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Pathogenicity and biological control of Bayoud disease by *Trichoderma longibrachiatum* and *Artemisia herba-alba* essential oil

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ABSTRACT

All the *Fusarium oxysporum* f. sp. *albedinis* (Foa) isolated from rachis were pathogenic to the date palm seedlings cultivar Deglet Nour, while *Fusarium* sp. isolated from soil (E1, E2, and E3) did not show any aggressiveness against these seedlings. In vitro antagonistic effect of *Trichoderma longibrachiatum* against three isolates of Foa tested by direct confrontation or remote confrontation on Potato Sucrose Agar (PSA) medium, revealed that the latest has inhibited mycelial growth of the pathogen by more than 60%, compared to the control and this after an incubation period of six days at 27 ± 2°C. Mycelial growth of *T. longibrachiatum* occupied the whole Petri dish after three days of incubation while the three isolates of Foa (T15D, M15A1, and O15T) occupied only 17%, 11%, and 20%, of the surface respectively. Biological control with essential oil of *Artemisia herba-alba* yielded important results with a minimum inhibitory concentration (MIC) ranging from 2.5 to 5 µl/ml and a minimum fungicidal concentration (MFC) of 80 µl/ml.

INTRODUCTION

The date palm *Phoenix dactylifera* L. is a monocotyledon dioecious plant belongs to the family of *Arecaceae* (Benabbes *et al*., 2015a). It has long been recognized as one of the most important resources of arid and saharan habitats because they represent an ecological, economic and social importance considerable for many countries in the arid zones, which are among the poorest in the world. In Algeria, the palm is considered as fortune of the most important economic resources, and this offers numerous benefits to the population of desert areas, especially in social terms. However, in recent years the date production has experienced a significant decline principally due to vascular fusariosis, known locally as Bayoud caused by a soil fungus *Fusarium oxysporum* f. sp. *albedinis* (Foa) (Benabbes *et al*., 2015a; Djerbi, 1983). Leading to huge economic losses from decreased crop yield and quality and since its appearance in Morocco, this disease has destroyed more than 10 million palms and three million in Algeria (Abdullah *et al*., 2010; Benabbes *et al*., 2015b). In most of the infested oases, the Bayoud has made disappeared the best variety such as Boufeggous, Medjhoul in Morocco and Deglet Nour in some oases in the south of Algeria (Djerbi *et al*., 1986).

The beneficial effect of soil-borne microorganisms in controlling pathogenic fungi and minimizing the use of pesticides has paved the way for several promising research (Caron *et al*., 2002). Furthermore, *Trichoderma* species are free-living fungi that are highly interactive in root, soils, and foliar environments, from their effective antagonistic capacity, these species are used as potential candidates for biological control of plant diseases (Kredics *et al*., 2003; Reino *et al*., 2008). Some strains of *Trichoderma* colonize the root surfaces and penetrate the epidermis, which improves root growth, productivity, and resistance to biotic and abiotic stress and assimilation of nutrients

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(Harman *et al.*, 2004). Similarly, the use of natural more eco-friendly products, like essential oils for the control of pathogenic fungi, has now become an obligation to limit the use of pesticides. Essential oils are volatile secondary metabolites produced in plants that confer protection to the plants from pathogens (Juárez *et al.*, 2015).

The objective of this work is to study the pathogenicity of Foa collected in different regions of southern Algeria, using two methods of biological control, firstly by its interaction with the antagonist *T. longibrachiatum* and secondly by application of *Artemisia herba-alba* essential oil and checking their potential inhibitory effect.

MATERIALS AND METHODS

Isolation of pathogenic fungi and antagonism

The 23 isolates used in this study were isolated in our previous work, 20 isolates from the spine were taken at the average crown of palms showing symptoms of Bayoud, and three isolates of *Fusarium* sp. near the rhizosphere of infected palms in three different regions (Adrar, Ghardaïa and Bechar) of the Southern Algeria (Sidaoui *et al.*, 2017).

As for the strain of *T. longibrachiatum*, it was isolated from the soil in the area of Aïn sefra, in the south-west of Algeria; it has been identified and sequenced by molecular methods.

