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IN VITRO STUDY OF BIOCONTROL POTENTIAL OF RHIZOSPHERIC MICROORGANISMS AGAINST *FUSARIUM OXYSPORUM* F.SP. *ALBEDINIS*

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A B S T R A C T

The present work investigated the ability of antagonistic microorganisms to inhibit the growth of pathogenic strains responsible for the vascular fusarium wilt of date palm or Bayoud's disease. The objective was to evaluate the antagonistic effect of native strains of *Trichoderma longibarchiatum, Penicillium* sp*., Fusarium* sp. and one isolate of *Bacillus* sp. against four isolates of *Fusarium oxysporum* f.sp. *albedinis* (Foa) under *in vitro* conditions. The isolates *Bacillus* sp. and *Fusarium* sp. were isolated from soil samples of the rhizosphere of date palm of Tamanrasset region in Algeria, these strains were morphologically identified, and a dual culture confrontation was carried out, placing the Foa in the center of the Petri dish and *Bacillus* sp. in the two cardinal points. For *T. longibarchiatum, Penicillium* sp. and *Fusarium* sp. they were confronted against Foa with a dual confrontation, the inhibition rates were determined, and a percentage of inhibition obtained varied from 64.29 to 65.75%; 57.14 to 64.29% and 36.36 to 52.05% for *T. longibarchiatum, Penicillium* sp and *Fusarium* sp. respectively. While *Bacillus* sp. exhibits the lowest percentage of inhibition in this study, and they were less than 16.67%. Comparison of mean value for each pair between *T. longibrachiatum, Penicillium* sp*., Fusarium* sp. on one hand and *Bacillus* sp. on the other hand concluded that there is a highly significant difference between these three fungal isolates and *Bacillus* sp. concerning inhibition rates with P-value (*P <0.0001*). Contrary, no significant difference was observed between *T. longibrachiatum* and *Penicillium* sp. with *P = 0.1895* and the two antagonists show the same letter A in the letter connection ratio, which means no difference in the level of inhibition.

Keywords: Algerian date palm, *Bacillus* sp., Bayoud, Confrontation, *Fusarium oxysporum* f.sp. *albedinis.*

INTRODUCTION

Plant diseases are the major problems in crop production and resulting in a large scale the loss of production and productivity worldwide. Unfortunately, the date palm has been decimated in recent years by Bayoud disease or vascular wilt of date palm, caused by the Ascomycete imperfect fungus, *Fusarium oxysporum* f.sp. *albedinis* (Foa). This disease is a serious threat for the palm groves of North Africa, consequently, the most susceptible varieties to the disease are destroyed, especially those that produce high quality of dates and in significant quantities (Deglet Nour, Bou Fegouss). Currently the Bayoud exist in Morocco, South and South-West of Algeria, some localities in the Mauritanian palm grove and stay always a menace to the unharmed groves palm of several countries (Oubella *et al.,* 2017; Essarioui *et al.,* 2018; Bahriz and Bouras, 2020; Hussain and Ismailli 2020; Metlo *et al.,* 2021). Effectively, Bayoud disease doesn't just causes a reduction in the production of dates, but also an imbalance of the oasis ecosystems, in particular the acceleration of desertification, this has forced farmers to

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migrate to cities and leave their lands neglected (El Modafar, 2010; Hussain and Ismailli, 2020). In fact, from his appearance in 1870 in the palms of North Africa, we estimated that it has caused the disappearance of 20 million palm trees, including three million of Algerian date palm especially in the regions of Adrar, Bechar, GHardaia and Biskra (Oubraim *et al.,* 2016; Jeger *et al.,* 2018; Metlo *et al.,* 2019; Hussain and Ismailli, 2020; Belhi *et al.,* 2020).

In this situation of great losses in palm groves due to Bayoud disease, it has become necessary to fight this epidemic using various methods, except that some of these methods such as chemical control have a harmful effect on human health and the biodiversity of ecosystems. Therefore, researchers are looking for other non-harmful alternatives capable of eliminating phytopathogens, among which figure biological control, using effective antagonistic bacteria and fungi. Currently it is the useful strategy to control plant diseases.

Owing to these aspects, the purpose of our work was to determine the inhibitory effect of some microorganisms isolated from the rhizospheres of date palm against the growth of *Fusarium oxysporum* f.sp. *albedinis* under *in vitro* conditions.

