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Biological Control Against Bayoud Disease of Date Palm (*Phoenix dactylifera* L.) using Antagonistic Fungi Species: Antibios and Mycoparasitism Studies

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Abstract: ***Fusarium wilt of date palm, also known as bayoud disease, is major disease in Algeria and Morocco. In this work, we studied a biological control of Fusarium oxysporum f. sp. albedinis, the causal agent of bayoud disease by using of an antagonistic fungi species (AFS), (Aspergillus niger, Fusarium oxysporum, Trichoderma harzianum, T. aureoviride and T. longibachiatum), for biological control test by antibiosis and mycoparasitism actions. We showed the antibiosis action by the inhibition of mycelial growth and sporulation inhibitions, and the mycoparasitism action by cytological alteration of Foa mycelia. All five antagonistic fungi showed significant effect (P < 0.05), of inhibition toward mycelial growth of Foa (60-75%), and its sporulation (80-85% of the control). The mycoparasitism action observed by AFS secretions of the anti-fungal lytic enzymes, chitinase and β-1,3-Glucanase, and also by microscopic observation showed clearly this action by coagulation and alteration of Foa mycelium and appearance of large vacuoles.***

Keywords: date palm, *Fusarium oxysporum* f. sp. *albedinis*, bayoud, biocontrol, antibiosis, mycoparasitism.

1. Introduction

The Fusarium wilt of date palm (*Phoenix dactylifera* L.), commonly named bayoud, caused by the fungus *Fusarium oxysporum* f. sp. *albedinis* (Foa), is the most destructive disease of date palm. The impact of this disease is most severe in North Africa, where 10 million of palm trees were destroyed in Morocco, and 3 million in Algeria[1].This disease appeared for the first time in 1870 in the Draa valley in the south of Morocco[2].The spread of this disease in Morocco can be explained by the advancement from one oasis to another along the river valleys, for example, from Morocco, the Bayoud entered in Algeria via oases close to the borders in Moroccan frontiers like Figuig oasis, by Zouzfana and saoura valleys. From there the fungus travelled via the caravan-routes in infested wood and branch to the oases of central Algeria [3,4].

The fighting strategies against this disease are very limited. Among these strategies, the deployment of Foa-resistant cultivars appear to be the most suitable and economic approach5,6, but natural resistant genotypes are scarce with a poor quality of fruits and the date palm breeding system is laborious and offered only as a long-term plan7. Alternative control measures such as the use of Foa antagonists are, therefore, necessary and need to be explored8.

Biological control using microorganisms represents a promising disease control alternative9. This kind of control is based on the screening of plant extracts or antagonistic bacteria and fungi that can confer protection against plant pathogens [9-11]. Several biocontrol agents were identified, including species of *Trichoderma*, *Penicillium*, *Gliocladium*, *Sporidesmium*, *Burkholderia*, *Bacillus*, *Serratia*, and many others. However, their efficacy has been often inconsistent when transferred into the field. Improving the success of such transfer relies on the elucidation of the mechanism of action and of how such mechanisms are affected by the environment [12].

Biocontrol agents achieve a better disease control via a microbial antagonism, which implies a direct interaction with the causal agent of disease and/or an indirect action involving the host[13,14]. The direct effect is usually a result of an antagonism between the biocontrol agent and the pathogen due to antibiosis or nutrient competition and/or parasitism, whereas the indirect interaction results from the enhancement of the plant defense mechanisms against the invading pathogen[15-19]. Hammerschmidt and Kuc20 reported that induced resistance in plants was associated with the enhancement of the cell wall lignifications, the stimulation of host-defense enzymes and the synthesis of pathogenesis-related (PR) protein. Concerning date palm-Foa interaction, El Hassni et al.11 reported that the inoculation of date palm seedlings with a hypoaggressive isolate of *F. oxysporum* provided a degree of protection against further attacks by an aggressive isolate of Foa. Jaiti et al. [21] tested the effect of root colonization with arbuscular mycorrhizal fungi, on the induction of date palm defense reactions against its pathogen. ElHadrami et al. [22] reported that the mycorrhizal plants showed a significant accumulation of non-constitutive hydroxycinnamic acid derivatives, such as the sinapic derivative I_2, known to play a crucial role in resistance of date palm to Foa.

