilized needle under a stereomicroscope and then putted on a new PDA medium (Zhang et al., 2007). The fungal strain was stored on PDA medium at 4°C in the dark.

Culture conditions of the bacterial strains

Bacterial strains were isolated from marine biofilms collected from Tunisian Coast (Hammam-chott). Marine biofilms were scraped with a cell scrapper and were directly inoculated on Luria-Bertani agar medium (Tryptone 10 g, Yeast extract 5 g, NaCl 10 g, Agar 20 g in 1 L of deionized water, pH 7.0±0.2). The agar plates were incubated at 30°C for 24 h. Bacterial colonies showing different morphologies were selected and purified on LB agar plates. Bacterial cultures were maintained at -80°C in LB liquid medium supplemented with 25% (v/v) glycerol.

Molecular identification of the bacterial strains

The 16S rRNA genes of each bacterium were amplified using primers fD1 (5'-AGA GTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGCTTAAGGAGGTGATCCAGCC-3') according to Mhamdi et al. (2002). The following cycling conditions were used: Initial denaturation at 95°C for 3 min; 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min; and final extension at 72°C for 5 min. The amplification products were purified from agarose gels using the Promega PCR purification kit (Wizard SV gel and PCR clean-up systems). Nearly full length 16S rRNA genes were sequenced by MACROGEN, Korea. Sequences were assembled by the CAP program available on the INFOBIOGEN site (http:// bioinfo.hku.hk/services/menuserv. html/) and checked manually. The FASTA program was used for sequence similarities in DNA databases. Nucleotide sequences were aligned using CLUSTALW software (Thompson et al., 1994). The nucleotide sequence data was deposited to the GenBank and accession numbers for the 2 strains were obtained.

In vitro assays

Dual-culture assay

The *in vitro* antagonistic assay was performed according to the dual culture method on PDA medium. A *R. solani* agar disk (6 mm) isolated from one day old culture was disposed at the center of Petri dishes and the bacterial strains were streaked in a square form around the agar disk at 2 cm distance. The antagonistic activity of the studied bacterial strains was estimated by the inhibition of the fungal growth in comparison to a solely cultivated fungal agar disk. The fungal growth was monitored by measuring the diameter in centimeter of the colony until 4 days at 25°C and 16 h photophase. Each bacterial strain was tested in three different plates and the experiment was carried out twice.

Potato tuber slice assay

Potato tubers were disinfected by alcohol (70%) and sodium hypochlorite (3%) solutions for 1-2 min each and then rinsed several times with sterile distilled water and were cut into rectangular slices (Length 6 cm and width 3 cm). The potato slices were immersed in the bacteria suspensions (10^8 cells / ml) prior to the inoculation with *R. solani* agar disk (6 mm of diameter). Potato slices soaked in LB medium and inoculated with *R. solani* were used as the controls. The treated potato slices were placed in Petri dishes that were lined with sterile moistened filter paper and incubated at 25°C. The result was monitored every day by measuring the radial growth of the fungus colony. This experiment was carried out twice.

Optimization of the antifungal activity

The antifungal activity was optimized by varying the composition of the culture medium. For this reason three liquid media were used including (i) LB medium, (ii) LB supplemented with glucose (4 g/ L) and 50% artificial sea water and (iii) M_2 medium (10 g Malt extract, 4 g glucose and 4 g Yeast extract in 1 L) with 50% artificial sea water (Fotso, 2005). The cultures were shaken at 150 rpm for 2 or 2.5 days at 27°C, after which the entire fermentation broth was freeze-dried and extracted with ethyl acetate. The extracts were then concentrated to dryness. The crude extract was dissolved in dichloromethane/methanol (9/1) at 50 mg/ml, in which the paper disks were dipped, dried under sterile conditions and put on PDA plates inoculated with the fungal agar disk. The plates were incubated at 25°C for 24 h after which the diameter of the inhibition zone was evaluated. This experiment was carried out twice

