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Confrontation of antagonistic fungi selected from the soil of the Figuig oasis to date palm Fusarium *f.sp. albidinis*

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Abstract

The date palm vascular fusarium wilt is mainly caused by a telluric fungus, *Fusarium oxysporum f.sp. albidinis*. This photogene is specific and exclusively a host. It is able to invade the entire vascular system of the plant, thus causing its obstruction, then the palms' wilt and eventually the plant's death. The pathogen's behavior in the soil is depending on its relation with the rest of the telluric microflora and its environment. Our work aims at finding selective antagonistic fungi from the soil's microflora of different plots sampled from Figuig palm grove, south east Morocco. The microflora samples have shown that these soils are rich in bacteria and fungi that can play a crucial role in the biological treatment as antagonists of this pathogen fungus. Several fungi have been isolated and purified, five of them have shown a great ability to inhibit the growth of Fusarium *oxysporum f .sp. albidinis in vitro* culture, via different mechanisms: by secreting mycotoxins and also by competing with the fungus.

Keywords: Date palm, vascular fusarium wilt, *Fusarium oxysporum f.sp albidinis*, biological treatment, antagonistic fungi.

Introduction

The challenges facing date palm cultivation are phytosanitary problems, which impede its development and its extension.

Considering the damages in the North African date palm groves, the date palm Bayoud or vascular fusarium wilt (*phœnix dactylifera*) caused by a telluric fungus, *Fusarium oxysporum f.sp. albidinis* (Killian & Maire, 1930), is the most destructive and the most threatening disease. This disease has taken an epidemic character: It has destroyed more than 10 million palm trees only in Morocco (Pereau-Leroy, 1958; Sedra 1995, 1999, 2003, 2005a). The disaster caused by the Bayoud is not restricted to the genetic erosion caused by the disappearance of numerous varieties among the best ones, but leads also to desertification and the impoverishment of the date palm farmers who end up migrating into town centers.

The first symptoms of the fusarium wilt could be detected by the wilt of the middle crown's palms which take on a whitish color (Djerbi, 1988). At the roots' level, the attack occurs mainly if there are injuries, the fungus gets then directly into the root in order to be lodged into the ligneous vessels (Djerbi, 1983).

Several methods of fight against that epidemic were considered. The use of chemical is sometimes satisfying because of its efficiency, but these synthetic products are unstable in the soil and pose the threat of favoring the selection of resistant strains (M.A.R.A, 1976) as well as spoiling the environment in the long term. The genetic and biological methods remain the most promising tracks, the first one, through the selection of varieties which would be in the same time resistant and with a good output potential, thanks to controlled crossbreeding (Sedra 1995, 1999), the second one through the selection of micro-organisms (bacteria or fungi) (Souna *et al.*, 2012), endowed with antagonist activity. The micro-organisms present in the compost are excellent candidates to that fight against the date

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palm fusarium wilt (Garbeva *et al.*, 2004; Chakroune *et al.*, 2008) and they are the subject of our study (or Close attention was directed to study highlight in details the behavior of these micro-organisms in our investigation).

Materials and methods

Fungus isolation

The samples studied were taken from various lots of Figuig's palm grove in Oriental Morocco, next to the Morocco-Algerian border (32°07'N - 01°14'W).

The samples were taken from a depth down to 8 inches. The sampling is carried out in a homogeneous way in different prospected gardens.

The density of the flora is estimated by the soil suspension and dilution method (Pochon, 1954) and by spreading on breeding grounds. Ten grams of soil are aseptically ground before being poured in suspension into 100 ml of sterile distilled water. From this parent solution, a series of dilutions to the tenth are prepared. Each dilution is repeated three times. The isolation of micro-organisms is achieved by the use of two selective medium environments: the PDA breeding ground enriched with antibiotics for fungi and the peptone based Mulller-Hilton milieu, for bacteria. After a week-long incubation in an incubator at 27°C, the AF labeled fungi are isolated, purified, bred and preserved in sterile sand at 4°C in darkness.

Pathogen isolation

The *Fusarium oxysporum f.sp. albidinis* strain used in this study was isolated from in the Bayoud infected rachis of a date palm. After isolation, purification and breeding, the pathogen is preserved in sterile sand at 4°C or in ammonium oxide, according to the Locke & Colhoun (1974) method.