MATERIALS AND METHODS

Obtaining of pathogenic and antagonistic fungal strains: In this study, four pathogenic strains of *Fusarium oxysporum* f.sp. *albedinis* (Foa) (E22, AS6, SG10 and B10/08) were used, these strains provided by the Applied Microbiology Laboratory, University of Oran 1 (Ahmed Ben Bella), Algeria. They were originally isolated in our previous work in regions of the Southern Algeria from infected rachis of date palm showing symptoms of Bayoud (Sidaoui *et al.,* 2017).

In addition to two antagonistic strains *Trichoderma longibrachiatum* and *Penicillium* sp., were selected from the laboratory collection.

Isolation of *Fusarium* sp. and *Bacillus* sp.: The two strains *Fusarium* sp. and *Bacillus* sp. were isolated from a soil samples by the following method: under aseptic conditions, a small amount of soil was collected from the rhizosphere of date palm in Tamanrasset region. Then, 3mg was sprinkled directly by sterile spatula in Petri dishes containing potato dextrose agar (PDA) culture medium and incubated at 27 °C for 3 to 7 days (Njenga et al., 2017).

Purification and identification of *Fusarium* sp.: After good growth of the fungal colonies around soil particles, a colony fragment was collected with a sterile platinum loop and successively transplanted in Petri dishes containing the PDA medium, to ensure the purity of colonies (Brown and Proctor, 2013). Then, the Petri dishes were incubated at 27 °C for 5 to 7 days.

After obtaining a pure culture of *Fusarium* sp., the identification was carried out firstly, by the macroscopic study on PDA medium (aspect of the colony, color and pigmentation, mycelium development, color of the reverse of culture and colony margin), and in a second step, a microscopic study of the morphological characters of asexual reproductive organs and the mycelium, by observation of a small fragment of culture aged of 5 to 7 days between blade and lamella directly under optical microscope and colored by the methylene blue, this method allows to observe the aspect of mycelium, the form and septation of macroconidia, presence or absence of microconidia and chlamydospores, and the form and size of the conidiophores (Brown and Proctor, 2013). The study of all these characters was based on the *Fusarium* identification key of Leslie and Summerell, (2006).

Purification and identification of *Bacillus* sp.: After isolating the bacterial strains on Nutrient Agar (NA) culture medium, we were re-streaked each colony successively, in Petri dishes containing 15 mL of NA medium by the quadrant method, which was done as described by Boughachiche *et al.* (2005) and Denis *et al.* (2016).

The identification of *Bacillus* sp. was based on two exams only:

Macroscopic observation: Observation of the strain with the nude eye and determine the various aspects such as (color, surface, diameter, margin…etc).

Microscopic observation: This exam was based on the observation of bacteria after Gram stain to determine the nature of the bacterial wall, the form, mode of grouping and presence or absence of spores.

Confrontation methods: Pathogens-antagonistic fungi confrontation: To evaluate the effect and ability of antagonistic fungi to suppress mycelial growth of the 4 pathogenic strains of Foa (E22, SA6, SG10 and B10 / 08), direct confrontation method using the *in vitro* co-cultivation assay were carried (Fig. 1A), according to Howell (2003), Sidaoui *et al.* (2018), Tian *et al.* (2020) and Chen *et al.* (2021) with slight modifications. In this test, two discs of 0.5 mm of diameter, one from 7 days aged culture of Foa, and another from antagonist, were deposited in Petri dishes containing 15 mL of PDA medium, which were placed opposite to each other in the Petri dishes on the same diagonal line with a distance of 1cm from the edge.

Petri dishes only with pathogen cultures (without antagonists), were served as control. Then, all the dishes were incubated at 27 ± 2 °C for 7 days.