Brine shrimp microwell cytotoxicity assay

The cytotoxicity of the crude extracts was determined against brine shrimps (*Artemia salina*) larvae using a microwell cytotoxicity assay (Takahashi et al. 1989). The mortality rate was determined by the following procedure: Dried eggs of *A. salina* (0.5 g) were added to a 500 ml separating funnel, filled with 400 ml of artificial seawater. The suspension was aerated by bubbling air into the funnel and kept for 24–48 h at room temperature. With a pipette, 40-50 shrimp larvae were collected and transferred to a deep-well microtiter plate. The dead larvae were counted (N), a solution of 50 µg of the crude extract in 10 µl of DMSO was added and the plate was kept at room temperature in the dark. A blind sample containing pure DMSO instead of a crude extract was used as a control. After 24 h, the number of the dead animals in each well was counted (A). The

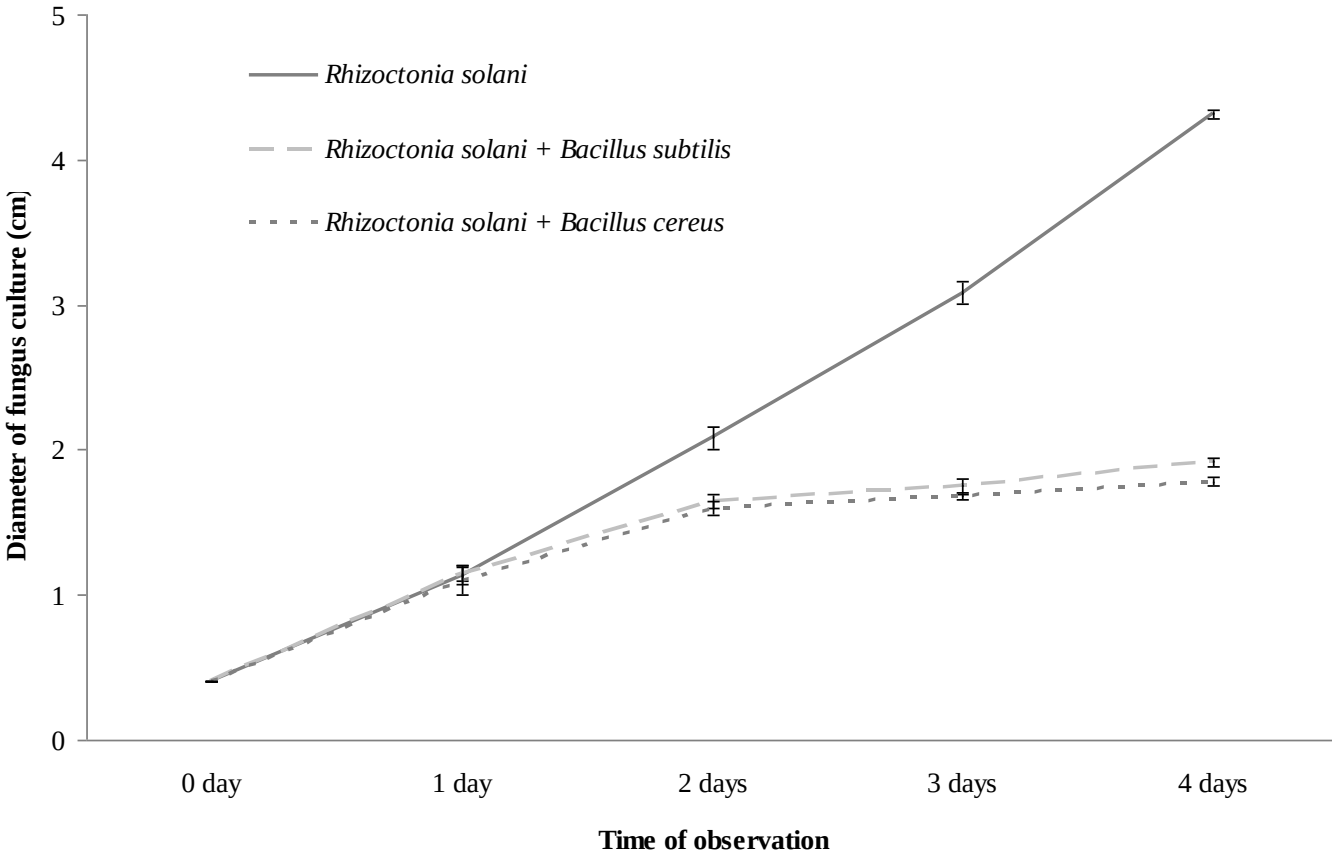

Figure 1. Radial mycelia growth of *Rhizoctonia solani* cultivated on PDA medium in presence or not of *Bacillus subtilis* (SE14) and *Bacillus cereus* (SE15).

