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In vitro Activity of Antagonistic Fungi towards *Septoria* spp

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ABSTRACT

Being aware of the importance of wheat in the Moroccan economy, its protection against biotic stress mainly wheat septoria is essential asrespect of the environment. Thus, biological control is one of the most interesting long term strategies. In this frame, an in vitro study of antagonistic effects in direct confrontation was conducted between 5 antagonistic fungi and 5 isolates of Septoria spp. After incubation on PDA medium at 22°C, tests revealed that all antagonists could inhibit mycelia growth of these pathogenic isolates. Diametrical growth was reduced with percentages up to 84.21%, 52.43% and 37.36% respectively for antagonists from Trichoderma genus, for Talaromyces flavus and for antagonists from Acremonium genus. Similar results were obtained while performing remote testing confrontation but with smaller reduction percentages.

Keywords: Wheat; *Septoria tritici*; *Septoria nodorum*; Antagonists; Biological control; Mycelia growth

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INTRODUCTION

Cereals can be described as the first speculation in Morocco. The special rank attributed to this kind of crops is revealed by the importance of areas that are covered by cereals. These areas have reached 4.7 million of ha during 2009/2010 agricultural season, which is equivalent to 70% of cultivated areas by year [1]. However, climatic conditions, presence of susceptible varieties combined to agricultural practices characterized by high density of culture and excessive nitrogen input lead to apparition of severe epidemics of septoria [2] that cause huge yield losses reaching 35-40% [3]. In order to fight against this disease, several control methods have been tested but only chemical control is considered as the most effective means [4]. Although use of chemicals lead to good results, it has adverse effects on the environment such as residues accumulation, soil pollution, ecological imbalance and resistance induction in pathogens [5]. Thus, other means need to be found. They should involve rationalization of agricultural practices, development of septoria-resistant varieties [6-8] and development of biological methods of control. On this basis, our work's aim is to evaluate *in vitro* antagonistic ability of 5 fungi against 5 isolates of *Septoria spp*.

EXPERIMENTAL SECTION

Biological Material

Pathogenic agents

This study was conducted with 3 isolates of *S. tritici* and *2* isolates of *S. nodorum*from 3 cereal areas in Morocco (Gharb, Doukkala and Zaër). These isolates had been purified and multiplied on PDA medium (Table 1).

Table 1: Origins and characteristics of isolates

Isolates (designations)	Origins	Crops	Species
E1	Gharb	Soft wheat	*Septoria tritici*
I29	Zaër	Soft wheat	*Septoria tritici*
D1	Doukkala	Soft wheat	*Septoria tritici*
A8	Gharb	Durumwheat	*Septoria nodorm*
A25	Zaër	Durum wheat	*Septoria nodorum*

Antagonistic agents

Five antagonistic fungi from the collection of the laboratory of phytopathology at the National Institute of Agronomic Research (INRA) Rabat had been used. They are:

Trichoderma viride (TV), Trichoderma harzianum (TH), Acremonium roseum (AR), Acremonium terricola (AT) and Talaromyces flavus (TF).

Methodology of Work

Assessment of antagonistic phenomena

In vitro antagonistic activity of the 5 antagonistic fungi towards *S. tritici and S. nodorum* had been studied according to 2 methods:

Direct confrontation: *In vitro* confrontations were performed according to Patel and Brown's method [9]. This method is also called << opposite growings technique >>. It consists in putting in 90 mm diameter Petri dishes that contain 15 ml of PDA, two 6 mm diameter explants, one carrying the antagonistic fungus to test and the another one carrying the pathogenic strain. They are placed along a diametrical axis, with 4 cm between them and equidistant from the center of the box. Controls containing only pathogenic fungi are included

Remote confrontation: Our protocol is based on the technique used by [10] with slight modifications. It consists in transplanting antagonistic and pathogenic strains in two separate dishes.

Control is formed by superposition of two boxes; the top one contains an explant of the pathogenic agent, while the lower contains only PDA medium.

Evaluation of the antagonistic effect

To assess the antagonistic effect, we used the technique indicated by [11]. It consists in measuring diametric growth according to 2 perpendicular axes: intersection point of these axes is the center of the explant which had been deposited on the Petri dish. Measures of mycelia growth are daily reported and the test ends when one of the strains covers the entire box.

Results Expression

Results are assessed by the reduction percentage of Septoria spp diametric growth. This percentage is calculated with the following formula:

$$\mathbf{RPDG} = \left(\frac{\emptyset T - \emptyset E}{\emptyset T}\right) \times \mathbf{100}$$

With:

RPDG: Reduction Percentage of Diametric Growth

$\emptyset T$:Average diameter of the control in mm.