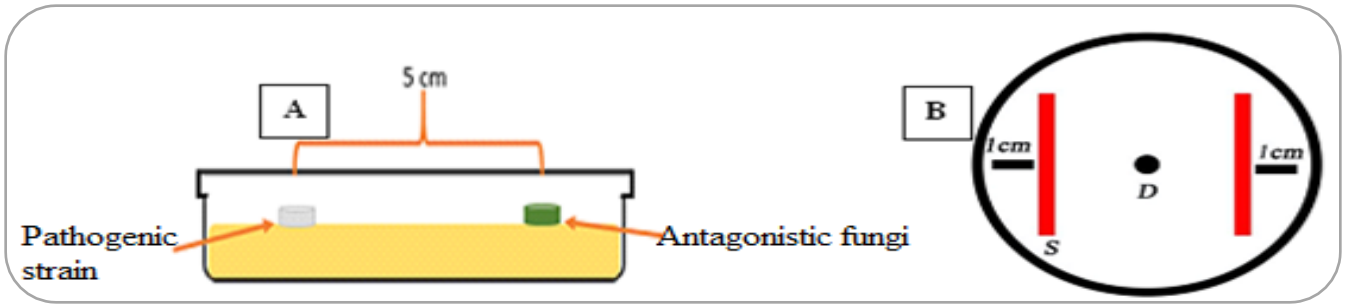

Figure 1. Confrontation methods (A: pathogens-antagonistic fungi ; B: bacteria-pathogens ; D: pathogen disc ; S: two streaks of *Bacillus* sp.).

Bacteria-pathogens confrontation: To evaluate the inhibitory effect of *Bacillus* sp. against four fungal pathogenic strains of Foa, a direct confrontation test was carried out under aseptic conditions. For this experiment, a bacterial isolate of *Bacillus* sp. 24h-old was streaked on Petri dishes, previously pre-poured by the PDA culture medium, in 2 equidistant streaks of 4 cm long on the two opposite sides of the edges of the Petri dish, Then, 5 mm mycelial disc of each pathogenic fungus was transferred and placed in the center of the Petri dish between the bacterial streaks (Fig 1-B). A disc of each pathogenic strain was placed in the center of the petri dish individually for use as control. Fungal pathogens growth inhibition was expressed as a percentage of radial growth inhibition relative to the control 7 days after incubation at 27 °C (Girish and Bhavya, 2018; Lahlali *et al.,* 2020; Marimuthu *et al.,* 2020; Kumar *et al.,* 2021; Mohiddin *et al.,* 2021).

Mycelial growth assessment: After 7 days of inoculation, the inhibition zones were measured and used to determine the inhibition rate using the formula below as described by Benouzza *et al.* (2020) and Chen *et al*. (2021):

$$I = \frac{(C - T)}{C} \times 100$$

I: rate of inhibition

C: radial growth of the pathogen in the control

T: the radial growth of the pathogen in the presence of the antagonist

STATISTICAL ANALYSIS

All experimental data collected from laboratory experiments were analyzed using JMP SAS Pro software (JMP®, Version <15>, and significant treatment results were determined by F values (*P≤0.05*).

RESULTS

Isolation of *Fusarium* sp. and *Bacillus* sp.: The isolation performed from rhizosphere samples of palm trees which were collected from Tamanrasset region in Algeria, showed that fungal colonies resemble to those of the genus *Fusarium*. Morphologically, these fungal colonies present a cottony aspect with pink color (Fig 2-A), and a rapid growth (7.5cm) after 7 days of incubation. Moreover, microscopic observation has allowed us to observe the presence of three types of conidia, macroconidia, microconidia and chlamydospores (Figure 2-B and C).

The bacterial strain isolated from the rhizosphere of date palm was characterized by colonies small size, rounded and regular shape, smooth aspect and a white color. After Gram staining, we observed the presence of rod shaped bacterium, isolated or grouped together in chains, these cells were revealed Gram positive (Figure 2-D).

