PHENYLACETIC ACID: A CHEMICAL MARKER OF *Fusarium oxysporum* f. sp. *albedinis* IN LEAFLETS OF SUSCEPTIBLE CULTIVAR OF ALGERIAN DATE PALM

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Abstract. This study aims to identify the secondary metabolites produced by *Fusarium oxysporum* f. sp. *albedinis* (Foa) in leaflets and roots of two date palm cultivars. One sensitive (Tgaza) and other resistant (Takerboucht) to fusariosis, as chemical markers for contamination. The determination of the chemical compounds was carried out by the gas chromatography coupled to mass spectrometry (GC-MS). The crude extracts of healthy leaflets contain low concentration of Phenylacetic Acid (PAA); however, the susceptible ones infested with Foa, contains high level of this compound. PAA was not detected in the roots of both healthy and diseased cultivars. We also tested the effect of this compound on mycelial growth and virulence factors (number of conidia and synthesis of fusaric acid) of Foa. At 10^{-2} g·L^{-1}, PAA exerts inhibitory effects on the growth of mycelia and on virulence factors. At 10^{-3} g·L^{-1}, phenylacetic acid stimulates the growth of mycelium from 1 to 7 days and fusaric acid is inhibited at the 10th day. In contrast, the PAA increases the number of conidia and the production of fusaric acid at 20 and 30th days. This investigation suggests that depending on the concentration and the incubation time, PAA could have an important role in pathogenesis. Thus, it is possible to conclude that the accumulation of PAA compound in date palm samples is a qualitative indication of contamination by *Fusarium oxysporum* f. sp. *albedinis*.

Key words: bayoud disease; *Fusarium oxysporum* f. sp. *albedinis*; GC-MS analyses; phenylacetic acid; *Phoenix dactylifera* L.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit crops in the Middle East and North Africa, esteemed for its delicious and nutritious fruit, commonly known as dates, which are enjoyed fresh or processed into various products [5]. In Algeria, date palm cultivation spans over 167,269 hectares, hosting approximately 18.5 million palm trees, yielding a production of over 1 million tonnes [18]. Despite the identification of more than 1000 cultivars, only a handful hold commercial significance, with the Algerian cultivar "Deglet Nour" dominating production, constituting about 53% of total date palm yield [25].

The cultivation of date palms faces significant challenges, particularly from the dangerous bayoud disease caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *albedinis* (Foa), which detrimentally impacts date production, a staple food for humans and animals in the North African desert. Notably, cultivars like Deglet Nour (DN) and Tgaza (TG) exhibit high susceptibility to Foa, whereas the Takerboucht (TK) cultivar demonstrates natural resistance against this pathogen.

Foa is classified as an "A2" quarantine pest by EPPO [22] and falls under the "A" category of pests requiring mandatory monitoring and control in Algeria [6].

Foa propagates through three types of asexual spores: microconidia, macroconidia, and chlamydospores, with its sexual or teleomorphic stage remaining elusive (Fig. 1). *F. oxysporum* can endure saprophytically in soil and plant debris in the absence of a chlamydospore host. Upon favorable conditions, chlamydospores germinate; invade the roots and traversing vascular tissues via cell wall-degrading enzymes (CWDEs). Within the vessels, the mycelium generates microconidia, transported upwards by the sap stream, ultimately leading to the demise of the tree upon the fungus and its toxins reaching the terminal bud [11, 14]. External symptoms of vascular wilt in date palms include unilateral bleaching and gradual leaf desiccation, resulting in tree mortality within 6 to 24 months (Fig. 2).

Throughout their development on host tissues or in culture, fungi can produce a wide range of toxic compounds with varied biochemical structures and modes of action [15].

Phytotoxins are some of the best studied virulence factors of phytopathogenic fungi and these secondary metabolites can be useful for the prediction of Foa virulence on date palm.

Many toxins (fusaric acid, succinic acid, 3-phenyllactic acid, peptidic toxins and phenylacetic acid) are *in vitro* synthetized by Foa [2, 17, 29]. Only the role of fusaric acid was determined in the pathogenesis process in date palm by Bouizgarne *et al*. (2004) [10].