Concerning the three modes actions of antagonistic fungi (antibiosis, mycoparasitism and competition), on Foa, there is no study at know confirm this action. The mycoparasitism for example is difficult to demonstrate *in situ* until very recently due the technical difficulties in making *in situ* microscopic observation (fluorescence imaging and differential staining)23. Antagonistic fungi species (AFS), can produce antifungal metabolites (Schirmböck et al.,

1994) [24] which may be competitor to fungal pathogens25,26, and also promote plant growth [27,28]. In addition, it has been demonstrated that a number of Trichoderma isolates are able to excrete hydrolytic enzymes such as chitinases and β-1,3-glucanases into the medium when grown in liquid media supplemented with laminarin or chitin29,30. Chitin and β-1,3-glucans are the main structural components of fungal cell walls[30-32]. It has been suggested that the hydrolases produced by AFS were likely involved in the degradation of fungal cells through alteration of their wall components [30].

In this case, the objectives of this work were to i) determine the inhibition effect by the *in vitro* test of five antagonistic fungi on mycelial growth and sporulation witch represents the antibiosis action, and ii) determine the mycoparasitism action by detection of accumulation of anti-fungal lytic enzymes and by microscopic observation of destroyed foa's mycelia by these antagonistic fungi species (AFS).

2. Materials and Methods

A total of twenty strains of five antagonistic fungal species were tested their efficacy of inhibition on mycelial growth of Foa for detect the antibiosis and mycoparasitism actions. These strains are collected from Saoura region of Algeria in the period September – November 2016. The test of biological control was carried out in our laboratory of phytopathology, in the period September 2016 - February 2017.

Table 1: Foa and antagonistic fungal species strains isolates with their origin

Foa isolates	Origin	Antagonistic fungal species	Strains
1	Saoura	*Aspergillus niger*	*Ag.n*
2	Saoura	*Fusarium oxysporum Trichoderma*	*F.ox*
3	Saoura	*harzianum*	*T22*
4	Touat	*Trichoderma aureoviride*	*T.aureo*
5	Touat	*Trichoderma longibachiatum*	*T. long*
6	Gourrara		
7	Gourrara		
8	Gourrara		
9	Mizab		
10	Mizab		

2.1. Foa isolates

Ten Foa isolates (Table 01), originated from Mizab, Saoura, Touat and Gourrara regions (South-West of Algeria). These isolates were grown on PDA medium (potato dextrose agar), at 22 °c for 5 days.

2.2. Antagonistic fungi species

Five antagonistic fungi species (AFS) strains were used for antagonism test, *Aspergillus niger*, *Fusarium oxysporum*, *Trichoderma harzianum*, *T. aureoviride* and *T. longibachiatum*, originated from Western Unity of Research, Algerian National Institute of Agricultural Research (INRAA-URO), Sidi Bel Abbes, Algeria.

2.3. Antibiosis action test

Antibiosis action of antagonistic fungi species is represented by the mycelia growth and sporulation inhibitions. Interactions between Foa and AFS were determinate by method of Küçük and Kivanç [33]. This method is based to calculate the growth inhibition rates (GRI%) of mycelia growth and sporulation of Fao by AFS effect. The inhibition percent was obtained using the formula:

$$\boldsymbol{GIR}\,\% = \frac{(\boldsymbol{R1}-\boldsymbol{R2})}{\boldsymbol{R2}} \times \mathbf{100}$$

Where GRI% represents growth inhibition rate, R1 means mycelia growth of Foa and R2 means mycelia growth of control [33].

2.4. Enzymatic assay (Mycoparasitism action test)

Antagonistic fungi species (AFS) were cultured at 22 °C on a synthetic medium containing (grams per litre of distilled water): glucose, 15; MgSO4 7H2O, 0.2; KH2PO4, 0.9; KCl, 0.2; NH4NO3, 1.0; Fe2+, 0.002; and Zn2+, 0.002. Flasks containing 50 ml of liquid SM were inoculated with 1 ml of a conidial suspension (106 conidia ml-1) of AFS. The glucose in the medium was substituted with one of the following carbon sources (each at 2 mg ml-1): laminarin, chitin or Foa cell wall preparation. Cultures were incubated at 28 °C in a rotary shaker. Supernatants were studied for enzymatic activities33. Foa was inoculated into 250 ml flasks with 50 ml of potato dextrose broth and incubated at 24 ± 2 °C for 6 days.