Essential oil isolation

The essential oil was obtained after hydrodistillation in a Clevenger apparatus of the aerial parts of *Artemisia herba-alba*, collected from the locality of Bouilef in Batna province, east of Algeria. The essential oil was dried under anhydrous sodium sulfate and kept in dark vials at 4°C until further use (Elhouitil *et al.*, 2017).

Pathogenicity screenings

Growth of date Palm seedlings

Seeds of dates Deglet Nour variety were obtained from two regions, Biskra and Ghardaïa soaked in the sodium hypochlorite at 12% chlorometric for 10 minutes and washed with sterile distilled water, then soaked for 20 minutes in 95% alcohol. After several washing steps with sterile distilled water, the seeds were placed in Petri dishes glass, padded with cotton, soused with sterile distilled water and placed in a stove at 38°C for two days, finally, the temperature was lowered to 28°C for one to two weeks.

When the radicle reached about 3 to 5 cm (Figure1), germinated seeds were transferred in transparent bags containing sterile mold and placed in a greenhouse at uncontrolled conditions.

Fig. 1: Pregermination of date seeds.

Preparation of inoculums

After three months, the seedlings reached the two-leaf stage (Figure 2) and were ready to be inoculated. For preparing the inoculum, six Petri dishes containing Potato Sucrose Agar medium were inoculated with a disc of 0.5 cm in diameter for each isolate, After seven days of incubation at 27 ± 2°C, 9 ml of sterile distilled water were poured into each dish and scratched the surface to recuperate the suspension which was then adjusted to 106 spores/ml.

Fig. 2: Plants after three months with two-leaf stage.

Inoculation of seedlings

Inoculation was carried out at the two-leaf stage in the greenhouse (Djerbi *et al.*, 1990) (Figure 2), without unearth the seedlings by injecting with a syringe devoid of a needle with 10 ml of the spore suspension in the bottom of the bag, where the young roots are grouped (Figure 3). This operation was carried

out without hurt the roots. We used 25 seedlings for each isolate. After the appearance of the first sign of the disease, we wrote down the notations and we stopped when the mortality rate caused by certain isolates had reached 100%. The test is valid when the mortality rate exceeds twenty percent (Karkachi *et al.*, 2014), while the control plants had presented no symptoms.

Fig. 3: Injection of spore suspension.

In vitro antagonism test

Antagonism between Foa and *T. longibrachiatum* was studied by the Howell method (2003), we deposited diametrically in Petri dishes containing 15 ml of PSA medium, two opposite explants of 0.5 cm diameter disc of the pathogen and the *T. longibrachiatum.* The controls are constituted only by the pathogen. The distance between discs was approximately six cm.

Evaluation of Mycelial Growth

Mycelial growth of Foa was evaluated by measuring the radius of the colony every day and this for seven days. The efficiency of *T. longibrachiatum* in suppressing radial growth was calculated as follows:

$$C– T/C. 100,$$

where C is radial growth measurement of the pathogen in the control and T is the radial growth of the pathogen in the presence of *T. longibrachiatum* (Benabbes *et al.*, 2015b; Rashmi *et al.*, 2016).

Antifungal activity of *Artemisia herba-alba* essential oil

The Minimum Inhibitory Concentration (MIC) was determined by the method of tube dilution assay as described by Saxena *et al*. (2012). With some modifications, serial dilution of essential oil was prepared in Dimethysulfoxide (DMSO) (1% v/v) to obtain a final concentration of 80, 40, 20, 10, 5 and 2.5 µl/ml. 1 ml of each concentration was added to tubes containing 1 ml of potato sucrose broth (PSB) medium and 1 ml of a fungal suspension, then incubated at 25°C for seven days. The test was done in duplicate. The MIC value was estimated as the lowest concentration that showed no visual growth comparing to the control tube. To determine the Minimum Fungicidal Concentration (MFC), samples (100 µl) were taken in duplicate from tubes presenting an absence of growth and transferred to Petri dishes on which seven ml of PSA medium were added. After incubation for five days at 25°C, the MFC was noted from the plates showed no visible fungal growth (Table 5).

