Rhizobacteria-mediated Induced Resistance in Barley against *Cochliobolus sativus* under Field Conditions

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The effect of four rhizobacterial strains on the severity of spot blotch disease caused by *cochliobolus sativus* was evaluated for two growing seasons under rainfed conditions. Three barley genotypes were used as host plant. All strains reduced *C. sativus* severity, with effect more pronounced when *Pseudomonas putida* BTP1 and *Bacillus subtilis* Bs2508 were used. The disease reduction was up to 56% in Arabi Abiad / *P. putida* BTP1. The grain yield was not obviously affected by the presence of the rhizobacteria, except some signifitive increase in season 2. Raising the resistance by soaking seed with rhizobacterial strains might be of ultimate value in agriculture.

Keywords: Barley (*Hordeum vulgare* L.), *Bacillus subtilis, Cochliobolus sativus, Pseudomonas putida* BTP1.

Some plant growth promoting rhizobacterial (PGPR) are able to stimulate inducible defense mechanisms that render the host plant less susceptible to a subsequent pathogen attack (Van Wees et al., 2008; De Vleesschauwer and Höfte, 2009; Mandal and Ray, 2011; Weller et al., 2012). Induction of enhanced defensive capacity can be systemic, as root treatment with such bacteria was shown to trigger protective effects on aboveground plant parts. This phenomenon, called induced systemic resistance (ISR), and has proved in several plant species against a wide range of bacterial, viral and fungal pathogens (Van Loon et al., 1998; Kloepper et al., 2004; Adam et al., 2008; Reglinski and Walters, 2009).

The necrotrophic fungus *C. sativus* (Ito and Kurib.) Drechsl. Ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], is a common foliar disease of barley, wheat and other cereals in warmer parts of the world. It reduces yield as well as quality of barley grain (Mathre, 1997; Kloepper et al., 2004; Da Rocha and Hammerschmidt, 2005; Ghazvini and Tekauz, 2008; Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009; Reglinski and Walters, 2009). Many different agents have been introduced in different planting materials and that can protect plants against various diseases by eliciting ISR (Kloepper et al., 2004; Da Rocha and Hammerschmidt, 2005; Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009; Reglinski and Walters, 2009). Strains of *Pseudomonas* and *Bacillus* species can be assumed plant resistance via directly antago-

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nize (Haas and Défago, 2005), and/or indirectly by inducing systemic resistance (Barker et al., 2013).

The knowledge about (PGPR) has been gathered from dicots, while the information about monocots still remains elusive (Van Loon, 2007; Vlot et al., 2008). The host-PGPR combination and the type of attacker have a huge influence on the potential resistance induced in monocots (De Vleesschauwer et al., 2006). The positive effect of PGPR against necrotrophic pathogens has been illustrated in a few cases (Van Wees et al., 2008; Pinedra et al., 2010).

The objective of the current research was to examine the biological potential of four known rhizobacteria strains (*Pseudomonas putida* BTP1 and *Bacillus subtilis* Bs2508, Bs2504, and Bs2500) differing in lipopeptides production (Ongena et al., 2007), against spot blotch disease on three barley cultivars under field conditions, and to determine possible consequences on plant growth and yield.

Materials and Methods

Bacterial cultures

Rhizobacterial strains (*Pseudomonas putida* BTP1, and *Bacillus subtilis* Bs2508, Bs2504, and Bs2500) were kindly provided by Dr. Philippe Thonart (Wallon Center for Industrial Biology, University of Liège, Belgium). They were maintained on Kings medium agar plates (King et al., 1954) at 4 °C before experimental use. *P. putida* BTP1 was grown on casamino acids (CAA) medium (Ongena et al., 2002). While the *B. subtilis* strains were grown on 868 medium (20 g/glucose, 10 g/L peptone, 10 g yeast and 15 g agar) (Jacques et al., 1999). All strains were incubated for 24 h at 30 ± 1 °C in the dark. Bacterial cells were collected and resuspended in 10 mM $MgSO_4$ to a final density of 10^8 colony-forming units (CFU) per mL before use.

