

Arbuscular Mycorrhizal Fungi Limit Incidence of *Fusarium oxysporum* f.sp. *albedinis* on Date Palm Seedlings by Increasing Nutrient Contents, Total Phenols and Peroxidase Activities

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Abstract: Date palm seedlings derived from Jihel (JHL), a susceptible cultivar to Bayoud disease (fusariosis caused by *Fusarium oxysporum* f.sp. *albedinis*, Foa), were subjected to root inoculation with an arbuscular mycorrhizal fungus (AMF) collected from south Morocco and multiplied on barley as host plant. Successfully colonized plants by mycorrhizal fungi (85 % of treated plants) produced typical intraradical structures (arbuscules, vesicles, hyphae). After ten months of colonization, mycorrhizal plants showed a significant increase in their growth expressed as shoot height, number of leaves per plant, shoot weight, root weight and the total biomass. Mycorrhizal and non-mycorrhizal (controls) date palm seedlings showed great differences in their leaf contents of phosphorus (P), potassium (K^+) and sodium (Na^+). When compared with controls, P increased more than two folds in mycorrhizal plants, while the values of K^+ and Na^+ doubled. When inoculated with Foa by injecting roots with a spore suspension, mycorrhizal (M + Foa) and non-mycorrhizal (C + Foa) date palm seedlings showed significant increases in their root total phenols and peroxidase activities during the first month after inoculation. The highest increases were found in mycorrhizal seedlings accompanied by limited plant death. Mycorrhization alone did not affect significantly total phenols and peroxidase activities during the first week of culture. Plant death decrease in plant lots subjected to root inoculation with the he AMF fungus. As revealed by mycorrhization of date palm seedlings, these results supported the hypothesis that induced resistance to Bayoud disease is mediated by high increases in phenolic compounds and peroxidase activities. These results highlight the importance of mycorrhizal fungi as biocontrol agents to combat Bayoud disease and improve date palm culture in infected palm groves.

Keywords: Arbuscular mycorrhizal fungi, Date palm, *Fusarium oxysporum* f. sp. *albedinis*, Bayoud, growth, mineral nutrition, phenolic compounds, peroxidase.

1. INTRODUCTION

The ubiquitous arbuscular mycorrhizal fungi (AMF) are an integral component of any soil system where they form obligate symbiosis with the roots of over 80 % terrestrial plant species [1].

This symbiosis is based on the beneficial exchange of reduced carbon from the plant and mineral nutrients, especially phosphate and nitrogen as well as water from the fungus [2, 3]. AMF are of particular interest because of their positive effects on plant growth, health and protection against biotic and abiotic stresses [4]. This role of symbiotic associations has been studied in the case of several host plants such as clover [5], *Ocimum basilicum* L. [6], sesame [7], cucumber [8], tomato (*Solanum lycopersicum* L.) [9] and also in the date palm [10-13]. For date palm, Bouamra *et al.* [11] and Apple [14] have reported the role of mycorrhizal symbiosis in improving the uptake of phosphorus, nitrogen and trace elements. Shabbir *et al.* [15] showed that the inoculation of One-year-old *in vitro* seedlings of the date palm variety 'Khenizi' with a commercial AM inoculum, stimulated growth of date palm under salinity conditions.

In the case of plant resistance against biotic stresses, AMF were found to be effective in reducing diseases caused by many pathogens such as *Cylindrocladium*, *Fusarium*, *Macrophomina*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotinium* and *Verticillium* [16].

In date palm (*Phoenix dactylifera* L.), *Fusarium oxysporum* f. sp. *albedinis* (Foa) causes a wilt disease called 'Bayoud'. It is a vascular pathogen that causes drastic reduction in cultivation and expansion of date palm in North Africa, leading to a considerable socio-economical impact. The control of Foa is difficult because chemical treatments are not effective and the prophylactic methods are not of interest due to the contamination of several palm plantations and to their non-durable impact. Therefore, planting resistant cultivars constitutes the only efficient and economic method to control Bayoud disease [17]. Oihabi [18] showed that the inoculation of date palm seedlings with *Glomus mosseae* reduced the Bayoud disease severity. Jaiti *et al.* [12] have also demonstrated the effectiveness of *Glomus monosporus*, *Glomus deserticola* and *Glomus clarum* in improvement of plant growth and reduction of Bayoud disease incidence. However, more data are needed about biochemical and physiological aspects of this interaction in date palm.