surviving larvae were killed by the addition of 0.5 ml methanol so that subsequently the total number of the animals could be determined (G). Actinomycin (10µg/ml) was used as a positive control which gives 100% mortality of brine shrimps larvae. The mortality rate (M) was calculated using the following formula:

$$M = \left[\frac{(A - B - N)}{(G - N)} \right] . 100$$

Where, M is the percent of the dead larvae; A is the number of the dead larvae; B is the average number of the dead larvae in the blind samples; N is the number of the dead larvae before starting the test and G is the total number of larvae.

RESULTS AND DISCUSSION

The *in vitro* antagonistic tests

The dual culture assay

Among the 30 bacterial strains isolated from marine biofilms, two strains exhibited potent antagonist effect against *R. solani* on PDA. The morphological identification showed that these two strains are bacilli Gram-positive bacteria (data not shown). They were identified as *Bacillus subtilis* (SE14) and *Bacillus cereus* (SE15) according to their 16S rDNA gene sequence. Gene sequences were deposited in Gen-Bank under the accession numbers bankit1208200 FJ908706 for *B. subtilis* and bankit1208201 FJ908707 for *B. cereus*. In the marine environment, 90% of bacteria are Gram-negative (Zobell, 1946) because they are much more adapted for survival in such an environment (Das et al., 2006). However, the two *Bacillus* species reported in this study are Gram-positive suggesting that their origin could be due to terrestrial run-off from rivers, as described by Asha et al. (2008). Antagonistic effect of these two strains was clearly observed due to the inhibition of *R. solani* mycelia growth since 2 days of incubation (Figure 1). After 4 days, the percentage of inhibition was about 44% for the two bacteria strains (Figure 1). This result was confirmed by co-culturing bacteria and fungi in liquid PDA medium (data not shown). According to Asak and Shoda (1996), *B. subtilis* might also act on pathogenic fungi by either producing antifungal substances or colonizing microsites faster than the surface fungi. As PDA medium

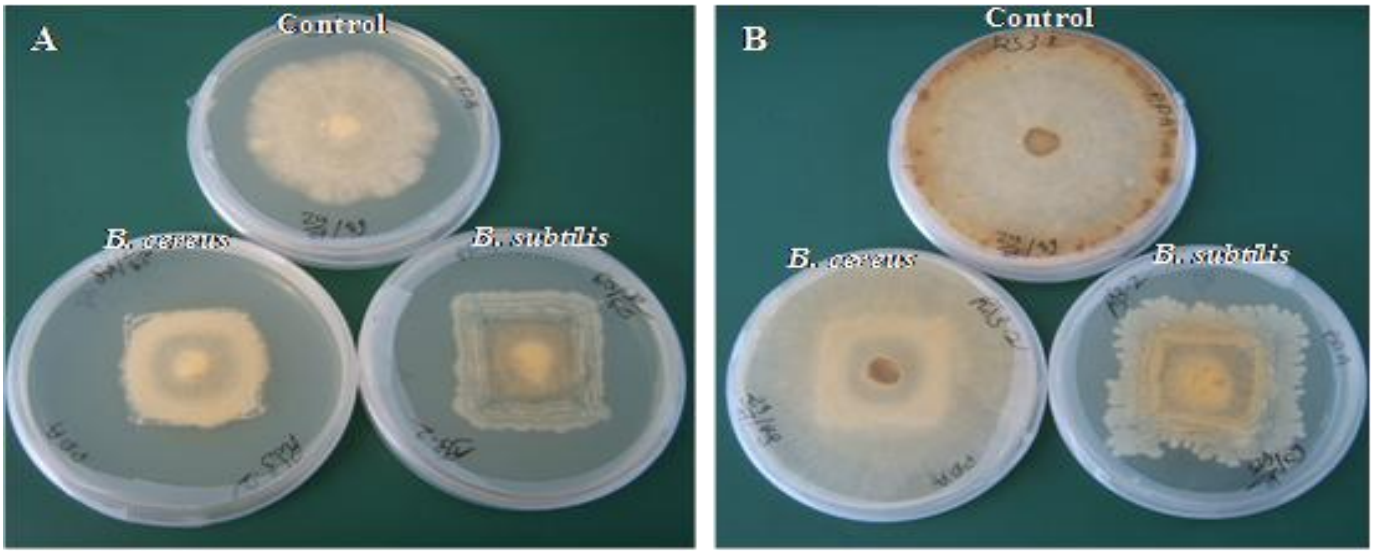

Figure 2. Dual culture assay of *Rhizoctonia solani* and two *Bacillus* species at 3 days (A) and 30 days (B) of direct contact.