RESULTS

Pathogenicity screenings

The average rate of seed germination was 98%, and we noticed that the appearance of symptoms begins at 15 days and death of all seedlings 2 months after inoculation, among 23 isolates we found that all the 20 isolates of Foa (Table 1) tested were pathogenic. The number of destroyed seedlings from 25 inoculated with an isolate of Foa was between 11 to 25. The percentage of seedling wasted away varied from 44% to 100% among isolates of Ghardaia (M15A1, M15G, M15D1, M15T1, M15D, I08G, M15A2 and M15D2), from 44% to 96% in isolates of Adrar (M15H, M15H1, M15T, T15H, M15T, M15D, O15T, M15A, O15H and O15D), finally, 96% and 100% mortality rates were noticed for two isolates of Bechar (B15F and B15H) respectively. While the three batches inoculated with *Fusarium* sp. isolated from soil (E1, E2, and E3) showed no aggressiveness against these seedlings. Comparison with the control plants remained healthy after two months of inoculation (Figure 4 and Table 1).

We observed the presence of brown necrosis in the roots of the inoculated seedlings; it is mainly based on host response and symptom development on different aerial parts of the plant (Figure 5).

Analysis of the variance (ANOVA) of the ratios of dead seedlings showed that there was no significant effect of geographic origin on the mortality ratio $P = 0.3042$ (Table 2).

The 20 isolates of Foa tested were isolated from at least eight date cultivars from Ghardaia, Bechar or Adrar. The results obtained showed that no correlation appears to exist between the pathogenicity of the strains and the geographical origin of the strain or the cultivar from which this strain was isolated. Isolates coming from Bechar showed the highest mortality rate (98.00 ± 2.83%) whereas these coming from Ghardaïa and Adrar showed the lowest mortality rates 74.63 ± 15.50% and 75.56 ± 22.84%, respectively. Moreover, the coefficient of variation (CV) in Bechar (2.89%) explained the homogeneity within isolates, on the other side the CV showed the highest variation within Adrar isolates (30.23%) and Ghardaïa isolates (20.77%) due to the heterogeneity in these two origins (Table 3).

In vitro antagonism test

The mycelial growth of the pathogen in the Petri dishes on PSA medium is slowed with the presence of the *T. longibrachiatum* antagonist with an almost similar rate of inhibition for the three isolates (63%) (Table 4) with a total halt of pathogen growth and the formation of a clear inhibition zone (Figure 7).

Table 4 showed the evaluation of the mycelial growth of the three isolates of Foa every day until the 7th day. The diameter of the pathogenic colony was 2.4 cm, 2.6 cm and 2.75 cm for M15A1, O15T, and T15D respectively in the presence of *T. longibrachiatum*, that is, for the same isolates; we have recorded 6.6 cm, 7.2 cm and 7.5 in the absence of *T. longibrachiatum.*

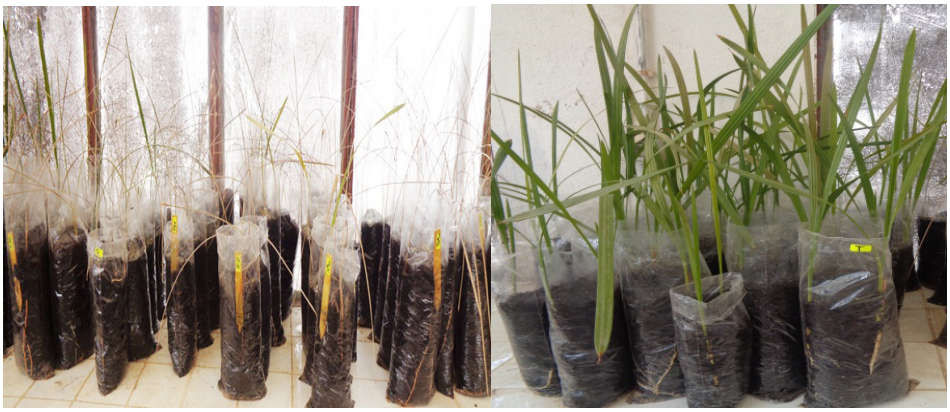

Fig. 4: Result of screening test (Left: Plants after two months of inoculation; right: control plants).

Table 1: Mortality rates by weeks and isolates.