Phenylacetic acid (PAA) is a volatile aromatic compound naturally produced by many organisms with antimicrobial, antifungal, and phytotoxic activities [30]. PAA is also endowed with hormonal activity similar to IAA in planta [28].

This compound is also isolated from the mycelium and culture filtrates of *F. oxysporum* Schlecht. Emend. Snyd. and Hans [1], *Rhizoctonia solani* [12], *Azospirillum brasilense* [27] and some species of *Bacillus* [23] as well as sclerotia of *R. solani* [4].

The results of Ait Kettout and Rahmania (2010) [2] showed that PAA was produced by strains of Foa, 22 days after inoculation of the medium Czapeck Glucose broth. It was absent in *Fusarium oxysporum* f. sp. *cubense* (NRLL 26022) and *Fusarium oxysporum* f. sp.

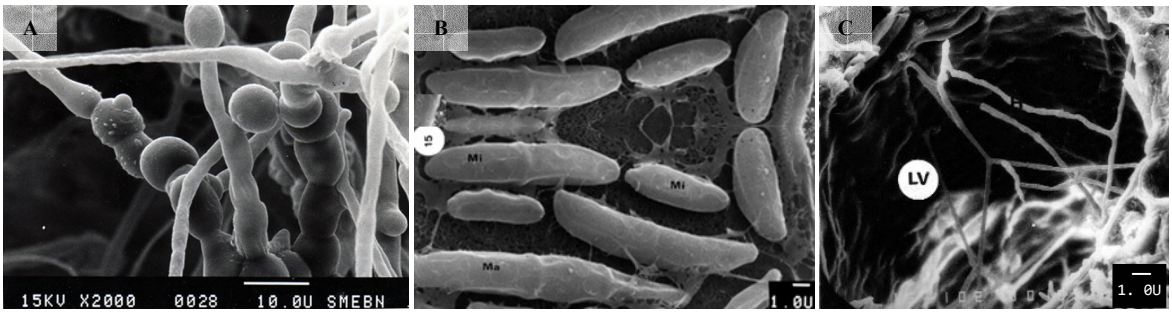

Figure 1. Reproductive organs of *Fusarium oxysporum* f. sp. *albedinis*. A: chlamydospores formed from mycelium (X 2 000), B: Microconidia (Mi) and Macroconidia (Ma) (X 6 000), C: multiplication of mycelium inside the vessels (X 1 000).

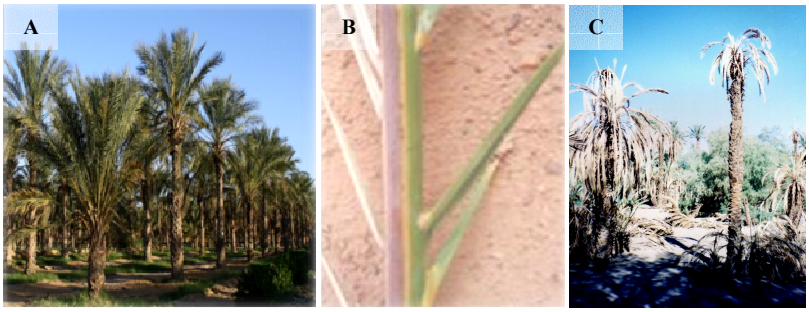

Figure 2. Symptoms of vascular fusariosis of date palm (Bayoud) infected by *Fusarium oxysporum* f. sp. *albedinis*. A: Healthy date palm, B: unilateral bleaching of a date palm leaflet**,** C: diseased date palm

lycopersici (NRLL 38554), pathogens of bananas and tomatoes respectively. There is little information to explain why fungi produce this compound, or what effect it might have at the cellular level.

Plant pathogens have evolved several strategies to manipulate the biology of their hosts to facilitate colonization, growth to high levels in plant tissues, and production of disease. One of the less well known of these strategies is the synthesis of plant hormones and hormone analogs, and there is growing evidence that modulation of host hormone signaling is important during pathogenesis [24].

In order to be able to detect these pathogens as soon as possible, we adopted by our laboratory a comparative profile between the host plant and the causative agent.