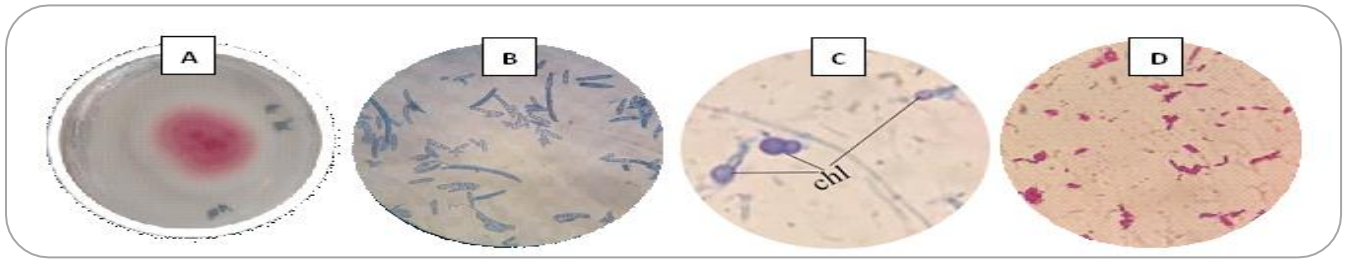

Figure 2. Morphological aspect of *Fusarium* sp. and *Bacillus* sp. isolated (A: Macroscopic observation on the PDA medium ; B and C: Microscopic observation ; ma: Macroconidia ; mi: Microconidia ; chl: Chlamydospores ; D: Microscopic observation of *Bacillus* sp. after Gram stain (Gx100).

Confrontation tests: Confrontation Pathogens-antagonistic fungi : The results of these tests showed rapid growth of *T. longibarchiatum, Penicillium* sp. and *Fusarium* sp. on the PDA medium, compared to

pathogenic strains of Foa. However, the highest inhibitory effect was noted by *T. longibarchiatum* with a rate of 65.75%. After three days of incubation, the plates were almost completely invaded by the antagonistic fungi. This observation was observed even in the presence of pathogenic strains.

Pathogenic strains growth was completely stopped with the formation of inhibition clear zone between the two colonies (antagonist-pathogen) in comparison to the control after 7 days of incubation by dual culture method, where inhibition percentage varied from 64.29 to 65.75%; 57.14 to 64.29% and 36.36 to 52.05% for *T. longibarchiatum, Penicillium* sp. and *Fusarium* sp. respectively (Table 1 and Fig 3).

Table 1. Evaluation of the inhibition rates (%) of the antagonists tested against the 4 pathogenic strains of Foa after 7 days of incubation.

	inhibition rates (%)			
	B10/08	SG10	SA6	E22
T. longibarchiatum	65.71	64.29	65.75	64.86
Penicillium sp.	57,14	64,29	61,64	60,81
Fusarium sp.	51.35	36.36	48.61	52.05
Bacillus sp.	9.46	14.55	16.67	15.07

B10/08, SG10, SA6 and E22 the pathogenic strains of Foa.