Antagonistic activity

The antagonistic activity was achieved in three Petri dishes containing 10 ml of PDA (Potato Dextrose Agar) in three time repetition. The test consists in the place of two agars patches (5 mm in diameter) in the same dish, one of them bearing the antagonistic fungus, the other one the pathogen, both placed on a diametric axis and in equidistance from the center of the dish. The subculturings are made at the same time. For the control, the PDA based Petri dish is inoculated only by the pathogen. The culture is carried out by one week long incubation at 27°C and for 3 or 4 days in continuous light (Benhamou & Chet, 1996) (Figure 1). When the mycelium filaments reach the periphery of the dish in the control dish containing the F.o.a, the inhibition rate is calculated according to the following formula:

$$\% \text{ Inhibition} = (Do - Dx)/Do \times 100$$

Do = diameter of the control F.o.a

Dx = Diameter of the F.o.a with the Afx antagonist.

This technique is inspired by the opposed culture technique, recommended by Patel & Brown (1969).

Evolution of the F.o.a in the substrate pre-incubated with the antagonists

From each PDA based Petri dish containing one of the Af antagonist to be studied, three to four one-centimeter patches are taken out and put in culture into two liters of Czapek liquid milieu. After 15-days of incubation at 27°C under stirring, the conidia of this culture were separated from the mycelium filaments by decantation. The conidia suspension is counted on Malassez cells under a microscope.

The multiplication on a liquid medium and the counting of the F.o.a are carried out in the same way as for the Afx antagonists.

Survey of the effect of the antagonists on the growth of F.o.a in substrate medium

For the five antagonists who are studied, 10 two-liter pots are prepared, each one containing peat, sand and

vermiculite according to the ratio: 1v peat, 1/2v sand, 1/2v vermiculite. The substrates thus prepared are humidified by sterile distilled water in order to get a homogeneous and non-compact substrate and sterilized at 120°C for 30 minutes. They were followed by the antagonists inoculated in the ratio of 150,000 CFU/g dry substrate, three days before being inoculated with the pathogen in the ratio of 150,000 CFU/g of dry substrate. For the controls, the substrates are inoculated only by the F.o.a humidity is maintained in each pot by adding 100 ml of distilled water every third day. In each pot, 10 g of substrate are picked up by core sampling and put in suspension into 10 ml of sterile distilled water and stirred for 30 minutes. The counting of the F.o.a is assessed by the method of suspension-dilution in Petri dishes containing 20 ml of agar F.o.a selective breeding grounds (KOMADA). 1 ml of the suspension is taken out and then put in culture in a Petri dish at 27°C for 7 days. The pathogen agent was identified according to the morphological and microscopically characteristics (thin, curly or grazing mycelium) and their pigmentation (salmon pink) (Sedra, 1993). The counting of the F.o.a is expressed in numbers of colonies per gram of dry matter (UFC/g M.S).

Results

Isolation of the pathogen from infected rachis

The strain of *Fusarium oxysporum f.sp. albidinis* (F.o.a) was isolated from the rachis of a contaminated palm of the Bouffagousse cultivar from the Figuig palm grove, which is a sensitive variety, attacked by the vascular fusarium wilt. The pathogen growth on the PDA medium is about 0.3 to 0.4 cm a day. In the more than 15-day old cultures, the mycelium tends to become Cottony. The macroscopic outlook of the culture is characterized by a thin curly salmon pink mycelium carpet (Photos 1a and 1b).

Photo 1a. Isolation of the pathogen from infected rachis.

Purification of the strains from the soil of the Figuig palm grove

The soil of the Figuig palm grove contains a rich and diversified population of micro-organisms. Several colonies are obtained by culture in different dishes according to morphological characteristics such as the color, the size or the general aspect. Each colony is picked up and put in suspension into sterile water. 1 ml of each one is spread independently in Petri dishes in the PDA medium. The culture is maintained at 27°C for 10 days, then, for a week under permanent light, until confluence. The isolates (from AF1 to AF12) are preserved in sterile sand for the confrontation study (photo 2).

The colonies have a rapid growth and a fleecy aspect, of different colors. These fungi belong to the Aspergillus and Penicillium genre. The growth of these fungi is entirely made of spore chains and reaches its maximum in the Petri dishes after 10 days of

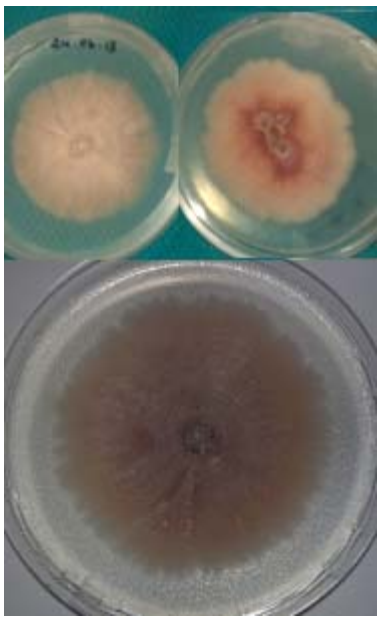


Photo 1b. Isolation of the pathogen in the KOMADA selective medium.