The chemical analysis of the compounds produced by the date palm, host plant of Foa, before and after infection was performed by Gaceb-Terrak and Rahmania (2010, 2013) [19, 20] and by Boucenna - Mouzali *et al*. (2018, 2021) [8, 9].

This investigation relates (i) the isolation and identification by GC-MS, of phenylacetic acid, from leaflets and roots of susceptible (Tgaza) and resistant (Takerboucht) cultivars growing in the uninfested and infested soils (ii) determination of effect of this secondary metabolite on the mycelial growth and on the virulence factors of *Fusarium oxysporum* f. sp. *albedinis.*

MATERIALS AND METHODS

Plant material

Leaflets, roots of healthy, and diseases female date palms 10 to 15 years old, were collected from the palm groves well irrigated and not subjected to any chemical treatment. The palm groves were located in the experimental station of INRA-Adrar, southwestern Algerian Sahara (27°54' North, 0°17'1" West).

Two date palm cultivars; Tgaza (TG), susceptible and Takerboucht (TK) resistant to Bayoud. The leaflets and roots of both cultivars were dried in the open air and at room temperature of 25 °C, at shade and then milled for the chromatographic analysis by GC-MS.

Pathogen Strains

The virulent strain of *Fusarium oxysporum* f. sp. *albedinis* (NRRL 38298), was isolated from rachis of fusariosis affected palms, and then stored on PDA medium at 4 °C. This strain was received from the Laboratory of Northern Region Research of United States Department of Agriculture (USDA).

Secondary metabolites

Three concentrations: 10^{-2}, 10^{-3} and 10^{-4} $g{\cdot}L^{-1}$ of PAA (phenylacetic acid, Sigma Aldrich 99%) were tested.

Culture media composition

Different culture media were tested: PDA medium (Potato-Dextrose-Agar) for seeding strain Foa (Table 1), Czapek Glucose Yeast Agar (CZGYA) to check the effect of phenylacetic acid (PAA) on the apical growth of the strain Foa, and Czapek Glucose Yeast Broth (CZGYB) medium to assess the effect of this metabolite on sporulation and production of fusaric acid (Table 2).

Table 1. Composition of Potato dextrose Agar medium (PDA)

Ingredients	Concentrations $g{\cdot}L^{-1}$
Dextrose	20
Potato extract	4*
Agar	20

*4g of potato extract is equivalent to 200g of potato infusion. Autoclave sterilization at 120°C for 20 minutes.

Table 2. Composition of Czapek Glucose Yeast Agar medium

Ingredients	Concentrations g·L^{-1}
$NaNO_3$	3
KH_2PO_4	1
KCl	0.5
$MgSO_4$, $7H_2O$	0.5
$FeSO_4$,$7H_2O$	0.01
Saccharose	30
Glucose	8
Yeast extract	2
Agar	20

Extraction and separation of organic substances

2.5 g of dry plant material was macerated in 75 mL distilled water on agitator Heidolph Promax 2020 at 148 rpm for 24 hours. After filtration, the organic phase was mixed with 3 g sodium hydroxide pellets (NaOH), this alkaline hydrolysis releases the organic substances from their ester combinations (-CO-O-C-); it is carried out for 2 hours at room temperature in the presence of nitrogen gas in order to avoid any oxidation of these substances in an alkalinemedium. After saponification of the organic phase, pure hydrochloric acid (HCl) is added to the filtrate in order to obtain a twice normal solution (2N). Acid hydrolysis breaks the acetal bonds (-C-O-C-) involved in heterosidic-type combinations [19]. The organic substances were separated from the aqueous phase with diethyl ether (40 and 30 mL) using a separatory funnel. The ethereal extract containing PAA is filtered and then evaporated; the dry residue taken up in 2 mL of pure methanol (MeOH) is intended for subsequent analysis by gas chromatography (GC) coupled with mass spectrometry (MS). Two chromatographic analyses are carried out per sample.