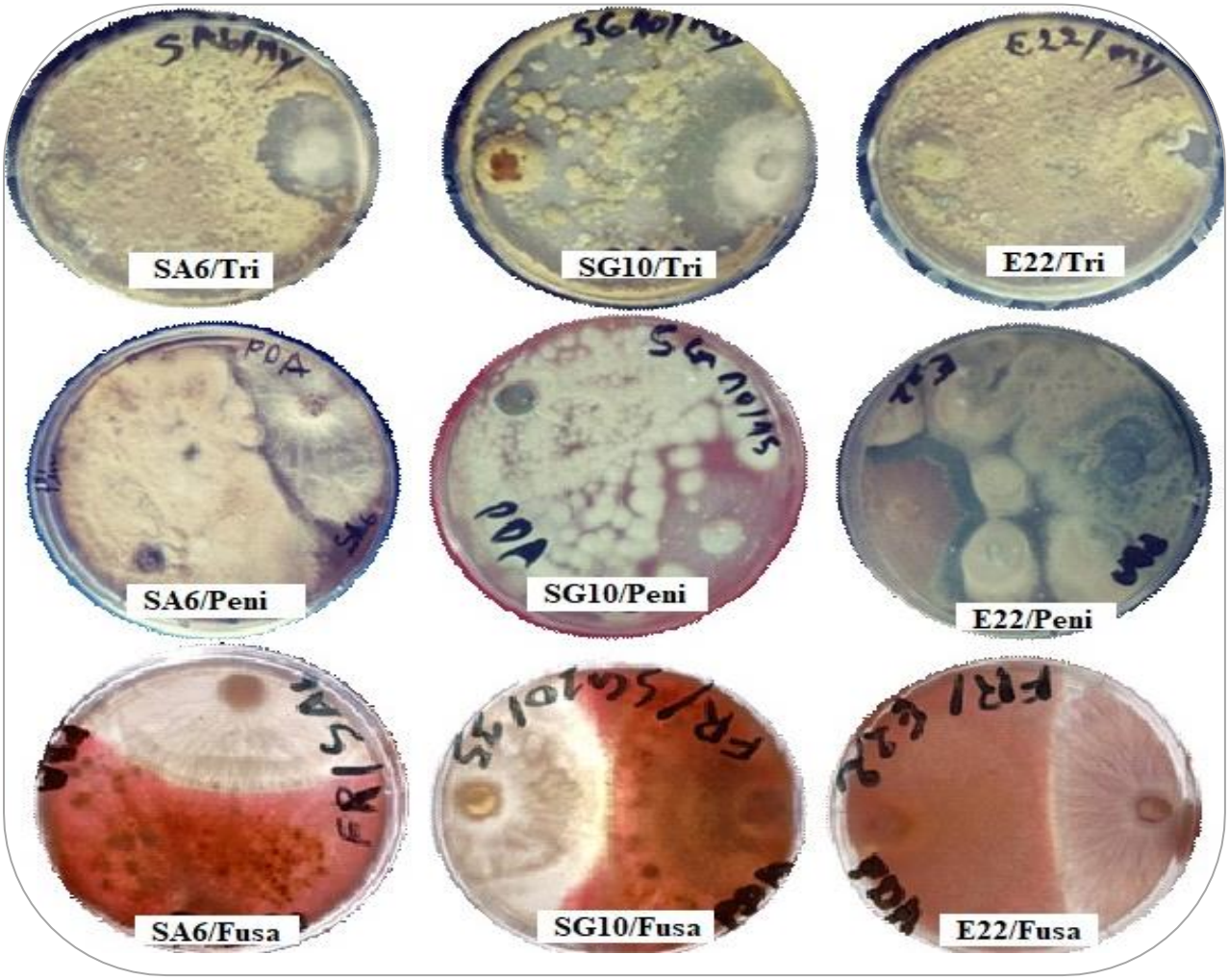

Figure 3 (a). Inhibitory effects of the antagonistic strains on Foa mycelial growth (Tri *T. longibrachiatum*. Peni *Penicillium* sp. Fusa *Fusarium* sp. *Bacillus* sp.).

Figure 3 (b). Inhibitory effects of the antagonistic strains on Foa mycelial growth (Tri *T. longibrachiatum*. Peni *Penicillium* sp. Fusa *Fusarium* sp. *Bacillus* sp.).

Confrontation pathogens-*Bacillus* sp.: According to the observed results, importance growth of Foa strains was noticed, which means that *Bacillus* sp. presents a very low inhibitor effect against these pathogens. After comparison with controls grown individually in the absence of *Bacillus* sp., we noticed a mycelial growth almost equal to that observed in presence of *Bacillus* sp. after 7 days of incubation (Table 1 and Fig 3).

Comparison of inhibition means: Comparison of means for each pair between *T. longibrachiatum, Penicillium* sp., *Fusarium* sp. on the one hand and *Bacillus* sp. on the other hand gives 51.22; 47.03 and 33.16 respectively with a standard error of the difference of 3.01 and a P-value (*P <0.0001*). This last we pushes to concluded that there are a very highly significant difference between the three fungi and *Bacillus* sp. concerning inhibition rates.

The limits of the confidence interval (lower and upper control limits) permit to determine precisely the difference inhibition between each pair. Examining the upper confidence limit of the difference (95%) and lower confidence limit (95%) of the difference (blue lines) in Figure 4.