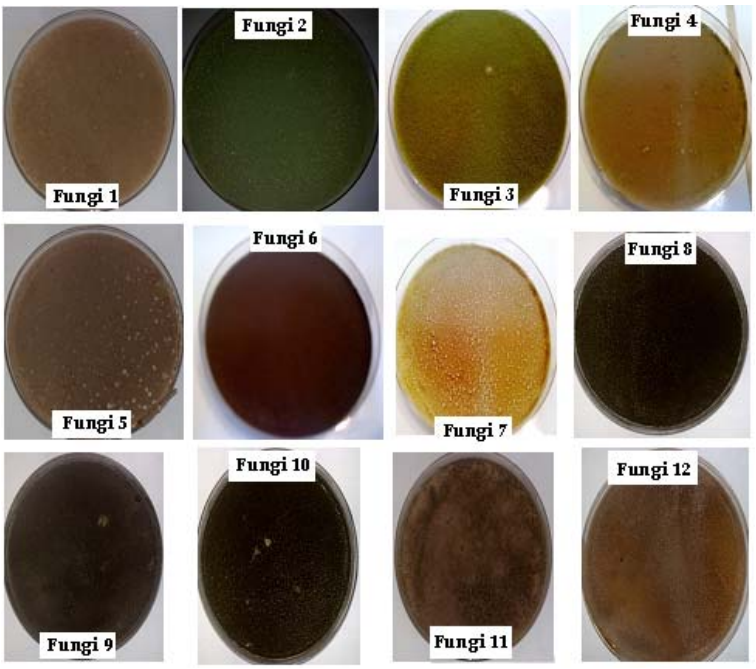


Photo 2. Purified fungi from the floor of the Figuig palm grove.

incubation. Only five fungi have displayed an antagonistic activity against *Fusarium oxysporum f.sp. albidinis* (F.o.a) on an PDA medium, the other fungi have produced a weak inhibition against the pathogen agent.

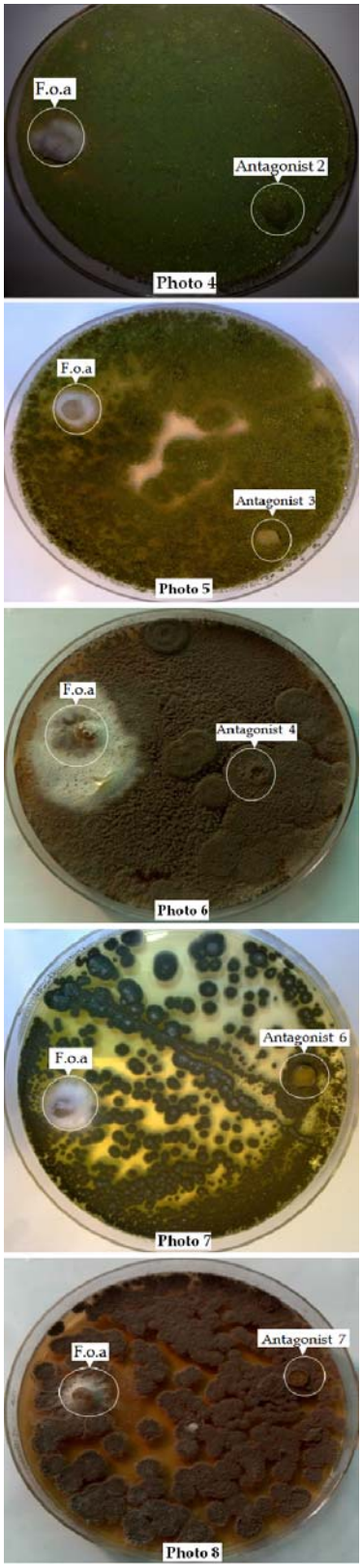
Direct confrontation test of the selected fungi on the mycelium growth of Fusarium

The mycelium growth of the pathogen in culture in the Petri dishes in PDA medium is slowed down in presence of some micro-organisms isolated in the soil of the palm grove. On the different antagonists tested, five have turned out to be efficient in inhibiting the growth of the F.o.a. The inhibition rates are varied according to the isolates and reach up to more than 80 %.

The AF3 strain turns out to be the most efficient, since it reaches an inhibition rate of 87% (Figure 2). The AF2, AF4, AF6 and AF7 antagonists have a respective inhibition capacity of 83%, 65%, 83% and 85% (photo 4-8). But the AF2, AF5 and AF13 fungi have displayed an inhibition capacity which does not exceed 20%. The other strains remain inefficient on the F.o.a (Table 1).