Mechanisms by which AMF improve resistance to biotic stresses are multiple and may include competition for infection site and host photosynthates, root damage compensa-

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tion, morphological changes in the host root, changes in microbial communities in the mycorrhizosphere and modifications in the phytohormone balance in the roots of the host plants, with respect to cytokinins, gibberellins, ethylene, abscisic acid (ABA) and jasmonates [19, 20]. Biochemical and physiological modifications may occur, following mycorrhizal colonization. These changes covered the production of phenolic compounds [21, 22], lignin formation [23] and induction of new isoforms of the hydrolytic enzymes, such as chitinase and glucanase [24].

Peroxidases (EC 1.11.1.7) are very active oxidoreductases studied in date palm considering their relationships with cultivar resistance to Bayoud disease [25, 26]. It has been reported previously that certain peroxidase fractions can modulate plant growth, in particular by their IAA oxidase activity [27, 28], while, other enzyme fractions could limit growth by tissue lignification and cell wall rigidification [29, 30]. Peroxidases were found to be involved in plant defenses against many pathogens. For example, reduction of the incidence of the banana *Fusarium* wilt was positively correlated with the induction of defense-related enzymes peroxidase and polyphenoloxidase [31]. As natural substrates of peroxidases and polyphenoloxidases, phenolic compounds and their oxidation products can play a major role in Ginseng resistance to root infecting *Fusarium* species [32]. Several reports reveal that typical plant defense responses against pathogenic microorganisms are also induced upon parasitic plant infection. These include an increase of peroxidase activity, lignification, and cell-wall phenolic deposition [33, 34].

Actually, more information is needed to understand the mechanisms by which AMF act as protectors of date palm against Bayoud disease. In a previous study, our team found that AMF stimulated growth of date palm seedlings derived from BFG cultivar [12] and induce plant protection against Foa by increasing peroxidase and polyphenoloxidase activities. Using another date palm cultivar (JHL), our present work plans to investigate the effect of AMF on plant nutrition and elucidate more details about the peroxidase-mediated protection of date palm against Bayoud disease, in term of root phenolic contents induced by the couple AMF-Foa.

The objective of this work is to study the beneficial role of complex of native AMF coming from the Aoufous date palm grove in the south of Morocco on the improvement of the growth and uptake of nutrient elements and to determine the potential induction by mycorrhization of some biochemical defense reactions that were previously shown to be involved in the mechanism of date palm resistance against *Fusarium oxysporum* f. sp. *albedinis*, causal agent of Bayoud disease, especially defense-related enzymes peroxidases and phenolic compounds [26, 35-38].

2. MATERIALS AND METHODS

2.1. Plant Material and Culture Conditions

Seedlings of cultivar Jihel (JHL) date palm were obtained from seeds disinfected and cultivated in plastic containers filled with a mixture of sterile sand and peat. Then the seeds were incubated for three weeks at 38 °C, before being

transplanted into plastic bags containing sterile soil in a greenhouse under a 16 h light regime at 25 °C. The soil sterilized by autoclaving (90 °C for 12 h) to eliminate native microflora and tested for its chemical and physical characteristics as follows: pH 8.7, humidity 11.03 %, conductivity 477.67 µs/cm, total nitrogen 0.0001 %, total carbon 0.61 %, available phosphor 0.00145 % and exchangeable potassium 0.072 %.

2.2. Biological Material, Growth Conditions and Mycorrhization of Date Palm Plants

The mycorrhizal inoculum consists of Aoufous complex (CAF) which was isolated from soil of palm Tafilalet in the south of Morocco [39]. CAF contains a mixture of native arbuscular mycorrhizal fungi: *Glomus* sp. (15 spores/g soil), *Sclerocystis* sp. (9 spores/g soil) and *Acaulospora* sp. and *Scutellospora* sp. (1 spore/g soil).

Barley was used as host plant for the multiplication of the mycorrhizal inoculums. Barley seeds were disinfected and germinated in plastic pots filled with soil containing mycorrhizal inoculums. After 3 months of culture mycorrhizal, the frequency of the mycorrhization of barley roots were estimated according to Phillips and Hayman [40]. The roots colonized by AMF were used as fresh mycorrhizal inoculums.