Detection and identification of phenylacetic acid by GC-MS method

Helium was used as a gas carrier and flowed at 1 mL·min-^{1} through a HP5 -MS capillary column (30 m x 0.25 mm x 0.25 µm, Hewlett Packard 6890). Analytical temperatures were set as follows: initial column temperature at 60 °C for 2 min, followed of 6 °C/min with a final temperature at 270 °C for 5 min. Compounds were further ionized at 70 eV and analysed by scanning mass spectrometry over a range of 40–600 m/z per scan using an ion trap mass spectrometer (5973 model).

The identities of compounds were verified by comparing them with the chemical databases deposited in the GC-mass spectrometry library and by referring the National Institute of Standards and Technology (NIST). Data processing was performed using the Origin 6.0 software (Data Analysis and Technical Graphics).

Thermostable volatile compounds are detected according to their elution order, retention time (RT) and recognition rate (%); the identification of the separated compounds is validated by mass spectra.

Measurement of Foa colony growth and sporulation

To determine the effect of PAA on mycelia growth of Foa (NRRL 38298) strain, a 5 mm disk is taken from a 7 day old PDA culture is inoculated into the center of a plate contained the Czapek Glucose Yeast Agar (CZGYA) media and PAA at 10^{-2} g·L^{-1}, PAA 10^{-3} g·L^{-1} and PAA 10^{-4} g·L^{-1}, or only of CZGYA as the control. These plates incubated at 27 °C for 6 days (d). Colony diameter was measured in two directions on each plate after incubation for 1 to 6 d. Three Petri dishes and two repetitions are performed for each concentration of PAA.

Sporulation is determined following the grown of *Fusarium oxysporum* f. sp. *oxysporum* in 250 mL flasks filled with 20 mL Czapek Glucose Yeast broth (CZGYB) and adjusted to pH 4.5, containing a 13 mm agar plug taken from a 7 day old PDA culture and PAA at concentration 10^{-2} g·L^{-1} and 10^{-3} g·L^{-1}. Incubation takes place at 27 °C. The broth was filtered to collect conidia. The number of conidia was counted the 10th, 20th and 30th days using the Malassez cell. Three flasks were used and three replicates were prepared for each PAA concentration.

Extraction and determination of fusaric acid

Fusaric acid (FA) production was determined from the growth of Foa (NRRL 38298) strains in Czapek Glucose Yeast broth the 10th, 20th and 30th days. For obtained the supernatant containing mycotoxin, each culture was filtred using whatman n°1. FA was sequentially extracted three times (3 min each) with 20 mL of ethyl acetate in a separating funnel. The ethyl acetate extracts were pooled and then evaporated by a rotator evaporator at 40 °C until dryness. The residue was dissolved with 3 mL of methanol (PA grade) and filtered by syringe fitted with a Poly-Tri-Fluoro-Ethylene (PTFE) filter 3 mm in diameter and 0.45 µm in porosity. FA in each sample were quantified with UV-Vis spectrophotometry (Shimadzu, Japan) at the absorbance λ = 270 nm, on the calibration curve of FA standard (25 mg·mL^{-1} of distilled water) according to the method of Edge (2005) [16].

Statistical analysis

All data were reported as means ± standard error of the mean (SEM). Differences between the control and each exposure group (tests) were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's HSD test at 5% level of significance using the computer software XL STAT 2021.2 for mycelial growth and student test for conidies number and production fusaric acid.

RESULTS

Identification of phenylacetic acid in date palm

The analysis of the leaflets and roots methanol extracts of date palm cultivars by GC-MS was performed using a gas chromatograph Hewlett Packard 6890 coupled to a mass spectrometer electron impact HP5973 type. The identification of chemical compounds was considered by comparing the mass spectra obtained with those of the NIST98 library (National Institute of Standards and Technology). Chromatograms obtained revealed several peaks labeled with their retention times. One of these peaks

corresponds to a phenolic compound, identified as Benzeneacetic acid, alpha., 3,4-tris [(trimethylsilyl)oxy]-,trimethylsilyl ester derivative of benzeneacetic acid or phenylacetic acid (PAA), with a retention time of 21.65 min (Fig. 3). This compound was more abundant in infected leaflets (10.5%) compared to the uninfected sensitive cultivar (1.4%), (Fig. 4). PAA was substantially similar in resistant leaflets of date palm obtained from infected and uninfected soils by Foa. This compound was not identified as well as in healthy than in diseased roots of two cultivars studied. In uninfected palm groves infested, the resistant date palm cultivar contained a weak level derived from PAA in leaflets. In the leaflets of susceptible cultivars with bayoud symptoms, we noted a remarkable increase of derived from PAA content during the infection.