$\emptyset E$: Average diameter of pathogenic strain facing the antagonistic strain in mm.

Statistical Analysis

Analysis of variance with three factors was performed (ANOVA3/factors: antagonist, isolate and confrontation) as well as multiple means comparison tests of SNK, Scheffe and Dunnett. These analyses were performed using statistical software packages SPSS 20 and SAS.

RESULTS AND DISCUSSION

1. *In vitro* antagonistic activity
2. Descriptive analysis
3. Direct confrontation

Figure 1 shows responses of *Septoria tritici* isolate (E1) against several antagonistic strains in direct confrontation after one week of incubation.

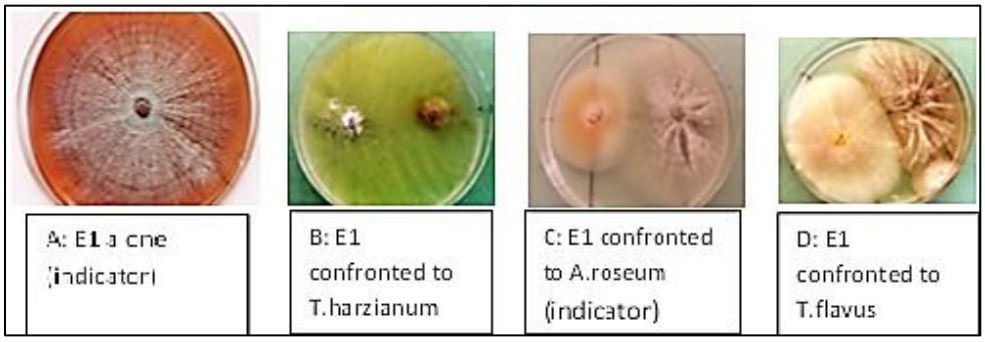

Figure 1: Direct confrontation test between E1 (*Septoria tritici*) and antagonists on PDA after 7 days of incubation. A: E1 alone (Control); B: E1 confronted with*T. harzianum*; C: E1 confronted with*A.roseum*; D: E1 confronted with *T.flavus*

Within 4 days of incubation, antagonistic strains from *Trichodermas* genus have a faster growth compared to *Septoria* isolates. After 96 hours of incubation, *Trichoderma harzianum* comes into direct contact with pathogenic colonies causing therefore growth arrest for all isolates tested. For *Trichoderma viride*, direct confrontation tests against *Septoria* isolates led to the appearance of an inhibition zone followed by growth inhibition.

Figure 2: Direct confrontation between A8 isolate (*Septoria nodorum*) and *Trichoderma*. A: *Trichoderma harzianum* mycelium covering A8 isolate; B: Appearance of a zone of inhibition resulting from direct confrontation between *Trichoderma viride* and A8 isolate

After 7 days of incubation, colonies of *T. harzianum* and *T. viride* cover pathogenic colonies on which they sporulate revealing their mycoparasiticpower (Figure 2). *Talaromyces flavus* and isolates of *Septoria* spp. grow at the same rhythm. Both antagonistic and pathogenic strains stopped their mycelia growth without any contact leading to the appearance of an inhibition zone without overlapping or sporulation on pathogenic colonies after 7 days of incubation. Antagonists from *Acremonium* genus grow slowly compared to the other antagonists and *Septoria* isolates.

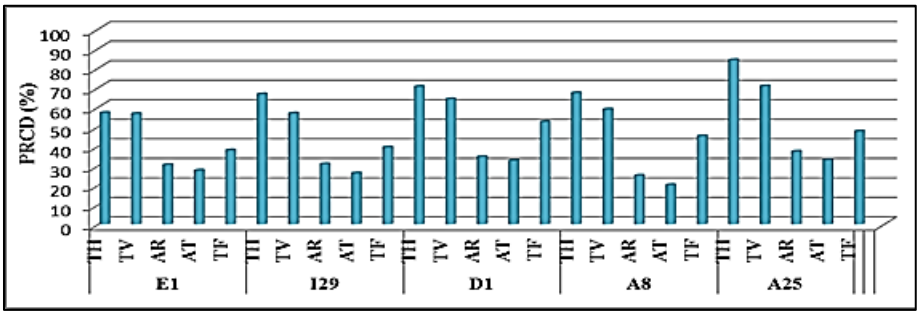

Figure 3: Effect of antagonists on mycelia growth of 5 septoria isolates in direct confrontation

Figure 3 shows that *Trichoderma harzianum and Trichoderma viridehave* a strong inhibitory activity with a RPDG greater than 50% whatever the pathogenic isolate is Strain A25 is the most sensitive with reduction percentages of mycelia growth equal to 84.21% and 70.87% when confronted with *Trichoderma harzianum* and *Trichoderma viride* respectively. Antagonists from Acremonium genus have led to less inhibitory activity with a RPDG varying

from 20.21 to 37.35% forisolates A8 and A25. RPDG are slightly more important for Acremonium roseum compared to *Acremonium terricola*. While for *Talaromyces flavus*, reduction percentage of mycelia growth doesn't exceed 50% just for isolate D1.