Mycorrhizal roots of barley are disinfected with a Strullu solution [41], containing streptomycin and chloramines T to eliminate other microorganisms than mycorrhizal fungi. They are then rinsed 3 times with distilled water and cut into fragments (1-2 mm in length). Then, those fragments (3 g per plant) were applied against the root of date palm plants to inoculate. The control date palm plants were cultivated without barley root fragments in the same conditions. Mycorrhizal plants and Control plants are grown in plastic bags with a diameter of 12 cm containing a soil and incubated under greenhouse conditions. Date palm plants were incubated in greenhouse under a 16 h light regime and 60-70 % RH at 25 °C and were irrigated weekly with 30 ml of Long Ashton-modified Nutrient Solution [42].

2.3. Estimation of AMF Colonization

After 10 months of growth in the greenhouse, the roots of date palm were removed from the soil. Samples of plants were tested for determination of root colonization by AMF. These roots are treated according to the technique of Phillips and Hayman [40]. They were initially placed in 10 % KOH for clearing and stained with 0.01 % trypan blue prepared in a solution of lactoglycerol. Roots were cut into 1cm segments and 150 randomly selected segments were examined under a microscope for the evaluation of frequency (% F) of AMF colonization as described by Trouvelot *et al.* [43].

2.4. Growth Parameters

The plant growth was estimated by calculating the height and the biomass production of shoots and the number of leaves formed per plant. The shoot and root weights of the fresh matter (FW) were measured before drying the material at 105 °C for 24 hours that leads to the weights of the dry matter (DW) for shoot and root.

2.5. Mineral Analysis

Samples for analysis (leaf pieces of mycorrhizal and control seedlings from JHL date palm cultivar) were grounded with porcelain crucibles (1g/ crucibles) and ashed at 450 °C for 4 hours in a mineralizer such as MS Labover Montpellier France followed by dissolution in 20 ml of 5 N hydrochloric acid and evaporated on a hot plate at low temperature. After evaporation, 2 ml of distilled water and 2 ml of 5N HCl were added. The solution was filtered through filter paper and the filtrate volume was adjusted to 50 ml with distilled water. The phosphorus content is measured by the spectrophotometer assay and the levels of sodium and potassium were determined by flame emission spectrophotometry using Flame photometer.

2.6. Effect of AMF Colonization on Induction of Biochemical Defense Reactions

After ten months of colonization of plant date palm by CAF, 30 plants (15 colonized and 15 non-mycorrhizal) were inoculated with the pathogen *Foa* and 30 others were kept as uninoculated healthy controls. The strain used is ZAG *Foa*; aggressive strain isolated from palm spine taken from infected foot with the palm of Bayoud in Draa [44]. Inoculation with *Foa* was performed by injecting 20 µl of a conidial suspension (107 spores /ml) into the main root of each plant. Control plants were maintained under the same conditions but injected with distilled water.

2.7. Analyses of Biochemical Parameters

The kinetics changes of enzyme activities and levels of soluble phenols in root samples were taken from inoculated plants and control plants at 0, 1, 7, 15 days and 1 month after inoculation with *Foa*.

2.7.1. Analysis of Soluble Phenols

Phenolic compounds were extracted and analysed as described by El Hadrami *et al.* [35]. 500 mg of fresh roots were homogenized in 2 ml methanol (80 %) at 4 °C and centrifuged 15 minutes at 9000 g. 50 µl of the phenolic extract were added to 250 µl of Folin-Ciocalteu reagent and 500 µl of a solution of calcium carbonate 20 %. The mixture was incubated in a water bath at 40 °C for 30 minutes and determined by spectrophotometry at 760 nm. The levels of phenolics were expressed as mg equivalent of catechin per g of FW. Three replicates were realized for each assay

2.7.2. Analysis of Peroxidases

Fresh roots (200 mg) were homogenized in 1 ml of Tris maleate buffer (0.1 M, pH 6.5) containing Triton X-100 (0.1 g/l) and centrifuged for 15 minutes at 9000 g. Peroxidase activity was assayed spectrophotometry at 470 nm using guaiacol as substrate; 20 µl of the enzyme extract were added to 2 ml of reaction mixture (Tris-maleate 0.1 M, pH 6.5 + 25 mM Guaiacol). Reaction were initiated with 20 µl of H₂O₂ (10 %) and followed for 3 minutes [12]. Three replicates were realized for each assay.

2.8. Statistical Analysis

The data were statistically analyzed by ANOVA with STATISTICA version 6 [45]. Duncan test [46] was used for

mean comparison. Statistical differences mentioned in the text are significant at $p = 0.05$.