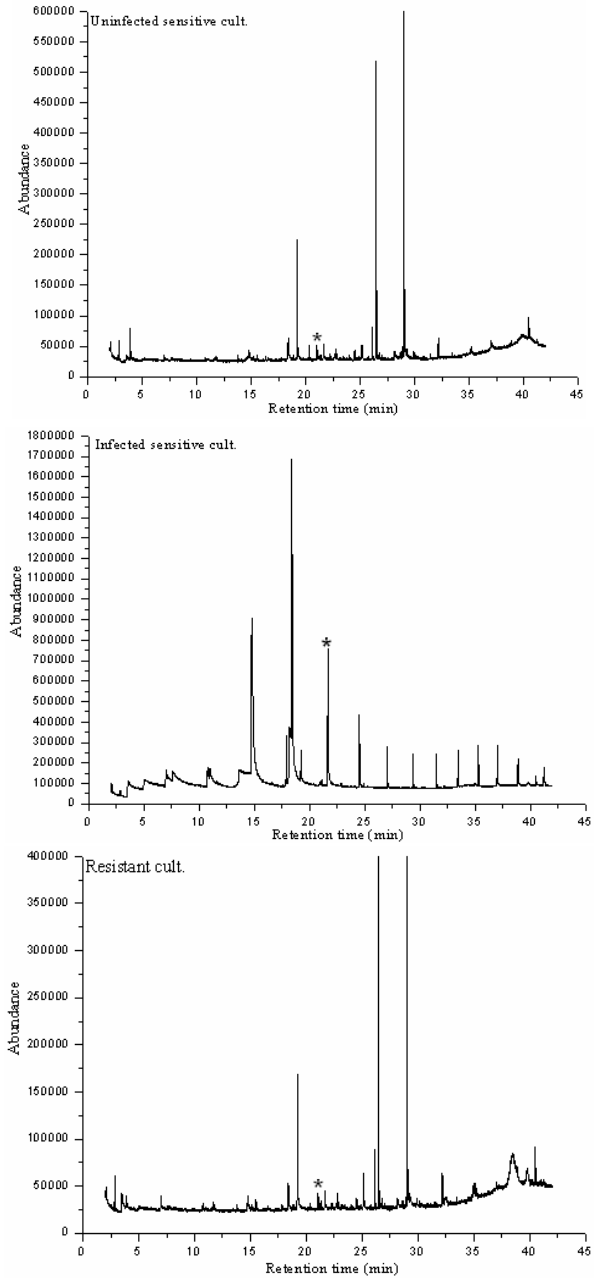

Figure 3. GC-MS chromatograms of leaflets extracts of sensitive and resistant cultivars. *: PAA; cult.: cultivar

Our findings indicate that PAA did not play an important role in date palm defense mechanisms against Foa, because its accumulation in susceptible cultivars as a response to pathogens did not block the progression of the parasite.

Based on the results obtained, we suggest that the accumulation of the PAA derivative in diseased leaflets of the susceptible cultivar would be of fungal origin.

Phenylacetic acid effect on the mycelial growth Foa

The growth of the fungal colony in the presence and absence of the phenylacetic acid (PAA) was measured daily for 6 days. The results were expressed in Figures 5 and 6. The inoculation of Foa on Czapek - Glucose Agar medium added with PAA at 10^{-3} g·L^{-1} showed that mycelia growth stimulates 1 at 6 d of incubation. However, in the presence of the PAA at 10^{-4} and 10^{-2} g·L^{-1} inhibited Foa growth. This inhibition was more severe at the high concentration.

These results show that the effect of phenylacetic acid on the mycelial growth is depending on the PAA concentration used in the culture medium.