	Mortality rate in%				
Isolates	**3 weeks**	**4 weeks**	**5 weeks**	**6 weeks**	**Total**
M15A1	16	40	24	16	96
M15G	04	24	20	24	72
M15D1	12	20	28	20	80
E1	00	00	00	00	00
B15H	16	24	32	28	100
M15H	04	12	20	16	52
T15H1	12	28	16	20	76
A15T	16	32	20	16	84
E2	00	00	00	00	00
T15H	20	28	24	24	96
M15T	00	16	13	24	53
M15T1	16	24	32	08	80
B15F	20	32	24	20	96
T15D	28	20	28	24	100
M15D	12	32	24	24	92
O15T	00	12	20	12	44
I08G	24	20	20	28	92
M15D2	12	16	28	20	76
E3	00	00	00	00	00
M15A	04	12	20	08	44
M15A2	06	14	18	06	44
O15H	08	24	28	28	88
O15D	04	16	20	12	52
control plants	00	00	00	00	00

Table 2: Analysis of variance on cumulated numbers of dead seedlings.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Geographic origin	2	940.63962	470.3198	1.2833	0.3042
Error	16	5864.0972	366.506		
C. Total	18	6804.7368			

Table 3: Means (± standard error) comparison of geographic origin on the mortality rate, according to least squares means (Student's t-Test).

Mortality rate (%)			
Geographic origin	**Range**	**Mean ± SD**	**CV (%)**
Bechar	96–100	98.00 ± 2.83 a	2.89
Ghardaïa	52–92	7463 ± 15.50 a	20.77
Touat	44–100	75.56 ± 22.84 a	30.23

Levels not connected by same letter not significantly different at P = 0.05.

Table 4: Evaluation of mycelial growth.

strains	T15D	M15A1	O15T	T1	T2	T3
1 day	0.2	0.1	0.1	.	.	.
2 day	0.8	0.3	0.5	.	.	.
3 day	1.5	1.0	1.7	.	.	.
4 day	2.5	1.2	2.2	.	.	.
5 day	2.55	2.2	2.5	.	.	.
6 day	2.65	2.4	2.58	.	.	.
7 day	2.75	2.4	2.6	**7.5**	**6.6**	**7.2**
R	**63.33**	**63.63**	**63.88**			

R = Inhibition rate; T1, T2 and T3 = Control after 7 days of incubation respectively for T15D, M15A1 and O15T.

Fig. 5: Left: the normal color of root "Control"; right: brown color of the root after inoculation.

Determination of the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC)

The results of MIC and MFC are summarized in Table 3, the lowest MIC of 2.5 µl/ml was noticed for the isolates T15D

and O15T, while the strain M15A1 showed a lower sensitivity and was inhibited with a concentration of 5 µl/ml. A fungicidal activity of the essential oil was achieved with a concentration of 80 µl/ml for all isolates.

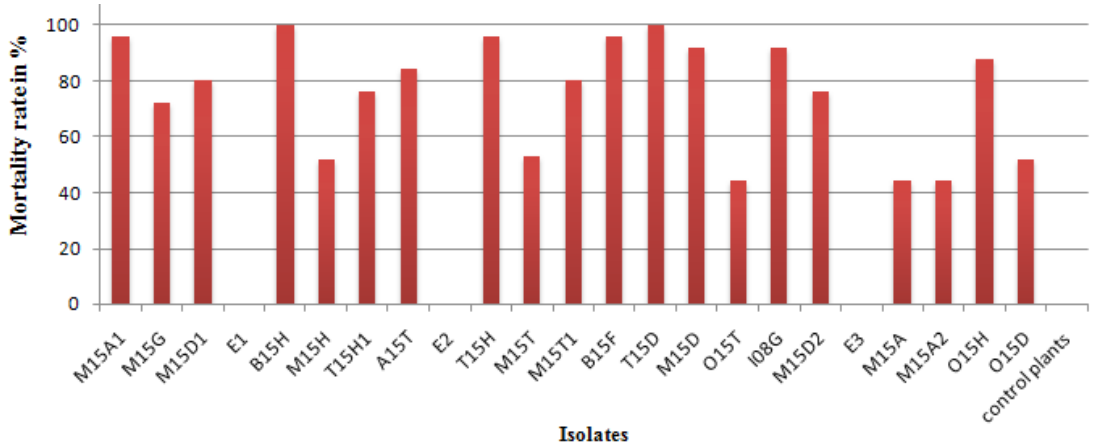

Fig. 6: Percentage of dead seedlings between the third and sixth week after inoculation by 23 *Fusarium* isolates (from M15A1 at 015D) and control plants.

