Bioefficacy of microbial antagonists against Zymoseptoria tritici on wheat

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Abstract. From our previous research of bio-control agents of the wheat pathogen Zymoseptoria tritici as an alternative to chemical control, one strain of Bacillus amyloliquefaciens and one strain of Trichoderma harzianum showed high antagonistic potential in vitro and in vivo as a foliar treatment on potted plants under greenhouse. The present work aimed to evaluate the antagonistic potential of these two strains of microorganisms in seed treatment. The results obtained showed that the two antagonists can reduce the severity of the disease assessed at three growth stages of the two wheat cultivars. B. amyloliquefaciens I3 reduced the severity of septoria leaf blotch by 56% and 58% compared to the checks on Aguilal and Karim respectively at the flag leaf stage, while in the case of T. harzianum A, this rate was 54% on Aguilal and 55% on Karim. These results suggest that the antagonistic potential is due to a distant mechanism of action such as induced systemic resistance. The viability tests of the two antagonists on coated seeds stored at 4 °C showed that they were viable after twelve months of conservation and preserved their antagonistic potential against Z. tritici.

1. Introduction

Septoria leaf blotch, caused by Zymoseptoria tritici (teleomorph: Mycosphaerella graminicola), is one of the most important foliar diseases of wheat worldwide, frequently causing severe yield reductions. Fungicides, including the triazole and strobilurin groups, are commonly used to control this disease. The risk of developing resistant strains to these fungicides and the potential danger of pesticide use on the health and the environment encourages the research of alternatives, including biological control using antagonists. Among these agents, there are species of non-phytopathogenic rhizobacteria of the genus Bacillus spp. such as B. amyloliquefaciens, B. licheniformis and B. subtilis. These bacteria colonize the roots of plants and produce lipopeptide molecules such as lipopeptide molecules (surfactins, iturins, and fengycins) that induce resistance in plants and increase their defensive capacities against subsequent infections through the phenomenon of induced systemic resistance [1]. In addition, these rhizobacteria could have strong direct antibacterial and antifungal activity [2,3,4]. Also, several strains of the filamentous fungi of the Trichoderma spp. have been used to control some telluric and aerial plant pathogenic fungi [5]. The biocontrol activity of these Trichoderma spp. Strains have been attributed to several mechanisms of action that act synergistically: competition for nutrients and space, antibiosis,
mycoparasitism through the production of specific cell wall degrading enzymes such as chitinases and proteases [6], induction of systemic resistance [7], and stimulation of plant growth [8].

In this study, the objective was to develop a method for coating wheat seeds with *T. harzianum* A and *B. amyloliquefaciens* I3, which have shown a high antagonistic potential *in vitro* and *in vivo* on potted plants against *Z. tritici*, and to study the efficiency of this mode of treatment on the control of this disease.

2. Methodology

2.1. Selection of antagonist and *Zymoseptoria tritici* strains

Based on our previous work [9], two efficient antagonist strains, *Bacillus amyloliquefaciens* I3 and *Trichoderma harzianum* A and two virulent *Z. tritici* strains (G1-1 and A5-1) were selected for this study. The G1-1 strain isolated from soft wheat was used for inoculation of the cultivar Aguilal (soft wheat), while the cultivar Karim (durum wheat) was inoculated with the A5-1 strain isolated from durum wheat as both varieties show specificity to *Z. tritici* strains [9].

2.2. Wheat cultivar selection and preparation of plants

The cultivars Aguilal and Karim were selected following septoria susceptibility tests conducted on a collection of cultivars used in Morocco. The sowing was performed in 9.5 x 10.5 cm pots with 5 seeds per pot and 5 pots (replicates) per treatment. The substrate used was composed of 1/3 sterile perlite and 2/3 peat. The pots were placed in the greenhouse until the 21 ZGS (main shoot and one tiller), 30 ZGS (beginning of stem elongation), and 39 ZGS (flag leaf) stage according to the Zadoks scale [10].

2.3. Preparation of antagonists inocula

The inoculum of *T. harzianum* A was prepared from cultures prepared on PDA (Potato Dextrose Agar) medium and incubated for 7 days at 25 °C in the dark. *B. amyloliquefaciens* I3 was cultured on Luria Bertani agar medium [11] and incubated 72 h at 27 °C in the dark.