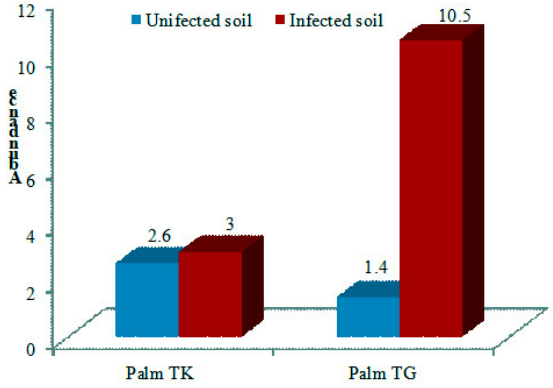

Figure 4. The phenylacetic acid rate in palms of date palm in presence or absence of Foa in soil. TK (r): Takerboucht resistant cultivar, TG (s): Tgaza sensible Cultivar

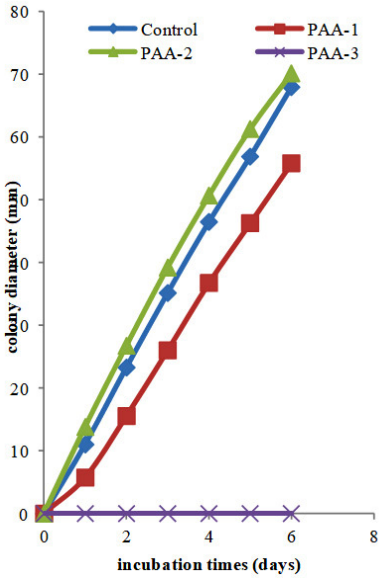

Figure 5. Effect of phenylacetic acid (PAA) on colony growth of *Fusarium oxysporum* f. sp. *albedinis* strains (NRRL 38298) for 7 days on in Czapek-Glucose-Agar plates. PAA-1:10^{-4} g·L^{-1}, PAA-2: 10^{-3} g·L^{-1}, PAA-3: 10^{-2} g·L^{-1}.

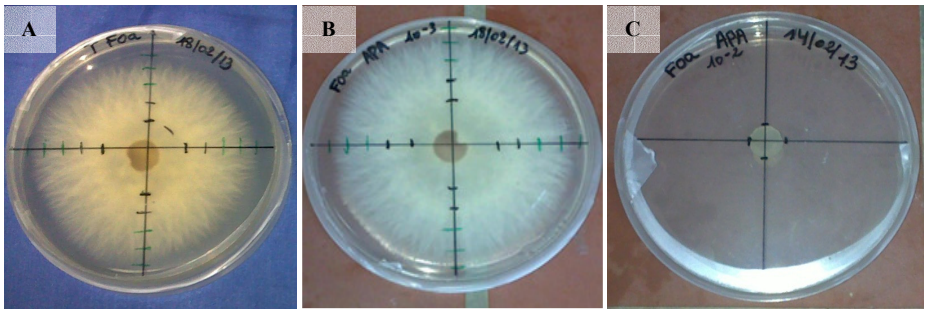

Figure 6. Diameter of Foa colonies with or without various concentrations of PAA in Czapek-Glucose Agar medium after 4 days of incubation at 27 °C, where: **A** - Control, **B** - PAA: 10^{-3} g·L^{-1}, **C** - PAA 10^{-2} g·L^{-1}.

Fungal growth data was recorded of three replicated cultures and four measurements per culture. The significant differences between treatments and control, were expressed by ANOVA Test ($\alpha = 0.05$)

Phenylacetic acid effect on sporulation

Figure 7 shows that in absence of PAA (Control), the conidia number was 58.10^3 conidia per mL at 10th, and then decreased progressively with the incubation time. In fact, it passed to 52.10^3 and then to 36.10^3 conidia per mL, at 20th and 30th days of incubation respectively. After addition of PAA at 10^{-3} g·L^{-1}, at Czapek-glucose-liquid medium, we observe a stimulating of 57% of conidiogenesis at the 30th day compared to the control. However, at 10^{-2} g·L^{-1}, this compound completely inhibited the sporulation.