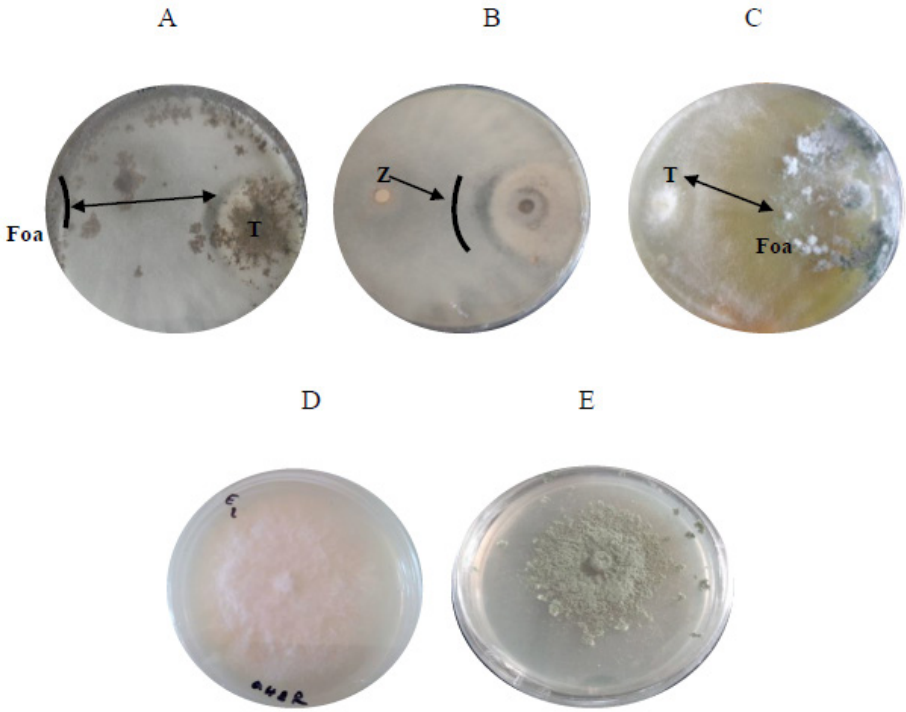

Fig. 7: A: Foa confronted with *T. longibrachiatum* (T) after three days; B: Zone of inhibition (Z) after seven days; C: The (T) mycelial occupy the whole surface and liberates the spores on the colony of Foa; E: *T. longibrachiatum*. colony; F: Foa colony.

Table 5: MIC and MFC values for treated isolates with *Artemisia herba-alba* essential oil.

	MIC (µl/ml)	MFC (µl/ml)
T15D	2.5	80
T15D	2.5	80
O15T	2.5	80

DISCUSSION

The high rate of mortality was recorded in plants inoculated with the 20 isolates of Foa, compared to seedlings inoculated by the three isolates of *Fusarium* sp. isolated from soil and control plants. A mortality rate of 70% was recorded, and this result is proximate to that reported by Karkachi *et al*. (2014) and Oubraim *et al*. (2016). The first symptoms of the disease were observed on the leaves of the inoculated seedlings, appearing with a yellow color and then these leaves are dried, which leads to the death of the latter and to sowing at the latest. Such symptoms have been shown to be similar to those previously reported for Bayoud disease (Zaid *et al.*, 2002; Elhassan, 2016). Khalil and Verreet (2003) had performed work on cotton diseased plants, they tested

the pathogenicity of 46 isolates of *Fusarium* sp. under glasshouse condition, and reported that 38 isolates (82.4%) were pathogenic to seedlings Giza 89. The same result was observed for six isolates of *Fusarium solani* which showed their pathogenicity to guava of Allahabad Safeda when tested in greenhouse (Gupta *et al.*, 2009).