was used for the dual culture assay, competition might be excluded as a variety in the mode of action of antifungal components according to Lee et al. (2008). In the dual-culture assay, the inhibition of the *R. solani* growth by the antagonistic bacteria occurred at 48 h (Figure 1) probably due to the late production of antifungal metabolites. This hypothesis is confirmed by an earlier study, in which we observed that *B. subtillis* SE15 activity was found within the supernatant with an optimum level at 48 h of incubation in LB medium (Elkahoui et al., 2011). This result corroborates with other works which found that *B. subtilis* produces lipopeptides belonging to the iturin family in the late phase of growth (Melo, 1998; Montealegre et al., 2003; Agarry et al., 2005). It may be presumed that the inhibition of *R. solani* growth by *B. subtilis* SE14 strain is due to the production of lipopeptides such as iturin and surfactin (Yu et al., 2002). In contrast to *B. cereus*, the antagonistic effect of *B. subtillis* on *R. solani* growth was persistent over 30 days of dual contact (Figure 2). This result suggests that the antifungal compounds produced by the two bacteria are different and the strain of *B. subtilis* produce stable and persistent secondary metabolites such as surfactin as demonstrated by Asaka and Shoda (1996). However, much more detailed analysis is needed to establish the actual biochemical nature of the persistent antifungal agent.

The potato tuber slice assay

Data of the *in vitro* assay carried out on potato slices are shown in Figure 3. The potato tuber slices inoculated with the pathogen showed typical *R. solani* growing mycelia on the surface which resulted in the maceration of the potato tissues around the fungal colony. A severe rotting on potato slices was observed after inoculation with *B. cereus* strain which may be due to the production of enzymes that degrade cellular structures of potato tissues. However, no visible symptoms of rotting were developed on potato slices inoculated with *B. subtilis* solely. Potato slices treated with *B. subtilis* and inoculated with *R. solani* agar culture showed less fungal growth and tissues maceration when compared with potato slices inoculated with the fungus only (Figure 3). The *in vitro* test on potato tuber slices developed in this study provides a powerful tool for the screening of potential biocontrol agents before their study in field experiments. In addition, this test proved to be the stringiest than the *in vitro* test on PDA medium. In fact, with the *in vitro* test on PDA medium we were able to discriminate only between antagonistic and non antagonistic bacteria, and for the *in vitro* test on potato tuber slices we were also able to discriminate between rotting and non rotting bacteria for potato tubers. So, the *in vitro* potato tuber slices can be used in com-plementation to the classical *in vitro* test on synthetic medium for the selection of antagonistic bacteria agents.

Effect of the culture medium and time on the antifungal activity and the cytotoxicity of the bacteria crude extract

The antifungal activity and the cytotoxicity of *B. subtilis* and *B. cereus* crude extracts were examined by using 3 media and 2 culture times (Table 1). The antifungal activity and the cytotoxicity of the two *Bacillus* strains