Remote Confrontation

Conversely to direct confrontation tests, results of remote confrontations don't show an inhibition of mycelia growth but this test led to a growth in a slow motion comparatively to the control. This effect was exerted by antagonists towards pathogenic strains. Figure 4 shows that antagonist's fromTrichoderma genius causes a reduction percentage of mycelia growth varying from 30.33 to 44.33% towards all septoria isolates. In contrast, Acremonium antagonists showed the lowest percentages of reduction in mycelia growth compared to the 3 other antagonists.

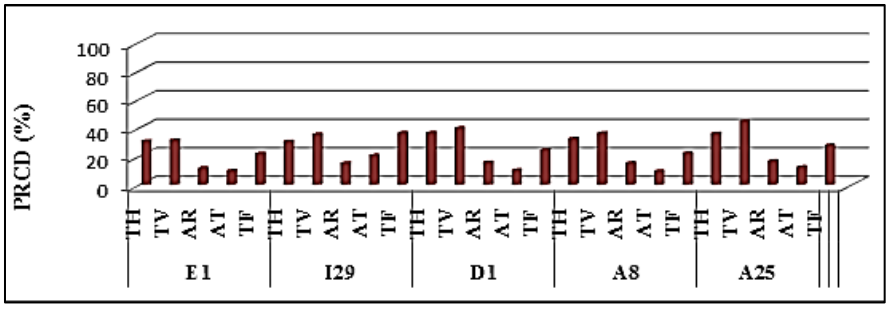

Figure 4: Effect of antagonists on mycelia growth of septoria's isolates in indirect confrontation test

In general, effect of volatile substances emitted by antagonists is relatively low. However, Trichoderma viride, shows more effectiveness with highest RPDG no matter the pathogenic isolate.

Statistical Tests

Variance analysis showed that factors: antagonists, confrontation and isolate have a significant effect at 5% (sig <0.05) on Septoria spp mycelia growth (Table 2).

Direct Confrontation

Dunett's test (Table 3) reveals that mycelia growths for the 5 isolates confronted with different antagonists are significantly different from controls (significance<0.05).

Table 2: Results of ANOVA 3 concerning effect of antagonists, isolate and confrontation on Septoria spp mycelia growth

Source	Sum of type 3 squares III	ddl	Square means	D	Sig
Adjusted model	9,632[a]	59	0.163	6,31,92,418	0
Ordinate at origin	36,263	1	36,263	14,03,73,05,944	0
Confrontation	0.133	1	0.133	5,12,97,755	0
Isolate	0.036	4	0.009	34,91,853	0
Antagonist	7,342	5	1,468	56,84,27,281	0
Confrontation * Isolate	0.111	4	0.028	1,07,27,620	0
Isolate * Antagonist	0.075	20	0.004	14,56,338	0
Confrontation * Antagonist	1,850	5	0.37	14,32,28,434	0
Confrontation * Isolat * Antagonist	0.085	20	0.004	16,38,583	0
Error	0	120	2.58E-03		
Total	45,895	180			
Corrected total	9,632	179			

Table 3: Dunett's multiple comparison test for average reduction of Septoria spp mycelia growth caused by the tested antagonists for each pathogenic isolate