The *Trichoderma* genus includes imperfect filamentous fungi with teleomorphs belonging to the hypocreales order of the *Ascomyceta* division (Kredics *et al.*, 2003). *T. longibrachiatum* grow rapidly on a culture medium which should be beneficial during the confrontation. Our results showed that the use of *T. longibrachiatum* reduce the growth of the three Foa tested with a similar rate of inhibition (63%) and the formation of a clear zone of inhibition. The results of confrontation have shown that the colony of *T. longibrachiatum* invade those of the Foa. If we compare our results with the ones published, it is close to that found by Bekkar *et al*. (2016), which demonstrated an inhibition rate ranged from 66% to 86% of 23 *Trichoderma* isolate against Foa. The same results were found by Souna *et al*. (2012) who reported that Foa has been inhibited by *T. harzianum* at a rate of 65%. According to Noveriza and Quimio (2004), when the value of inhibition percentage was higher than 60%, the antagonist was regarded as promising biocontrol agent. Hibar *et al*. (2005) carried out that the antagonist *T. harzianum* showed that an inhibition of the growth of *F. oxysporium* greater than 65%. The inhibitory activity of *Trichoderma* species on the growth of *F. oxysporum* was observed by Dabire *et al*. (2016), with an inhibition rate of 78% for *Trichoderma* (ThTab). Several species of *Trichoderma* sp. have been known as effective biological control agents and many strains registered for commercial purposes in the context of plant protection (Nicolás *et al.*, 2014).

The importance of using extracts of medicinal plants in biological control against pathogens is currently the closest solution to maintain the health of plants and the environment, so the use of *Artemisia herba-alba* essential oil in this work, has allowed to determine the MIC and the MFC, the lowest MIC of 2.5 µl/ml was noticed for the isolates T15D and O15T while the strain M15A1 showed a higher resistance and was inhibited with a concentration of 5 µl/ml. A fungicidal activity of the essential oil was achieved with a concentration of 80 µl/ml for all strains. This result is different from the one published by El Houiti *et al*. (2016), who recorded an MFC of 10 µl/ml for essential oils extracted from the stems and flowers of *Rhanterium adpressum* against Foa strain. The same results were found by Takhi *et al*. (2011) for the essential oil of *Pistacia lentiscus*. Also an approximal MIC of 2 µl/ml for *Hertia maroccana* (Batt.) essential oil was noticed for the Foa strain (Bammou *et al.*, 2016), while, in the previous study done by Khaldi *et al*. (2015), a lower MIC of 0.66 µl/ml was observed for *Anethum graveolens* L. essential oil against Foa. Another study was conducted by Benabbes *et al*. (2015a) on powdered extracts of three medicinal plants (pomegranate, rosemary, and oleagenous) to test the sensitivity of Foa strain, the result gave the following percentages of inhibition (97%, 85%, and 70%) respectively. Similarly, Singh *et al*. (2006) showed that the 6 µl dose of the *star anise* essential oil prevented the growth of *F. moniliforme*. In the same trend, Hathout *et al*. (2015), reported that rocket and *star anise* extracts showed a high antifungal activity and a complete inhibition of *F. solani* at a concentration of 100 ppm, whereas, *F. oxysporum* was completely inhibited at a lower concentration (50 ppm). A similar result demonstrated that leaf extracts from *Santalum album* showed a significant reduction in the radial growth of *F. proliferatum* (80%) for 100% of concentration, another study carried out on Mustard oil as antifungal, with 25% of concentration, Mustard oil reduced mycelial growth of *F. proliferatum* with 82% (Bhale, 2015).

CONCLUSION

After our study on pathogenicity, our results showed the aggressiveness of Foa against date palm seedlings, with varying mortality rates; and this explains the absence of correlation between the geographical origin and the pathogenicity of the isolates.

The use of *T. longibrachiatum* and the *Artemisia herba-alba* essential oil, in biological control of the Foa, enabled us to record important results for inhibiting the capacity of the mycelial growth of the Foa in direct confrontation. The follow-up of this study is necessary to test *T. longibrachiatum* and *Artemisia herba-alba* essential oil in vivo on date palm seedlings, with well-controlled conditions to confirm the results.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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