Evolution of the F.o.a in the substrate pre-incubated with the antagonists

In this experience, we have studied the effect of the five antagonistic fungi on the evolution of the Foa in the substrate mixture (sand/peat/vermiculite: 1/3, 1/3, 1/3).



Photos 4-8. Results of direct confrontation test of selected fungi.

A dramatic decrease of the population of Fusarium *oxysporum f.sp. albidinis* appears in the substrates in the presence of each studied antagonist (Table 2, Figure 3) is noticed. In the control, which is the substrate without any antagonist, a proliferation phase, which can last for 30 days, can be observed, followed by a very small decrease. On the contrary, in the presence of different antagonists, a decrease of the F.o.a proliferation is noticed only after a month of culture in the inoculated these substrates. That decrease is prolonged until the total or near total disappearance of the pathogen.

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Discussion

The aim of this study is to look for micro-organisms with an antagonistic activity to the date palm pathogen that is *Fusarium oxysporum f.sp. albidinis*, from the soil of the Figuig palm grove. Five strains of fungi of the Aspergillus and Penicillium genres, once isolated and purified, have displayed an inhibiting activity of the F.o.a in culture in a PDA medium. These selected fungi were assessed to display an inhibiting rate of the mycelium growth higher than 65%, with a highly significant rate for the AF3 and AF7 antagonists: respectively 87% and 86%; the smallest reduction being noticed with AF2.

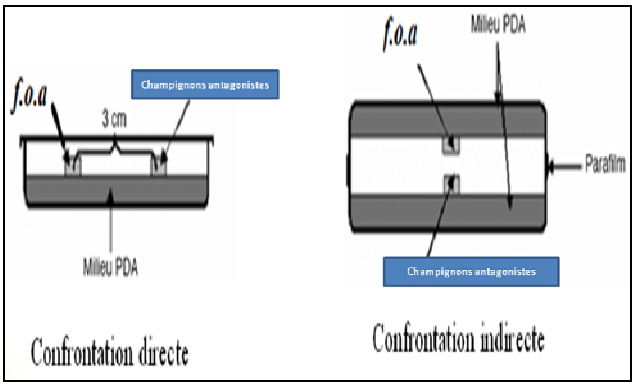

Figure 1. Testing direct confrontation and indirect.

Amik *et al.* (1996) have isolated soil micro-organisms with varied antagonistic levels to the phytopathogen isolates.

Similar results were obtained by Rouxel (1976) and Hjeljord *et al.* (2001), who have evidenced an inhibition of the attacks from pathogens of the cultivation of strawberries and melons. *Fusarium oxysporum f.sp. albidinis* is a telluric fungus able of maintaining itself in the soil for long periods of time and also in palm debris, thanks to its chlamydospores which have thick and resistant membranes. It is important to notice that the F.o.a's saprophyte phase can unfold itself in the soil while being able to infect the plant. Following the selection of these strains which induced an inhibition of the growth of fusarium on breeding grounds, we have tried in our further experiences to explain through which way that inhibition took place.

The mixing of the fusarium and the antagonists in a sterile substrate, composed of a mixture of 2/3 peat and vermiculite and 1/3 of soil, enabled us to follow the growth of that fungus for 3 months. Our results have evidenced a continuous disappearance of the pathogenic agent.

These results are similar to those of the direct confrontation test of the micro-organisms present in the compost that we carried out in our laboratory (Chakroune, 2006). These observations evidenced the inhibiting effect of the antagonistic fungi in the elimination of *Fusarium* oxysporum *f.sp albidinis*, since, for the control, the autoclave of the substrate and the absence of antagonistic fungi have left the pathogen profit at the maximum from favorable conditions, inducing a strong proliferation due to the absence of competition, the presence of essential nutrients and favorable physicochemical conditions.

In these conditions, the pathogen multiplies more easily since the suppressing effect of the antagonists is nil. Whereas, in presence of the latter, the density of the pathogen decreases because the antagonists compete with the pathogen for nutrients and/or vital space. This technique is efficient against pathogens, like fusarium, depending on external sources of carbon. The bioprotection which is provided by these numerous fungus isolates relies on the modes of action developed by these antagonistic fungi. On the whole, the beneficial effects of these isolates involved in the control of the vascular fusarium wilts can be explain by three major mechanisms : competition for the nutrients and the non infection sites, strong root colonization and systemic resistance of the plant (Bensaid *et al.*, 2005); Snissi *et al.*, 2006).