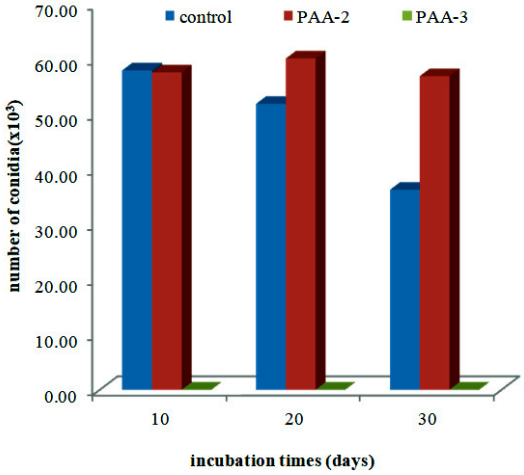

Figure 7. Effect of phenylacetic acid (PAA) on sporulation by *Fusarium oxysporum* f. sp. *albedinis* strains (NRRL 38298) after liquid Czapek- Glucose culture for 6 days. PAA-2: 10^{-3} g·L^{-1}, PAA-3: 10^{-2} g·L^{-1}. Data are represented as the mean conidia production of three replicated cultures.

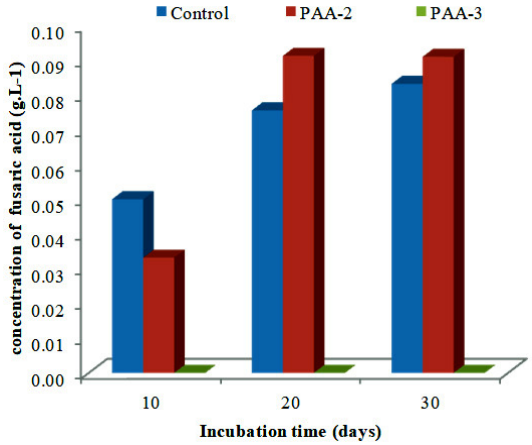

Figure 8. Effect of phenylacetic acid (PAA) on Fusaric acid (FA) production by *Fusarium oxysporum* f. sp. *albedinis* strains (NRRL 38298) after liquid Czapek-Glucose culture for 6 days. PAA-2: 10^{-3} g·L^{-1}, PAA-3: 10^{-2} g·L^{-1}.Data are represented as the mean of mycotoxin production of three replicated cultures

Phenylacetic acid effect on fusaric acid production

The variation of the production of fusaric acid by Foa on Czapek-Glucose-liquid medium with time (Fig. 8), showed a value of 0.08 g·L^{-1} at 30th day of growth. Similar results were obtained by treatment with PAA at 10^{-3}g·L^{-1} with 0.09 g·L^{-1} at 20th and 30th days.

These observations informed us that the use of PAA 10^{-3} g·L^{-1} reduces the production of fusaric acid by 40% at 10th day, and then, stimulates it with 12% at 20th and 30th days of incubation compared to control (Fig. 8). PAA has a biostatic effect on Foa. However, at 10^{-2} g·L^{-1}, this compound completely inhibited the production of fusaric acid.

DISCUSSION

Throughout their co-evolution, plants and pathogenic microorganisms have developed complex relationships resulting from a constant exchange of molecular information. Pathogens have developed a whole range of offensive strategies to parasitize plants, and in return, plants have deployed a defensive arsenal.

PAA has a broad distribution and has been studied in bacteria, fungi, algae and land plants. To our knowledge, limited information is available on the specific role of this compound in the development of the disease symptoms and pathogen host response. PAA is a volatile aromatic compound naturally produced by many organisms with antimicrobial, antifungal, and phytotoxic activities [13].

In this work, GC-MS analysis identifies the phenylacetic acid (PAA) derivative from the crude extracts of healthy and diseased leaflets of sensitive cultivar (Tgaza) and of resistant (Takerboucht).