Plant materials

Two spring barley types [Arabi Abiad (Landrace) and WI2291 (improved cultivar)] and one winter type (Banteng) were chosen for their different reaction to *C. sativus* (Arabi and Jawhar 2012a, 2012b).

Assays of induced resistance with rhizobacteria

Seeds of three genotypes (Arabi Abiad, WI2291 and Banteng) were soaked for 15 min in each rhizobacterial strain suspension at a concentration of 10^8 CFU/ml prior to sowing in the field in the second week of October, where the soil temperature ranged from 22–27 °C. The experiments were conducted under natural rainfed conditions (450 mm annually) in Dobaya at 20 km west of Damascus, using a randomized complete block design with three replicates (50×50 cm each) during two growing seasons. Treatments were as follow: 1) inoculations with *C. sativus*. 2) inoculations with *C. sativus* and soaking with one of rhizobacterial strain. 3) soaking with a rhizobacterial strain. 4) plant control (free of disease and rhizobacteria). Soil fertilizers were drilled before sowing.

Fungal inoculation

For the inoculation process, the most virulent pathotype of *C. sativus* in Syria, Pt4 (Arabi and Jawhar 2003; 2007), was used in this study. The isolate was grown in 9 cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) and incubated for 10 days, at 22 ± 1 °C in the dark to allow mycelial growth.

Plants were inoculated at growth stage (GS) 12 (Zadoks et al., 1974). Spore suspension (approximately 2×10^4 spores/mL) containing the surfactant Tween 20, was uniformly sprayed onto plants, then they were covered with polyethylene for 3 days to maintain a relative humidity (RH) up to 80–90% for infection and disease development.

Disease rating and yield performance

Infected and healthy plants were counted in each replicate. The infection response based on the measurement of individual lesion size (dimension; mm) for each second leaf was assessed 10 days after inoculation according to Fetch scale (Fetch and Steffenson, 1999). Inoculated and non-inoculated (control) plants were harvested at maturity. Grain yield was determined on individual plants.

In vitro *antagonistic test*

Zero point one ml of spore suspension (approximately 2×10^4 spore/ml) of *C. sativus* was spread onto Petri dish containing PDA medium by sterile glass spreader. Then, four holes were made in each Petri dish, for each bacterial strain (BTP1, Bs2500, Bs2504 and Bs2508). A 0.1 ml of bacterial suspension (10^8 CFU/ml) were transferred onto a hole. While 0.1 ml of distilled water was transferred onto the fourth hole as a control. All Petri dishes were incubated at room temperature (25 ± 1 °C) in dark for 4–5 days. The experiment was repeated three times.

Results and Discussion

In vitro antagonistic test between the four rhizobacterial strains (BTP1, Bs2500, Bs2504 and Bs2508) and *C. sativus* showed that the two strains (BTP1 and Bs2500) did not inhibit *C. sativus* growth compared with the control. There was non-antagonistic effect of these two strains against *C. sativus* (Fig. 1). This is in agreement with previous studies on tomato, which showed that *P. putida* did not produce or secret any fungi toxic compound (Ongena et al., 1999), and the surfactins produced by Bs2500 strain had not fungitoxic properties (Maget-Dana et al., 1992; Peypoux et al., 1999). In contrast, the two strains Bs2504 and Bs2508 (producing fengycins only or both fengycins and surfactins lipopeptides, respectively) showed a high ability to inhibit *C. sativus* growth. There was direct antagonism between them (Fig. 1). This result is supported by precedent work that showing that fengycins are fungi toxic compounds (Vanittanakom et al., 1986). Ongena et al. (2007) showed that fengycins or the producing strains did not migrate through the plant from inoculated roots to the infected leaves. Since both agent, the pathogen (fungus) and inducer (bacteria), remained spatially and temporally separated on different plant or-
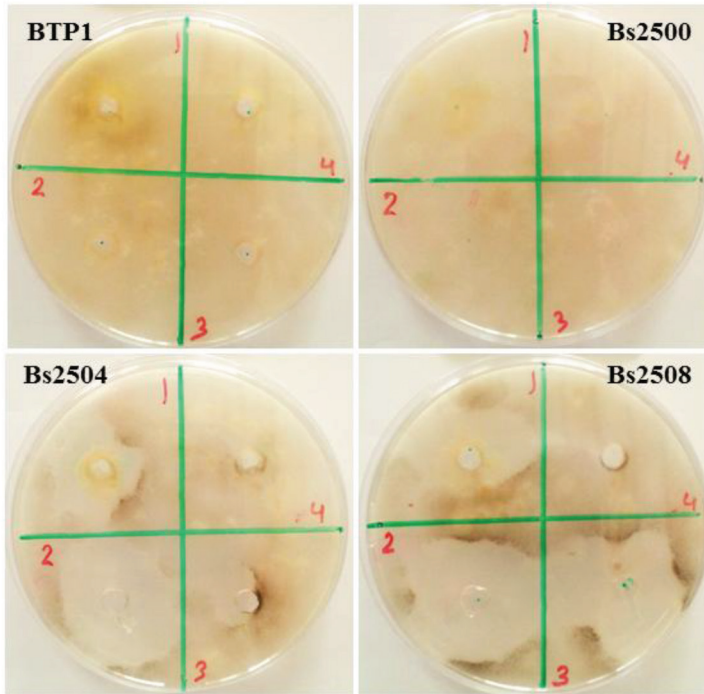
Fig. 1. *In vitro* antagonistest of four rhizobacterial strains against the virulent pathotype of
Cocholobolus sativus in Syria (PT4), with four replicates per Petri dish (1, 2, 3, 4, in red).