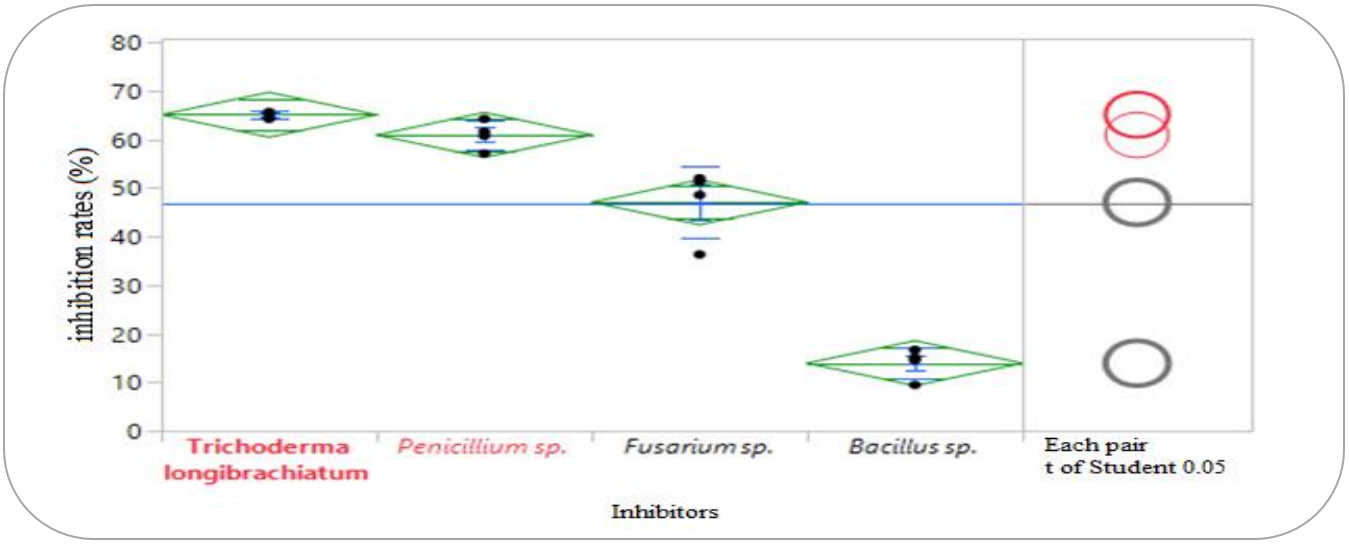

Figure 4. ANOVA- One-factor inhibition rates versus inhibitor

We were conclude that the means inhibition rates of *T. longibrachiatum, Penicillium* sp. and *Fusarium* were superior than 44.66 to 57.77; 40.48 to 53.58 and 26.60 to 39.71 to that of *Bacillus* sp. respectively (Table 2). Contrary, no significant difference was observed between *T. longibrachiatum* and *Penicillium* sp. with *P = 0.1895* and the two antagonists show the same letter A in the letter connection ratio, which means no difference in the level of inhibition (Table 3).

Table 2. Ratio of ordered differences of pairs

Level	- *Level*	Difference	Standard error of the difference	lower control limits	upper control limits	P-value
T. longibrachiatum	*Bacillus* sp.	51.22	3.01	44.66	57.77	<,0001
Penicillium sp.	*Bacillus* sp.	47.03	3.01	40.48	53.58	<,0001
Fusarium sp.	*Bacillus* sp.	33.16	3.01	26.60	39.71	<,0001
T. longibrachiatum	*Fusarium* sp.	18.06	3.01	11.51	24.61	<,0001
Penicillium sp.	*Fusarium* sp.	13.88	3.01	7.33	20.43	0.0006
T. longibrachiatum	*Penicillium* sp.	4.18	3.01	-2.37	10.73	0.1895

Table 3: Letters connection report

Level	Averages	Letters
T. longibrachiatum	65,15	A
Penicillium sp.	60,97	A
Fusarium sp.	47,09	B
Bacillus sp.	13,94	C

DISCUSSION

It is necessary to use biological control methods against phytopathogenic diseases which affect the economy of several countries and to avoid chemicals which have serious effects on the health of humans, animals and the environment. Indeed, the use of fungal and bacterial species with very strong antagonist activity is currently presents an interest in the biological, agronomic and biotechnology fields.

In this study, we were noticed that the *T. longibrachiatum* strain has a very high efficiency for inhibited the growth of Foa after 7 days of incubation on the PDA medium. The following inhibition rates 64.29; 64.86; 65.71 and 61.75% were recorded respectively for SG10, E22, B10/08 and SA6 strains. Similar results were obtained by Sidaoui *et al.* (2018) and bekkar *et al.* (2016). Their results indicated that *Trichoderma* strains reduce the mycelial growth of Foa isolates tested, with inhibition rates of 63% and 65% respectively. Due to their rapid development and their production of bioactive substances, several *Trichoderma* species limited the growth of *Fusarium* species and considered as a powerful microbial antagonists and can be used as a bioprotector or as a plant biostimulant (Sghir *et al.,* 2016; Benouzza *et al.,* 2020; Haouhach *et al.,* 2020; Bustamante *et al.,* 2021; Pani *et al.,* 2021; Hammad *et al.,* 2021). In the same context, the efficacy *in vitro* of the inhibitory activity of *T. harzianum* against *F. oxysporum* were assessed by Hibar *et al.* (2005) who recorded inhibition rates greater than 65%, after a direct confrontation on the PDA medium. In addition, Tiru *et al.* (2021) have reported an antagonistic effect of *T. asperellum* strain with percentages of inhibition ranging from 65.71 to 82.85% against *F. moniliforme*.