3. RESULTS

3.1. Frequency of Mycorrhization. Microscope Observations

As checked by microscope observations, all mycorrhizal plants were successfully colonized by AM fungi complex *Aoufous* and produced typical intraradical structures (arbuscules (A), vesicles (V), hyphae (H)) as shown in Fig. (1).

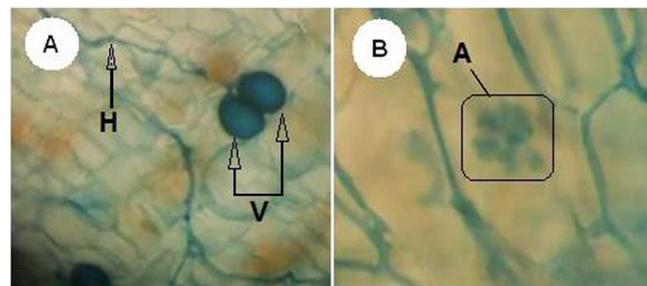


Fig. (1). Observations by light microscopy of date palm seedling roots colonized by AMF (GX 40). **A:** vesicle (V) and hyphae (H) structures; **B:** arbuscule structure (A).

The frequency of mycorrhization of all treated plants reached 85 % and the control plants (non-inoculated) are actually non-mycorrhizal.

3.2. Growth Parameters

After 10 months of mycorrhization, mycorrhizal plants significantly increased shoot height and number of leaves per plant as compared to the control plants (Table 1).

Table 1. Measurements of Shoot Height, Shoot Weight, Root Weight, Total Biomass and Number of Leaves in Date Palm Seedlings from JHL Cultivar Colonized by AMF (Mycorrhized Plants) and Non-Mycorrhized Seedlings (Control Plants) after 10 Months of Mycorrhization with CAF (*Aoufous* Complex). Shoot Weight and Root Weight are Expressed on the Basis of Fresh Matter (FW) and Dry Matter (DW)

Treatments		Control Plants	Mycorrhized Plants
Shoot height (cm)		37.16±0.49 a	44.12±0.56 b
Shoot weight (g)	FW	20.91±2.4 a	30.06±3.2 b
	DW	7.03±0.4 a	11.12±0.5 b
Root weight (g)	FW	12.04±1.2 a	15.39±1.6 b
	DW	3.26±0.2 a	4.04±0.3 b
Total biomass (g)	FW	32.95±3.6 a	45.45±4.8 b
	DW	10.29±0.6 a	15.16±0.8 b
Number of leaves per plant		5.88±0.10 a	6.36±0.08 b

Values are means ± standard error of three replicates from two experiments. For each growth parameters, values followed different letters (a–b) are significantly different at $p = 0.05$ level.

In addition, mycorrhizal plants showed a significant increase in weights of the fresh matter (FW) and dry matter (DW) of shoots (30.06 and 11.12 g) compared with the control plants (20.91 and 7.03 g). A same trend was obtained for roots. Concerning the total fresh and dry biomass, mycorrhizal plants had values (45.45 and 15.16 g) significantly higher when compared with controls (32.95 and 10.29 g) (Table 1).

3.3. Mineral Analysis

Analysis of leaf mineral compositions showed a significant increase in phosphorus (P), potassium (K⁺) and sodium ion (Na⁺) in mycorrhizal plants when compared with control plants (Table 2). The highest increase was obtained for the phosphorus mineral, where mycorrhizal plants showed more than twofold the phosphorus content of control plants.

Table 2. Determination of Total Phosphorus, Potassium and Sodium in Date Palm Seedlings of JHL Cultivar Colonized by AMF and Control

Treatment	[P]	[K+]	[Na+]
Control JHL	0.533±0.118 a	3.645±0.499 a	0.628±0.075 a
mycorrhizal JHL	1.193±0.183 b	5.319±1.312 b	1.076±0.069 b

Values are means ± standard error of three replicates from two experiments. For each treatment, values followed by the same letters (a-b) are not significantly different at p = 0.05 level.