gans, this could suggest that diseases incidence reduction is probably due to resistance induced in plant (Fig. 1).

Student–Newman–Keuls test on severity of *C. sativus* disease values, showed significant ($p < 0.05$) interactions between rhizobacterial strains and barley genotypes (Table 1). Growing season had significant ($p < 0.05$) effect on disease severity, indicating that both rhizobacterial strains and genotypes differ in ability to induce resistance and genotypes resistance. Compared with the control, all strains had a positive effect in reducing disease severity (Table 1). As it presented in Table 1, Arabi Abiad and WI2291 (spring barley) were very susceptible genotypes to *C. sativus* (rating 48.3% and 62.8%, respectively) whereas Banteng was more resistant genotype (winter barley). *P. putida* BTP1 was the best strain in reducing disease severity with mean values 19.7 and 25.9% in seasons 1 and 2, respectively. This reduction achieved 51.6 and 44% for the above two seasons. Mean genotype response to strain treatment was significantly different. The resistant genotype Banteng had 16.2% and 16.35% mean disease rating in the first and second season, respectively. In the opposite, the susceptible genotype WI2291 was the most susceptible one with mean disease rating of 33.1 and 42% in seasons 1 and 2, respectively.

The same trend in increasing resistance was observed vis-à-vis the three genotypes. The susceptible landrace Arabi Abiad exhibited a clear and significant induction of resistance by *P. putida* BTP1 treatment with disease decreasing by 56.3 and 45.9% in seasons 1 and 2, respectively. Results obtained for WI2291 indicate the same trend as those for

Arabi Abiad in the two growing seasons (Table. 1). The resistant barley genotype Banteng exhibited less increase in resistance with 41.8 and 42.5% in the same growing seasons. The resulting resistance in our assays can be lasting against *C. sativus,* where the rate of disease reduction ranged between 19.9% (Banteng/Bs2500) and 56.3% Arabi Abiad/ *P. putida* BTP1 (Table 1).

In general, there were no significant differences (main effect) in grain yield between the two growing seasons. The grain yield was not affected by rhizobacterial strains in season 1 for all genotypes used in this work (Table 2), while it was significantly affected by rhizobacterial strains in season 2. Compared with the diseased control, all bacterial strains had a positive effect in increasing grain yield vis-à-vis the genotypes used here. This is in agreement with the precedent work of on barley showing that there was no clear effect of induced resistance on yield (Reglinski et al., 1994).