Tested isolates	(I) Treatment		Mean differences (I-J)	Standard error	Signification	Confidence interval at 95%	
						Lower limit	Upper limit
E1	TH	Control (E1)	0.8573128*	0.0018021	0	0.852085	0.86254
	TV	Control (E1)	0.8528359*	0.0018021	0	0.847608	0.85806
	AR	Control (E1)	0.5850824*	0.0018021	0	0.579854	0.59031
	AT	Control (E1)	0.5538754*	0.0018021	0	0.548647	0.5591
	TF	Control(E1)	0.6642133*	0.0018021	0	0.658985	0.66944
I29	TH	Control (I29)	0.956000*	0.001217	0	0.95247	0.95953
	TV	Control (I29)	0.856000*	0.001217	0	0.85247	0.85953
	AR	Control (I29)	0.590000*	0.001217	0	0.58647	0.59353
	AT	Control (I29)	0.538000*	0.001217	0	0.53447	0.54153
	TF	Control (I29)	0.679667*	0.001217	0	0.67614	0.6832
D1	TH	Control (D1)	0.997667*	0.004023	0	0.98599	1.00934
	TV	Control (D1)	0.929000*	0.004023	0	0.91733	0.94067
	AR	Control (D1)	0.794000*	0.004023	0	0.78233	0.80567
	AT	Control (D1)	0.607667*	0.004023	0	0.59599	0.61934
	TF	Control (D1)	0.810000*	0.004023	0	0.79833	0.82167
A8	TH	Control (A8)	0.963000*	0.001599	0	0.95836	0.96764
	TV	Control (A8)	0.875333*	0.001599	0	0.8707	0.87997
	AR	Control (A8)	0.524333*	0.001599	0	0.5197	0.52897
	AT	Control(A8)	0.466333*	0.001599	0	0.4617	0.47097
	TF	Control (A8)	0.738000*	0.001599	0	0.73336	0.74264
A25	TH	Control (A25)	1.162000*	0.001915	0	1.15644	1.16756
	TV	Control (A25)	1.001000*	0.001915	0	0.99544	1.00656
	AR	Control(A25)	0.657667*	0.001915	0	0.65211	0.66322
	AT	Control (A25)	0.611667*	0.001915	0	0.60611	0.61722
	TF	Control (A25)	0.764333*	0.001915	0	0.75878	0.76989

According to SNK's test (Table 4), mycelia growth for the 5 isolates confronted with antagonists form four distinct subgroups:

- Subgroup I: Formed by the control;
- Subgroup II: Formed by antagonistsfrom Acremonium genus (AR and AT), mycelia growth of the isolates was slightly inhibited;
- Subgroup III: Formed by antagonist Talaromyces flavus (TF) and
- Subgroup IV: Formed by antagonists from Trichoderma genus (TH and TV) that induce a very important inhibition of mycelia growth compared to that generated by the other antagonists tested.

This can be explained by genetic diversity and origins of Septoria's isolates; 3 isolates from Septoria tritici and 2 from Septoria nodorum.

Table 4: SNK and Scheffe multiple comparison testsfor averages of Septoria spp mycelia growth reduction caused by the tested antagonists

	Antagonist	N	Subgroups for alpha=0.05			
			1	2	3	4
Student-Newman-Keuls	Control	15	0			
	AT	15		0.5555		
	AR	15		0.6302		
	TF	15			0.7313	
	TV	15				0.9028
	TH	15				0.9872
	Significance		1	0.139	1	0.062
Scheffe	Control	15	0			
	AT	15		0.5555		
	AR	15		0.6302		
	TF	15			0.7313	
	TV	15				0.9028
	TH	15				0.9872
	Significance		1	0.139	1	0.062

Remote confrontation

Dunnett's test (Table 3) reveals that all treatments performed by antagonists on Septoria mycelia growth are significantly (significance<0.05) different from the control.

According to SNK's test (Tables 4-6) mycelia growths for each isolate facing each antagonist form six distinct homogeneous subgroups.

Table 5: Dunnett's multiple comparison tests for inhibition averages of Septoria spp mycelia growth caused by antagonists (remote confrontation)

Tested isolates	(I) antagonist		Mean Differences (I-J)	Standard error	Significance	Confidence Interval at 95%	
						Lower limit	Upper limit
E1	TH	Control(E1)	0.586667*	0.001155	0	0.58332	0.59002
	TV	Control (E1)	0.593000*	0.001155	0	0.58965	0.59635
	AR	Control (E1)	0.350333*	0.001155	0	0.34698	0.35368
	AT	Control (E1)	0.318000*	0.001155	0	0.31465	0.32135
	TF	Control (E1)	0.636667*	0.001155	0	0.63332	0.64002
I29	TH	Control (I29)	0.583000*	0.001036	0	0.57999	0.58601
	TV	Control (I29)	0.637000*	0.001036	0	0.63399	0.64001
	AR	Control (I29)	0.399333*	0.001036	0	0.39633	0.40234
	AT	Control (I29)	0.340000*	0.001036	0	0.33699	0.34301
	TF	Control (I29)	0.470667*	0.001036	0	0.46766	0.47367
D1	TH	Control (D1)	0.648000*	0.001232	0	0.64442	0.65158
	TV	Control (D1)	0.682333*	0.001232	0	0.67876	0.68591
	AR	Control (D1)	0.407000*	0.001232	0	0.40342	0.41058
	AT	Control (D1)	0.325333*	0.001232	0	0.32176	0.32891
	TF	Control (D1)	0.516000*	0.001232	0	0.51242	0.51958
A8	TH	Control (A8)	0.605000*	0.000962	0	0.60221	0.60779
	TV	Control (A8)	0.646000*	0.000962	0	0.64321	0.64879
	AR	Control (A8)	0.400667*	0.000962	0	0.39787	0.40346
	AT	Control (A8)	0.314000*	0.000962	0	0.31121	0.31679
	TF	Control (A8)	0.491000*	0.000962	0	0.48821	0.49379
A25	TH	Control (A25)	0.642333*	0.000882	0	0.63977	0.64489
	TV	Control (A25)	0.728667*	0.000882	0	0.72611	0.73123
	AR	Control (A25)	0.421000*	0.000882	0	0.41844	0.42356
	AT	Control (A25)	0.360333*	0.000882	0	0.35777	0.36289
	TF	Control (A25)	0.549000*	0.000882	0	0.54644	0.55156