Table 3. Total Phenol Contents in Roots of Mycorrhizal Date Palm Seedlings from JHL Cultivar (M) and Control Plants (C), Compared with Total Phenols Obtained 0, 1, 7, 15 and 30 Days after Foa Inoculations of Mycorrhizal (M + Foa) and Non-Mycorrhizal (C + Foa) Plants, Showing Different Levels in Plant Death

Treatment	Total Phenol Content (mg/g FW)				
	0 day	1 day	7 day	15 day	30 day
C	34.85±5.67 a	31.44±8.94 a	28.80±10.27 a	32.00±6.99 a	33.05±3.03 a
M	32.27±8.32 a	36.65±8.99 a	25.64±5.78 a	41.47±21.04 ab	39.03±5.73 a
C+Foa	41.76±4.93 a	42.01±14.63 a	44.18±6.07 a	58.85±11.94 bc	97.86±4.18 b
M+Foa	43.41±11.55 a	48.65±7.33 a	54.60±11.59 a	106.35±7.91 c	136.43±11.51 c
Plant death (C+Foa)	0 % (-)	0 % (-)	0 % (-)	4.6% (+)	10.3 % (++)
Plant death (M+Foa)	0 % (-)	0 % (-)	0 % (-)	3.2 % (+)	4.8 % (+)

Values are means ± standard error of three replicates from two experiments. For each treatment, values followed by the same letters (a-c) are not significantly different at p = 0.05 level.

Table 4. Peroxidase Activities in Roots of Mycorrhizal Date Palm Seedlings from JHL Cultivar (M) and Control Plants (C), Compared with Peroxidase Activities Obtained 0, 1, 7, 15 and 30 Days after Foa Inoculations of Mycorrhizal (M + Foa) and Non-Mycorrhizal (C + Foa) Plants

Treatment	Peroxidase Activity (U/g FW)				
	0 day	1 day	7 day	15 day	30 day
C	161.05±10.22 a	239.89±61.63 a	384.84±130.01 a	372.53±24.31 a	297.10±43.35 a
C+Foa	210.76±66.25 a	321.42±52.88 a	332.00±96.85 a	522.67±64.49 ab	631.93±10.92 ab
M	243.12±178.93 a	236.84±118.8 a	382.98±46.56 a	465.57±16.56 bc	443.37±140.27 b
M+Foa	293.59±202.86 a	367.62±11.62 a	457±111.91 a	661.53±5.28 c	1031.95±32.42 c

Values are means ± standard error of three replicates from two experiments. For each treatment, values followed by the same letters (a-c) are not significantly different at p = 0.05 level.

Percentages of plant death are the same as presented in Table 3.

3.4. Total Phenols and Peroxidase Activities. Effect of Inoculating Mycorrhizal and Non-Mycorrhizal Date Palm Seedling with *Fusarium oxysporum* f. sp. *albedinis*, the Causal Agent of Bayoud Disease

Results presented in Table 3 showed that mycorrhizal and not mycorrhizal date palm seedlings exhibited no substantial changes in their root contents of total phenols during the first month of culture. However, Fifteen days after Foa inoculation, seedling roots started to show significant increases in the amount of total phenols in mycorrhizal and non-mycorrhizal plants. In addition, this period was marked by the appearance of external symptoms typifying Bayoud disease (whitening and wilting leaves). This increase is more important in the mycorrhizal plants and was more pronounced 30 days after the pathogen infection where The Total phenol content reached 136.43 mg/g FW in mycorrhizal plants and only 97.86 mg/g FW in non mycorrhizal plants. Limited plant death observed in mycorrhizal seedlings, coincided with high root phenolic contents induced by Foa inoculation.

Regarding root peroxidase activities, the tendency of changes was found to be similar to root phenolic contents. Thus, peroxidase activities of date palm seedlings were not significantly affected by mycorrhization (Table 4). However, Foa infection significantly enhanced this activity in mycorrhizal plants (M + Foa) than non-mycorrhizal (C +

Foa) ones. Thus, 15 days after Foa inoculation, mycorrhizal plants high peroxidase activity (661.53 U /g FW) than non-mycorrhizal seedlings (522.67 U /g FW). This difference becomes higher after 1 month of Foa inoculation, when enzymes activities reached 1031.95 and 631.93 U/g FW for mycorrhizal and non-mycorrhizal plants, respectively. As mentioned for total phenol variations (Table 3), limited plant death fits with high levels of peroxidase activities induced by Foa in mycorrhizal plants.

4. DISCUSSION

Considered as a contribution to protect date palm (*Phoenix dactylifera* L.) against Bayoud disease, this study highlights physiological and biochemical aspects of date palm AMF mycorrhization, which could enhance resistance through the improvement of growth (nutrient supply) and the activation of the defense mechanisms of host plants against *Fusarium oxysporum* f. *albedinis* sp, the causal agent of Bayoud disease.