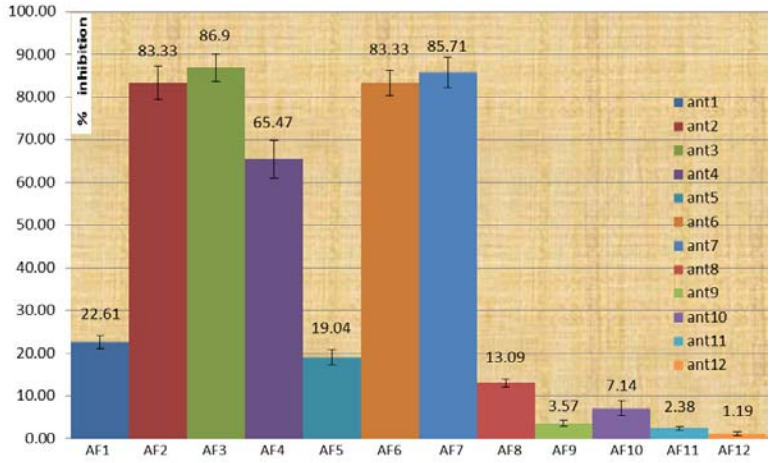

Figure 2. Inhibition percentage of antagonistic fungi selected on the growth of F.o.a.

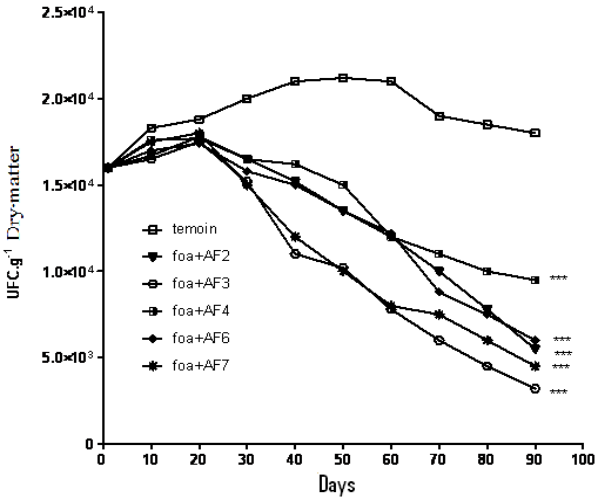

Figure 3. Evolution of the F.o.a in the substrate pre-incubated with the antagonists.

Table 1. Effect of antagonists on the inhibition rate growth mycelian F.o.a. (Control F.o.a. Diameter = 8.4 cm)

Diameter (cm)	Fungi antagonist	1	2	3	4	5	6	7	8	9	10	11	12
	Test F.o.a.	6.5	1.4	1.1	2.9	6.8	1.4	1.2	7.3	8.1	7.8	8.2	8.3
	% Inhibition	22.61	83.33	86.9	65.47	19.04	83.33	85.71	13.09	3.57	7.14	2.38	1.19

Table 2. Enumeration of the population of F.o.a. (*10^4)

Days	1	10	20	30	40	50	60	70	80	90
Control	1.60	1.83	1.88	2.0	2.1	2.12	2.1	1.9	1.85	1.8
foa+AF2	1.60	1.67	1.78	1.65	1.52	1.35	1.2	1.0	0.78	0.55
foa+AF3	1.60	1.65	1.75	1.52	1.10	1.02	0.78	0.60	0.45	0.32
foa+AF4	1.60	1.76	1.77	1.65	1.62	1.50	1.20	1.10	1.00	0.95
foa+AF6	1.60	1.70	1.74	1.58	1.50	1.35	1.22	0.88	0.75	0.60
foa+AF7	1.60	1.75	1.80	1.50	1.20	1.00	0.80	0.75	0.60	0.45

Conclusion

The present paper is an attempt to explain in details the effort done to the isolation, purification and use of telluric fungi in the biological control of the vascular fusarium wilt of the date palm.

The present work has aimed at the isolation, purification and use of telluric fungi in the biological control of the vascular fusarium wilt of the date palm. This study enabled us to select five fungi which revealed a good inhibiting capacity of the mycelium growth of the pathogen in direct confrontation. We have made it clear that these antagonists induce a reduction of *Fusarium oxysporum f.sp. albidinis.* The follow-up of this study on these antagonists, by *in vivo* tests carried out on the spot and controlled, aiming at elaborating a biopesticide, is necessary, on one hand, to confirm the results we have obtained and, on an other hand, to study their potential efficacy under different conditions of the environmental conditions and also to clarify the cellular mechanisms and molecular antagonistic plant-pathogen interactions involved in the protection (A.Snissi, L.Ezzouhri, B.D.Rossi, H.K. Lairini 2006).

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