The mycelium was then collected by filtration and thoroughly washed with sterile distilled water and homogenised for 5 min at the highest speed. The mycelial suspension was centrifuged at 30000 × g at 4°C for 20 min. The pellet was resuspended in distilled water and sonicated on ice 4 times for 5 min using a sonicator (Sonics & Materials Inc., USA). The suspension was centrifuged at 800 x g at 4 °C for 10 min [34]. Chitinase (E.C. 3.2.1.14) activity was determined by following the release of GlcNAc from colloidal chitin. Specific activity was defined as units of enzyme activity per milligram of protein. The activity of β-1,3-glucanase [E.C. 3.2.1.39] was determined by following the release of free glucose using the glucose oxidase reagent. Specific activity was expressed as mmol glucose h-1 (mg protein)-1. Protein was determined by the method described by Küçük et al. (2007) [34].

2.5. Statistical analysis

All the experiments schemes were randomized complete. At least two replicates per treatment were performed and each whole experiment was repeated independently two times. The collected data were submitted to analysis of variance (ANOVA) by one factor and was performed for the data on antibiosis action was represented by AFS's inhibitions rates of mycelial growth and sporulation. The data on enzymatic activity were subjected to mycoparasitism action of AFS. The F-values were calculated at $p<0.05$. Standard errors has been calculated and marked in tables. The significance of differences among treatments was recorded at $p<0.05$ by the

experimental statement named global randomized with one studied factor (inhibition rates)33,34, and multiple comparisons of the means were conducted according to the Newman–Keuls test at $p<0.0533$. Multiple replicates of the same experiment showing no significant differences were combined within the same analysis unless stated. All data were transformed to ensure the variances homogeneity. Multiple comparisons of the means were conducted according to the Newman–Keuls test at $p<0.0533$. The StatBox software v. 6.4 (Grimmersoft, ed'04, USA) was recorded at $p<0.05$, by the experimental statement named global randomized with one studied factor (inhibition rates) [33,34].

3. Results

3.1. Antibiosis test

The mycelial growth of Foa was inhibited by all AFS isolates (Fig. 1), with zone inhibition formation. These AFS exhibited an excellent antagonistic activity against mycelia growth of Foa with GIR% value more than 70% (Table 2). The isolate T22 was identified as the strongest antagonist against Foa with significantly high of GIR% value (80%). Other four isolates of AFS exhibited GIR% value between 60 and 75% (Table 2). The ANOVA indicated significant differences at 1% level than the control among isolates for inhibition rate (Table 2).

Table 2: Mycelial growth inhibition rates (%) of *Fusarium oxysporum* f. sp. *albedinis* (Foa) on PDA medium in presence of five antagonistic fungi species (AFS) after 7 days of incubation

Foa isolates	Growth inhibition rate (GRI) (%)				
	T. harzianum (T22)	*T. longibachiatum*	*T. aureoviride*	*Aspergillus niger*	*Fusarium oxysporum*
1	75a	72a	72a	65b	65b
2	75a	73a	71a	63b	66ab
3	74a	72a	72a	64b	66ab
4	75a	71a	72a	61c	65b
5	75a	72a	73a	60c	64b
6	75a	74a	72a	65b	63b
7	73a	73a	71a	63b	63b
8	75a	73a	72a	61c	61c
9	75a	74a	71a	62bc	62bc
10	72a	73a	73a	64b	63b
Foa control	0d	0d	0d	0d	0d
GRI global (%)					
LSD	5.04*				
C. V. (%)	22.6				
Sporulation inhibition (%)	85	82	81	80	80

c.v., Coefficient of variation; a, b, c, d, Ranking of the inhibition rate of mycelial growth is presented in four groups, when the values within a column labelled by the different alphabets are significantly different at $p<0.05$; LSD, Least significant difference at $p<0.05$; *, significant effect at 5% level.