Table 6: SNK's multiple comparisons test for averages inhibition of Septoria spp mycelia growth caused by tested antagonists (remote confrontation)

	Antagonist	N	Subgroups for alpha = 0.05					
			1	2	3	4	5	6
Student-Newman-Keuls	Control	15	0					
	AT	15		0.33153				
	AR	15			0.39567			
	TF	15				0.53267		
	TH	15					0.613	
	TV	15						0.6574
	Signification		1	1	1	1	1	1

DISCUSSION

The use of specific microorganisms that interfere with pathogenic agents is an ecological and nature-respectful approach to overcome problems caused by conventional chemical methods to protect plants. Scientific research has repeatedly shown that various fungal microorganisms can act as natural antagonists of different plants pathogenic agents [12]. In this work, the potential of 5 antagonistic fungi was evaluated *in vitro* against 5 isolates of Septoria spp. Results showed that antagonists Trichoderma grow faster than pathogenic isolates in direct confrontation. An inhibitory effect on mycelia growth over than 50% towards all tested pathogenic isolates was revealed. These results are coherent with several studies that have shown effectiveness of the antagonists *Trichoderma* against many plant mycoses [13-15]. After 96 hours of incubation, a growth arrest for all isolates tested was caused by *Trichoderma harzianum*that come into direct contact with pathogeniccolonies. Then, *Trichoderma harzianum* covers the colonies of pathogens and it sporulates on these colonies. Invasion of pathogen's mycelium by *T. harzianum* was also

observed with [16] by direct confrontation test on culture medium between this antagonist and a soil fungus, *Pythium ultimum* after 4 days of incubation [17] described the parasitic action of *T. harzianum* on *Rhizoctonia solani* and *Sclerotium rolfsii* which attacks its host by wrapping its mycelium around the host's hyphaes. Eventually, the mycoparasite enters host cells and consumes cytoplasm content. According to [18], this interpenetration promotes the action of enzymes such as chitinase ß1-3 glucanase that lead to lysis of parasite's mycelium. Direct confrontation tests between pathogenic isolates and *Trichoderma viride* were characterized by the appearance of an inhibition zone followed by an inhibition of mycelia growth. This can be explained by other studies that have shown that, before interaction between fungi mycelia, *Trichoderma viride* produces small amounts of extracellular exochitinases [19]. The remote inhibitory activity of 5 antagonists towards the five Septoria spp isolates was noted for all antagonists with a decreased diameter of pathogenic colonies. *Trichoderma viride* was the most efficient antagonist followed by *Trichoderma harzianum*, *Talaromyces flavus* and finally *Acremonium* species towards the 5 pathogenic isolates [20] were able to isolate an antibiotic from *T. viride* (Trichodermin), these authors do not exclude the possibility of production in this species, other antibiotics such as the gliotoxin and the viridin discovered previously by other researchers. The results of experiments performed by [21] *in vitro* have shown that *T. flavus* produce hydrogen peroxide and acts by antibiosis to control *Verticillium dahliae*.

CONCLUSION

Biological fight against wheat's septoria by antagonistic fungi has led to satisfactory results in order to control the disease. The tested antagonistic microorganisms have proved effectiveness *in vitro* and *in vivo.*
Trichoderma species were more efficient than *Talaromyces flavus* and *Acremonium* in reducing mycelia growth of pathogenic isolates. This difference in sensitivity of different isolates of *Septoria spp*. towards antagonists suggests the need to look for more efficient antagonists. In the context of a more integrated approach to solve the problem, treatments with *Trichoderma* spores could be a promising way to control the disease in field and to reduce amount of fungicide residues. Finally, from this study, we can deduce that Moroccan species *Trichoderma harzianum* offers a promising material that could probably protect effectively wheat against *Septoria* caused by *Septoroa tritici*.

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