Penicillium sp. was effective in reducing mycelial growth of Foa during tests *in vitro* by an inhibition rate approximately 60% with the formation of a clear zone of inhibition between the two colonies pathogen-antagonist. This result is consistent with that found by Benabbes *et al.* (2015) after confrontation tests between Foa and antagonist strains belong to the genera *Aspergillus* and *Penicillium*, which showed an inhibition rate of 65%. Indeed, our findings upper those of Rajathi *et al.* (2020) who found minimum zone of inhibition

after direct *in vitro* confrontation for both *Penicillium* sp. against *F. moniliforme.* In fact, several strains of the genera *Penicillium* and *Alternaria* have been isolated and reported as potentially active agents in the biological control of certain plant diseases (Pecundo *et al.,* 2021; Chen *et al.,* 2021). In addition, a large part of the species of the genus *Penicillium* has several action mechanisms for plant protection (production of antibiotics, induction of resistance and the establishment of mycoparasitic interactions) against pathogenic microorganisms (Nicoletti and De Stefano, 2012).

In our study, after 7 days of incubation, the colonies of Foa were fully invaded by *T. longibrachiatum* and *Penicillium* sp. with intensive sporulation adhering to pathogen colonies.

We also were conducted a confrontation assay to test the ability of *Bacillus* sp. to be potential biocontrol agents against Foa. In this test we were noticed a very low inhibition rate14% of *Bacillus* sp. against the pathogenic strains of Foa after the confrontation on the PDA medium, and the pathogens continued their development over the entire Petri dishes. Similar results were obtained by Lounaci and Athmani-Guemouri (2014) who found an inhibition rate of 13%, after the confrontation on the PDA medium of certain genera of *Fusaruim* with *Paenibacillus polymyxa*. In the same way, Dukare and Pual (2021) published an inhibition rate of 27% as a result of *Bacillus* sp. against *F. udum*. Different results were revealed by Ben Slama *et al*. (2019) who demonstrated percentage of inhibition round 55% of *F. oxysporum* f.sp. *albedinis* by *Bacillus* strains isolated in the semi-arid regions in Tunisia and Algeria. In addition, Harba *et al.* (2020) showed that several *Bacillus* species, having significant antagonist activities against *F. culmorum* and *F. solani.* Indeed, *B. subtilis* provided the most remarkable result with an inhibition rate of 97.2% against *F. solani,* and *B. tequilensis* was the most efficient in the formation of the inhibition zones against *F. culmorum*. In addition, Jangir *et al.* (2018) published that 4 strains of *Bacillus* isolated from the rhizosphere show the ability to inhibit the growth of *F. oxysporum* with inhibition rates varying between 79.37 and 87.5%. The inhibitory efficacy of *Bacillus* sp. on the *Fusaruim oxysporum* growth was recorded by Lui *et al.* (2020). These authors found varying inhibition rates between 40 and 52% after direct confrontation tests on PDA medium. It also reported that *B. subtilis* W3.15 exhibits high inhibition rate of 66.5% against *F. oxysporum* mycelial growth in 6 days of incubation (Putri *et al.,* 2021).

CONCLUSION

From this study, we were concluded that the possibility to control the Bayoud disease using antagonistic microorganisms isolates obtained from the rhizosphere of the host plants. Data has shown that biocontrol agents assessed *in vitro* have inhibitory activity of *T. longibrachiatum, Penicillium* sp*.* and *Fusarium* sp. against Foa growth, except *Bacillus* sp. which showed a low rate of inhibition. Their excellent biocontrol results mean that *T. longibrachiatum* and *Penicillium* could be used as the preferred strains for the biocontrol of vascular wilt of date palm and other vegetable crops. However it is necessary to combine the research involving *in vitro* and *in vivo* in order to be tested the strains with experimentation on date palm seedlings under field conditions.

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