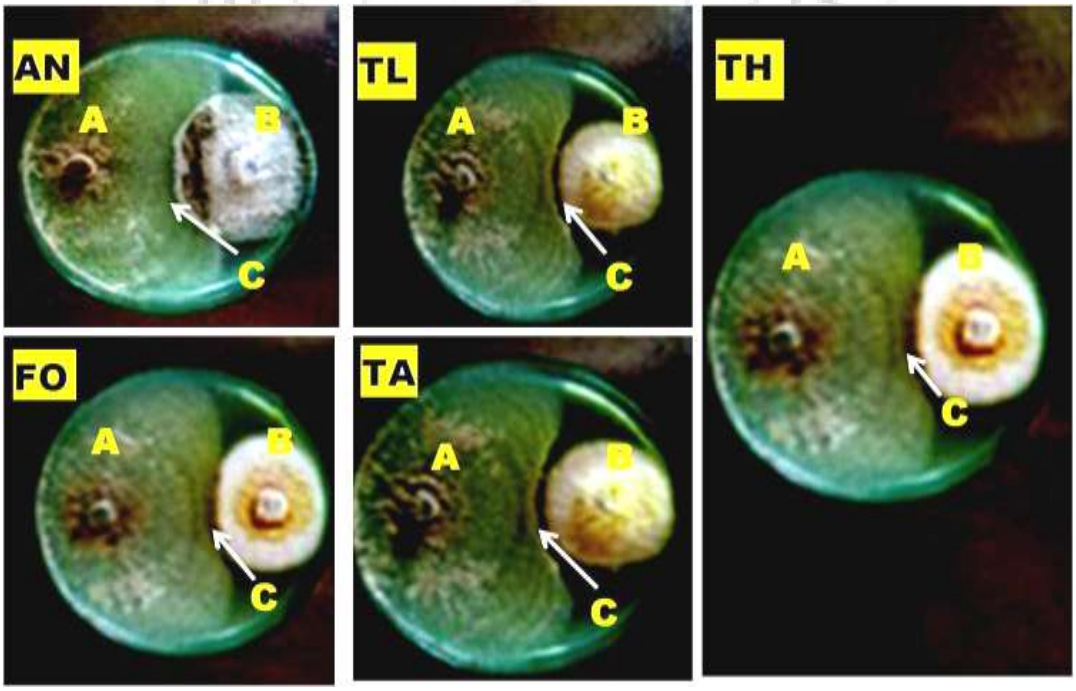

Figure 1: Anatgonistic fungi species effect on mycelial growth of Foa with inhibition zone formation. TH, Foa confrontation with T. harzianum; TA, Foa controntation with T. aureoviride; TL, Foa confrontation with T. longibachiatum; AN, Foa confrontation with Aspergillus niger; FO, Foa confrontation with soil's *Fusarium oxysporum*. A, Foa colony; B, antagonistic fungi strain colony; C, inhibition zone formation.

3.2. Mycoparasitism test

All tested isolates of AFS showed glucanase and chitinase activity when grown in presence of cell wall constituents of the pathogen. Of the lytic enzymes produced by these isolates, β-1,3-glucanase activity was much higher than chitinase activity (Table 3).

Mycoparasitism action was observed also by microscopic observations of the mycelium harvested in the periphery of Foa colonies on the side of the most effective antagonists revealed generally a coagulation of the fungal cytoplasm, characterized usually by the presence of small vesicles and very often by the appearance of large vacuoles (Fig. 2).

Table 3: Lytic enzymes activity produced by AFS isolates.

AFS strains	Chitinase accumulation (µmol GIcNAc h-1 mg × protein-1 (Cell wall of Foa)	Glucanase accumulation (µmol h-1 mg × protein -1) (Cell wall of Foa)
Aspergillus niger	4.07	64.02
Fusarium oxysporum	4.5	70.01
Trichoderma harzianum	8.02	100.1
Trichoderma aureoviride	6.01	87.9
Trichoderma longibachiatum	6.0	85.9
ANOVA		
LSD isolates 5%	0.4*	2.7*
LSD treatments5%	0.23*	2.1*
LSD interaction5%	0.76*	6.6*

*, Significant at 5% level.