2.4. Coating seeds with the antagonists

The biomass of each antagonist was mixed with a nutrient solution prepared with 12% gelatin, 0.1% sucrose, 1.5% cellulose, and 10% clay (kaolinite and montmorillonite). The volume of the suspension used is 6 ml for 50 g of seeds. The suspension was adjusted to 10^8 cfu/seed. The coating is performed in a liquid seed treatment machine HEGE 11 which allows having a homogeneous pulverization of the suspension on the wheat seeds. Then, the clay was added in dry powder form, and the seeds were dried for 48 hours at ambient temperature and stored at 4 °C. For the control, the seeds were coated with the same nutrient suspension but without an antagonist.

2.5. Production of septoria inoculum and plant inoculation

Production of *Z. tritici* inoculum was performed on PDA medium. The plates were incubated at 19°C in the dark for 7 days. The dishes were scraped after the addition of 3 ml of sterile distilled water. The suspension was filtered and diluted to adjust the concentration to 10^6 pycnidiospores/ml using mallassez cell, followed by Tween 20 at 0.02% of the total suspension volume. Plants were inoculated extensively by foliar spraying of the *Z. tritici* suspension. The plants were placed under controlled conditions (photoperiod 12h/12h and relative humidity from 95% to 100%) for three days; then, they were placed under greenhouse at 95% relative humidity. This trial was repeated three times.

2.6. Evaluation of disease severity

Three weeks post-inoculation, the severity of the disease was assessed on the leaves of two randomly selected plants per pot using the Ziv and Eyal key [12], which is based on the size of the area of pycnidia-containing lesions relative to the total leaf area, expressed as a percentage. The following formula estimated the percentage of septoria severity reduction by antagonists:
Percentage of severity reduction = \((S1 - S2) / S1\) \times 100 \quad (1)

S1: disease severity on leaves of control plants inoculated with septoria and not treated with antagonists.
S2: disease severity on leaves of plants treated with antagonists and inoculated with septoria.

2.7. Statistical analysis

The severity values expressed as percentages were transformed into decimal logarithms. The data were analyzed with the SPSS software version 21. The factors studied were: the treatments (B. amyloliquefaciens I3, T. harzianum A, and negative control) and vegetative stages (21 ZGS, 30 ZGS, and 39 ZGS [10]). The data of this experiment were analyzed using the two-factor analysis of the variance model. The comparison of means was performed with the Duncan test.

3. Results and discussion

3.1. Adhesion of the product on treated seeds

The coating technique adopted was selected following several comparison trials involving the nature and relative quantities of each constituent (12% gelatin, 0.1% sucrose, 1.5% cellulose, and 10% clay) of the medium used as a substrate for the preparation of the antagonist suspensions used for the seed treatment. This formulation allowed the best adhesion of the product on the seeds after treatment.

3.2. Effect of seed coating with antagonists on the reduction of septoria severity

From the results obtained on the soft wheat cultivars "Aguilal" and "Karim" of durum wheat, it appeared that both B. amyloliquefaciens I3 and T. harzianum A reduced the severity of septoria. Statistical analysis showed a very highly significant difference between treatments (B. amyloliquefaciens I3, T. harzianum A, and the control) on both wheat cultivars and between vegetative stages on the cultivar Karim. At the same time, there was no significant difference between vegetative stages on the cultivar Aguilal (Figures 1, 2, and 3).

Results of the main shoot and one tiller stage showed that B. amyloliquefaciens I3 reduced symptoms by 47% on the cultivar Aguilal and 53% on the cultivar Karim compared to the controls, while the rate of symptom reduction by T. harzianum A was 43% on Aguilal and 45% on Karim (Figure 1). At the beginning of the stem elongation stage, the reduction of septoria severity was 55% on Aguilal and 58% on Karim in the case of B. amyloliquefaciens I3 treatment; for T. harzianum A, the reduction rates were 54% and 52% on Aguilal and Karim respectively (Figure 2). Regarding the flag leaf stage, the reduction rates of septoria severity by B. amyloliquefaciens I3 were respectively around 56% and 58% on Aguilal and Karim, while in the case of T. harzianum A treatment, the rates did not exceed 54% on Aguilal and 55% on Karim (Figure 3).
Figure 1. Effect of seed coating of Aguilal and Karim cultivars by *Bacillus amyloliquefaciens* I3 and *Trichoderma harzianum A* at the 21 ZGS stage [10] in the control of *Zymoseptoria tritici* strains G1-1 (on Aguilal variety) and A5-1 (on Karim variety). Bars indicate standard errors of means. The letters indicate the homogeneous groups according to Duncan’s test at *p*=0.05.