Induced resistance is a host genotype response, and the environment generally influences its expression under field conditions. It is known that the expression of induced resistance varies in barley genotypes (Tucci et al., 2011; Walters et al., 2011). Our re-

Table 1

Mean disease severity (%) of three barley cultivars inoculated with *C. sativus* PT4,
soaked with rhizobacteria during two growing seasons

Treatment	Season 1			Mean	Season 2			Mean	Mean
	Arabi Abiad	Banteng	WI2291		Arabi Abiad	Banteng	WI2291		effect
C. sativus PT4	48.3a*	21a	52.7a	40.7a	52.3a	23.7a	62.8a	46.3a	43.5a
BTP1	21.1c	12.2b	25.7b	19.7d	28.3d	13.6b	32.7c	25.9d	22.3b
Bs2500	29.9b	16.8ab	31.4b	26b	36.5bc	14.9b	38.5b	30bc	28b
Bs2504	24.6bc	16.1ab	29.1b	23.3bc	41.3b	16.2b	38.8b	32.1b	27.7b
Bs2508	23.6bc	14.8b	26.5b	21.6cd	34cd	14b	37bc	28.4c	25b
Mean	B29.5	C16.2	A33.1		B38.5	C16.5	A42		
Mean effect		B26.3				A32.3			

*Mean followed by different small latters (column) and preceded by different letters (line) differ significantly at (p < 0.05) according to the Newman–Keul's test.

Table 2

Effect of *C. sativus* / rhizobacteria strains interactions on grain yield (g/plant)
of three barley cultivars during two growing seasons

Treatment	Season 1			Mean	Season 2			Mean	Main
	Arabi Abiad	Banteng	WI2291		Arabi Abiad	Banteng	WI2291		effect
C. sativus PT4	4.31a*	3a	5.17a	4.17b	4.1c	2.81b	5.3b	4.1c	4.12b
BTP1	5.58a	4a	6.4a	5.34a	4.57b	3.4a	5.71ab	4.56b	4.65a
Bs2500	5.22a	3.62a	5.86a	4.9ab	4.87b	3.28a	5.88ab	4.65b	4.77ab
Bs2508	4.82a	4.1a	5.83a	4.91ab	5.33a	3.47a	6.1a	4.96a	4.94a
Bs2504	5.44a	3.87a	6.11a	5.18a	5.63a	3.13ab	6a	4.92a	5a
Mean	B5.1	C3.73	A5.87	B4.9	C3.22	A5.78			
Main effect	4.9A	4.63A							

*Mean followed by different small latters (column) and preceded by different letters (line) differ significantly at (p < 0.05) according to the Newman–Keul's test.

sults are in concordance with the results found by the two previous authors. Therefore, landrace Arabi Abiad was significantly more responsive than the other two genotypes (WI2291 and Banteng). Arabi Abiad present a high susceptibility in the diseased control and had lower yield than the other spring barley (WI2291), meaning a potential for improving its resistance after rhizobacterial treatment. Subsequently, there was in general a stability of induced resistance and yield. This result suggest that, either the plant use resources diverted from growth to defense, or they possess sufficient resource to assist both of them (Córdova-Campos et al., 2012). Our experiments were conducted under similar conditions, where rainfall during the two growing seasons ranged between from 400 and 450 mm, and the same design. This could suggest the hypothesis of balance between plant defense and growth.

Conclusion

The present study showed from one side that the rhizobacterial strain *P. putida* BTP1 could not inhibit *in vitro C. sativus* growth. This result is in concordance with those obtained by Ongena et al. (1999) on *P. putida* BTP1. On the other side, barley seeds were soaked with one of the rhizobacterial strain before sowing, and the plants were inoculated with *C. sativus* Pt4 at growth stage (GS12) (Zadoks et al., 1974), i.e. There was no contact between the pathogene and the this strain. All barley bacterial strains have reduced the spot blotch caused by *C. sativus,* with effect more clear when *P. putida* BTP1 was used. Recent studies showed that *P. putida* BTP1 induced systemic resistance in tomato against *Botrytis cinerea*. This stimulation was related to induction of the lipoxygenase (LOX) pathway (Adam et al., 2008; Mariutto et al., 2014). In a future work, we will focus on identifying and quantifying compound fundamental for the ISR activity of the four rhizobacterial strains used in this study.

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