Ten months after installation of mycorrhization, microscope observations confirmed the success of plant colonization by AM fungi complex Aoufous. Typical intraradical structures (arbuscules (A), vesicles (V), hyphae (H)) were observed as shown in figure 1. Date palm seedlings derived from JHL cultivar (susceptible variety to Bayoud disease), showed a high frequency of mycorrhization (85 %). This data is in accordance with previous results obtained by our team who reported a frequency of mycorrhization of the same AMF up to 83.33 % obtained for BFG, another Bayoud-susceptible date palm cultivar [12]. These data reflect the ability of species forming the complex CAF to colonize the root system of date palm.

After ten months of colonization, mycorrhizal plants showed a significant increase in their growth expressed as shoot height, number of leaves per plant, shoot weight, root weight and the total biomass. This plant response to mycorrhization has been obtained for many other plant species, such as *Cupressus atlantica* [47] and *Citrus tangerine* [48]. The stimulated growth of Mycorrhizal date palm seedlings was accompanied by increases in their leaf contents of phosphorus (P), potassium (K⁺) and sodium (Na⁺). This positive effect of AMF on the various parameters of growth can be explained by improving the nutritional status of date palm, including mineral nutrition. This growth stimulation was also reported in other plants such as strawberry [49], licorice [50] and alfalfa [51]. It was attributed to increases in the capacity of host plants to explore a larger soil volume leading to more nutrient assimilation [52].

In the second part of this work, we reported for the first time that limited death of mycorrhizal seedlings derived from the date palm cultivar JHL and inoculated with Foa (the causal agent of Bayoud disease), was found to be associated with significant increases in root phenolic contents. Changes in phenolic contents have been reported before in other fusariosis, such as the tomato wilt [53], where *Fusarium oxysporum* strain CS-20, previously shown to reduce the incidence of *Fusarium* wilt of tomato (biocontrol) through an uncharacterized host-mediated response, acted by alterations in tomato secondary metabolism (phenolic compounds) that

are typical of resistance responses against *Fusarium oxysporum* f. sp. *lycopersici*.

Studying the effects of mycorrhizal fungal inoculations of *Glomus* species on phenolic compounds and pathogenesis related proteins in pepper - *Phytophthora capsici* that plant pathosystem, Ozgonen *et al.* [54] found that total phenolic compounds increased in all treatments as compared to non mycorrhized non pathogen treated control, but was highest when plants were inoculated with both, the mycorrhizal fungi and the pathogen.

The present work showed that changes in the activities of peroxidase extracted from roots of the date palm cultivar JHL followed a same trend as changes of phenolic contents. This finding complete data previously published by our team [12], where changes in phenolic contents have not been studied. However, qualitative aspects of induced phenolic compounds (hydroxycinnamic acid derivatives) have been reported in mycorrhized date palm seedlings challenged with Foa [38]. The increased level of phenolics could provide an adequate substrate to oxidative reactions catalysed by POD and/or PPO that, consuming oxygen and producing fungitoxic quinones, make the medium unfavourable to the further development of pathogens, as suggested by Lattanzio *et al.* (2006) [55]. In the infectious process, plant cells usually respond by increasing the level of pre-existing antifungal phenols at the infection site, after an elicited increased activity of the key enzymes (PAL and chalcone synthase) of the biosynthetic pathway [55, 56]. It was demonstrated that Stems of resistant flax lines contained a higher content of total phenols than susceptible ones upon pathogen challenge. The activity of peroxidase, polyphenyl oxidase, catalase enzymes as well as proline content were significantly increased in powdery mildew infected leaves of flax lines as compared with either resistant or susceptible parents [57].

In conclusion, these results are indicative of the important role played by the AMF associated with date palm seedlings in their development and their resistance to Foa. The effectiveness of the CAF mycorrhizal association acted on two levels; i) Enhancing nutrient supply, in particular P and K elements that stimulate growth and, ii) Stimulation of defense mechanisms through increasing total phenols and peroxidases activities. This mycorrhizal association may be used as a promising strategy to develop tools to fight against Bayoud disease.

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CONFLICTS OF INTEREST

The manuscript submitted has been prepared according to the journal's 'Aims and Scope' and 'Instructions for Authors', and checked for all possible inconsistencies and typographical errors.

On submission of the manuscript, the authors agree not to withdraw their manuscript at any stage prior to publication.

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