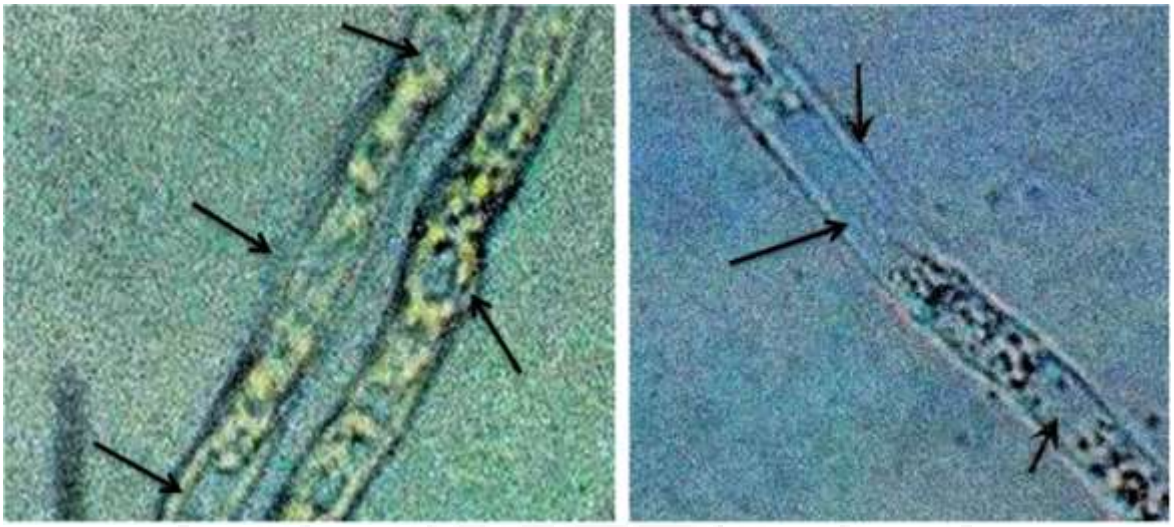

Figure 2: Microscopic observations of *Fusarium oxysporum* f. sp. *albedinis* (Foa) mycelia with vacuolization formation by mycelium presence of antagonistic fungi strains

4. Discussion

This study was the determination of the AFS efficacy against Foa by the antibiosis and mycoparasitism actions. The impact of the intensive use of chemical pesticides on the environment has resulted in the increased interest in biocontrol strategies for plant disease management[35-40]. The biological control importance based antibiosis and mycoparasitism actions has not been documented widely8, all works of biological control on induction of plant resistance, phenolic compounds and antagonistic bacteria[10-12, 29, 41, 42]. In this work, it was tested five antagonistic fungi species (AFS) strains, for their potential to control Bayoud in date palm, a disease caused by Foa.

Hydrolytic enzymes, such as chitinase and glucanase are thought to be closely related to the mycoparasitism [26,43,44,45].
The potential of AFS as biological agents of plant pathogens was first recognized against soil-borne root pathogens like *Rhizoctonia solani*, *Pythiul ultimum*, *Fusarium oxysporum*, *Fusarium solani*, *Sclerotium rolfsii* and *Cochliobolus sativus* [46,47].

The *in vitro* antibiosis tests on mycelia growth and sporulation have shown that simultaneous co-culture of these microorganisms and Foa does not stop completely its growth. This inhibition effect of Foa might be the result of antifungal compounds released by the antagonists into the culture media. In some cases of interaction with Foa antagonists, the sporulation of Foa colonies was highly reduced particularly in the border where the inhibition is stirring8,12.

The mycoparasitism potential of AFS wasn't well documented23. Hydrolytic enzymes, such as chitinase and glucanase are thought to be closely related to the mycoparasitism26,43,44,45. The secretion of hydrolytic enzymes, such as chitinase and glucanase are thought to be closely related to the mycoparasitism26,43,44,45,48. All tested isolates showed glucanase and chitinase activity when grown in presence of cell wall constituents of the pathogen (Table 2). Of the lytic enzymes produced by antagonistic fungi isolates, β-1,3-glucanase activity was much higher than chitinase activity (Table 2). Similar variations in different isolates have been observed for various lytic enzymes in *Trichoderma*32,49,50,51. This result is similar to that by reported Innocenti et al. (2003) 30, in which antagonist microorganism produced high levels of enzymes when grow on *Rhizoctonia cerealis* mycelia, and by Kuçuk et al. (2007)34 on *Ascochyta rabiei*, causal agent of ascochyta blight on chickpea (*Cicer arietinum* L.).

Further studies are conducted in order to highlight the *in planta* effect of these antagonists on the endophytic development of Foa, and to set up the ecological conditions required for their settlement within the date palm rhizosphere.

5. Conclusion

We showed in this study the presence of lytic enzymes chitinase and β-1,3-glucanase secreted by AFS for mycelia alteration of Foa and inhibition of growth by antifungal compounds inhibitors. In this study we have investigated the selection of a set of antagonistic fungi species that can act as antagonists against Foa based on their ability to inhibit more or less strongly its mycelial growth and/or sporulation, by antibiosis and mycoparasitism actions. In Algeria, the biological control against bayoud disease should provide a potential effective approach for the prevention of healthy groves (South-East of Algerian desert), and increase the levels of these AFS and their specific activities could be a selective advantage in antagonism and which is able to control the bayoud of date palm in oases is being evaluated.

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