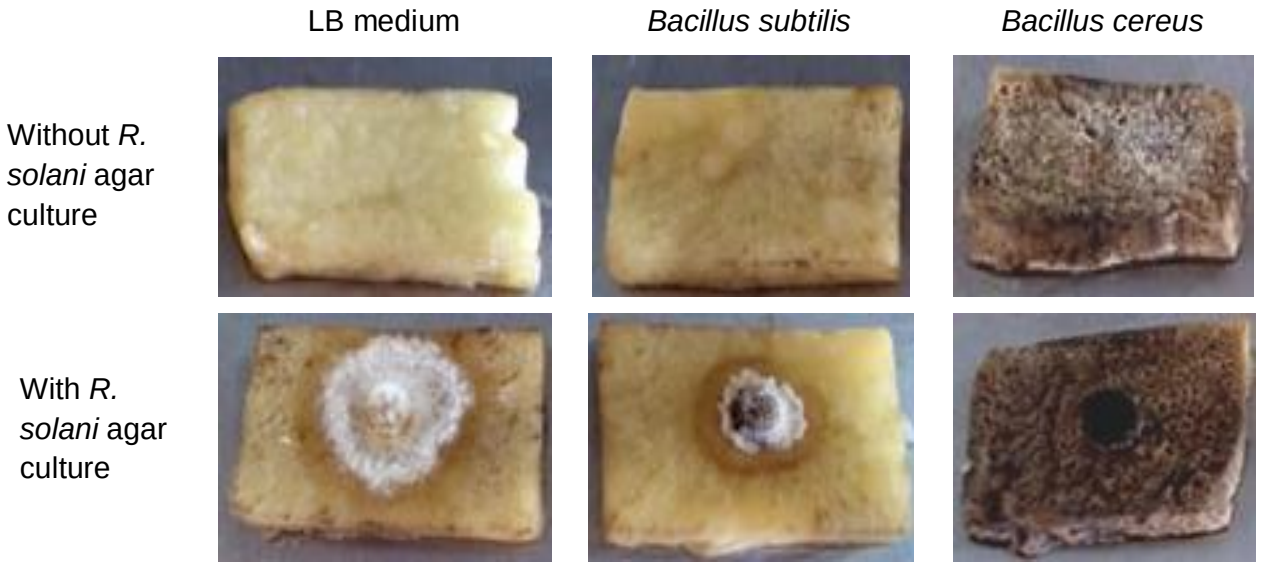

Figure 3. In vitro antifungal activity of *Bacillus subtilis* and *Bacillus cereus* against *Rhizoctonia solani* on potato tuber slices 4 days after inoculation. Top left: control slices tuber treated with LB medium; Top middle: slices tuber received only *B. subtilis*; Top right: slices tuber received only *B. cereus*; bottom left: slices tuber received *R. solani*; bottom middle: slices tuber received both *R. solani* and *B. Subtilis*, bottom right: slices tuber received both *R. solani* and *B. cereus*.

Table 1. The antifungal activity against *Rhizoctonia solani* and the cytotoxicity test of the methanolic extract from *Bacillus subtilis* and *Bacillus cereus*.

Antagonistic bacteria	Culture period (h)	Medium	Level of antifungal activity *	Cytotoxicity (%)
Bacillus cereus	48	LB	+++	0
		LB + Glucose + 50% sea water	++	9
		M2 + 50% sea water	-	26.5
	60	LB	++	0
		LB + Glucose + 50% sea water	+	11.5
		M2 + 50% sea water	-	4
Bacillus subtilis	48	LB	+++	0
		LB + Glucose + 50% sea water	+	50.5
		M2 + 50% sea water	-	100
	60	LB	++	0
		LB + Glucose + 50% sea water	+++	100
		M2 + 50% sea water	-	100

*: '–' absence of inhibition, '+' low inhibition, '++' moderate inhibition, '+++' high inhibition of the fungal growth.

extracts were dependent from the medium composition and the culture time (Table 1). The two bacteria cultivated in LB medium at 48 h of incubation showed the maximum biomass accumulation (data not shown), a high antifungal activity and no cytotoxic effect on Brine shrimp larvae (Table 1). Thus, this combination appeared to be the best for the production of the antifungal compounds with high activity against *R. solani* and no deleterious effect for animals. The cultivation of the two *Bacillus* bacteria species on LB and M2 media supplemented with sea water gave the maximum levels of cytotoxicity against brine shrimp larvae (100% of mortality). These results

indicate the importance of the choice of the culture medium and time for the production of powerful and safe antifungal compounds, that can be used in an integrated management system in agriculture as indicated by Akpa et al. (2001).

The evidence in this study suggest the importance of the developed *in vitro* antagonistic assay on potato tubes slices for the screening of bacteria for their antagonistic activities and for avoiding strains with a rotting effect on plant tissues. In addition, it is essential to note the importance of the choice of the culture medium and time for the production of powerful and safe antifungal compounds. Finally, the *B. subtilis* strain SE14 constitutes a promising biocontrol agent which produces persistent antifungal compounds against *R. solani.* Our future vision will be the use of the SE14 strain under greenhouse and field conditions to study its effect in reducing *R. solani* attack in potato crops.

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