Figure 2. Effect of seed coating of Aguilal and Karim cultivars by *Bacillus amyloliquefaciens* I3 and *Trichoderma harzianum A* at the 30 ZGS stage [10] in the control of *Zymoseptoria tritici* strains G1-1 (on Aguilal variety) and A5-1 (on Karim variety). Bars indicate standard errors of means. The letters indicate the homogeneous groups according to Duncan’s test at *p*=0.05.
Figure 3. Effect of seed coating of Aguilal and Karim cultivars by *Bacillus amyloliquefaciens* I3 and *Trichoderma harzianum* A at the 39 ZGS stage [10] in the control of Zymoseptoria tritici strains G1-1 (on Aguilal variety) and A5-1 (on Karim variety). Bars indicate standard errors of means. The letters indicate the homogeneous groups according to Duncan's test at $p=0.05$.

3.3. Effect of seed storage on the viability of antagonists

The viability tests performed on wheat seeds coated with coated *B. amyloliquefaciens* and *T. harzianum* showed that seed storage at 4°C did not affect the efficacy and viability of the antagonists after 12 months (Figure 4).

Figure 4. Viability test on seeds coated by *Bacillus amyloliquefaciens* I3 (A) and *Trichoderma harzianum* A (B) after twelve months of storage at 4°C.

The present study showed that the two antagonists used as a seed treatment to control septoria leaf blotch in wheat provided a significant reduction of *Z. tritici* symptoms on the leaves of wheat plants of the two susceptible cultivars used (Aguilal and Karim). The best levels of protection were attained with *B. amyloliquefaciens* I3 at the flag leaf stage of the Karim cultivar (up to 58% reduction in disease severity compared to the control). This suggests that *B. amyloliquefaciens* I3 and *T. harzianum* A can act as inducers of induced systemic resistance (ISR) in wheat plants by the presence of these microorganisms or their secondary metabolites in the rhizosphere. These results are in agreement with those of [13], who confirmed the activation of salicylic acid (SA) and jasmonic acid (JA) signaling pathways by a *T. harzianum* strain on cucumber plants against *Fusarium oxysporum f. sp. radicis*.
cucumerinum. Similar results were obtained by [7], who showed that T. harzianum to hydroponic cucumber culture under aseptic conditions increases chitinase and peroxidase activity, thus inducing systemic resistance in plants. Also, [14,15] demonstrated the ability of a Bacillus subtilis strain to induce systemic resistance in the pathosystems Pythium ultimum/beans, Botrytis cinerea/apples, Colletotrichum lagenarium/cucumbers, and Pythium aphanidermatum/tomato. Similar results were obtained by [1,16,17], who showed that the overproduction of mycosubtilin by B. subtilis significantly increases the protective effect of the strain against the disease caused by Pythium aphanidermatum.

Also, showed that foliar application of cyclic lypopeptides (fengycins, surfactins, and mycosubtilins) extracted from Bacillus subtilis strain BBG131 on a susceptible wheat cultivar ''Alixan'' protected the plants up to 35% against Z. tritici. More recently, [18] reported that treatment of wheat cultivar "Avatar" with surfactin extracted from B. amyloliquefaciens S499 stimulates SA and JA pathways on wheat plants confirmed by the expression of PR5 and LOX2 genes. The results obtained also show a progression in the level of protection with the age of the plants, which can be explained by the multiplication and adaptation of antagonists at the rhizosphere of the wheat plants. However, the medium used in the treatment is a combination of sugars as a nutrient source, gelatin to ensure the attachment of conidia and bacterial cells, and clay (kaolinite and montmorillonite) as adsorbent; this enhanced the adhesion of the antagonists after the coating and also the colonization and maintenance of these microorganisms in the rhizosphere after sowing. Thus, the coating of seeds by antagonists by antagonists appears to be an up-and-coming method to control septoria leaf blotch, mainly as the two antagonists survived more than twelve months at 4°C.

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