In plants, PAA acts as a growth promoter and typically has a positive role in plant development. In contrast to this, PAA has been described as a phytotoxin [13]. Indeed, the results obtained have shown a high accumulation of PAA in the diseased leaves of Tgaza compared to healthy ones. The high

level of this compound was implicated as the phytotoxic substance in date palm fusariosis disease. Similar results were obtained by Kawazu (1996) [23] on *Pinus thunbergii*. Park (1977) [26] shown that PAA causes degenerations of organelles in leaf cells of Japanese Pear, which would explain probably the bleaching of the date palm leaves. It is difficult to assert that PAA alone is responsible for date palm wilt disease.

However, Akram *et al*. (2016) [3] suggested that PAA plays a key role in the induction of plant systemic resistance, not demonstrated in this study.

According to the results obtained, the accumulation of PAA is of fungal origin. We can assume that the phenylacetic acid produced by the fungus modifies the endogenous level of free IAA (indole acetic acid) and/or salicylic acid in the diseased plant, which reduced its defense mechanisms. In effect, PAA is a secondary metabolite derived from the phenylalanine, the precursor of indole acetic acid (IAA) and salicylic acid.

In several cases, the pathogen itself produces auxin, and in these interactions, auxin can be viewed as a virulence factor. However, in other interactions, the pathogen stimulates auxin accumulation or auxin signaling in the host through the action of virulence factors that have evolved to modulate host auxin biology [24]. PAA has been recognized as natural auxin [28]. Elevated auxin levels can antagonize plant defense reactions and promote pathogens infections [30].

It is possible that the accumulation of PAA in the leaves of the susceptible cultivar stimulates the virulence factors of Foa. So, we tested *in vitro* the effect of PAA on mycelial growth, the number of conidia and the production of fusaric acid by Foa.

Our results show that the effect of phenylacetic acid on mycelia growth, sporulation and fusaric acid production is depending of the PAA concentration used in the culture medium. PAA at 10^{-3} g·L^{-1} stimulates the colonies diameter of Foa, conidia and the phytotoxin fusaric acid production.

We can therefore assume that the PAA increases the risk of infection in the host (date palm) by the pathogen. These results are similar to those of Bartz *et al.* (2012) [7] who revealed that PAA metabolic is involved in *Rhizoctonia* disease development. This result is in agreement with the established ability of the fungus to produce and metabolize PAA [2].

PAA at 10^{-2} g·L^{-1} completely inhibits the mycelia growth, sporulation and fusaric acid production. Similar results were obtained by Bartz *et al*. (2012) [7] and Hwang *et al*. (2001) [21]. Hwang *et al*. (2001) [21] have indicated that the growth of *Rhizoctonia. solani* was completely inhibited by 0.4 mM PAA, then 73.5 mM can inhibit the growth of *Pythium ultimum*, while concentrations upper than 7.35 mM have no effect on *Alternaria mali*. Bartz *et al*. (2012) [7] have shown that 7.5 mM of PAA had a significant effect on mycelial dry weight of *Rhizoctonia solani* with the mean percent reduction exceeded 50% in mycelial dry weight.

The antimicrobial properties of PAA, suggests that this compound might affect the disease ecology of Foa via multitrophic interactions, conferring a competitive advantage to Foa over other microbes of the rhizosphere. Note that the action of phenylacetic acid on date palm-Foa pathosystem depends on its concentration in their cells.

The occurrence of plant diseases is the consequence of plant–pathogen interactions. Pathogen effectors that modulate plant immunity or interfere with plant cellular functions are considered to be the main mediators of host–pathogen interactions [30].

The results obtained showed that Foa use many strategies to promote infection date palm. One of them is the secretion of Phenylacetic acid which mimics auxin and hijack the cellular machinery to disrupt date palm cell functions.

Also, Foa synthesizes of PAA, non host specific phototoxic to stimulate mycelial growth, conidia and fusaric acid mycotoxin production. Accumulation of PAA in planta can antagonize plant defence reactions and promote pathogen infection. It would be interesting, to study the effect of PAA on the reactions of defences of host plant date palm to understand the interaction date palm- pathogenic agent, this work is in progress. Thus, it is possible to conclude that the presence of PAA in diseased date palm organs is a qualitative indication of contamination by *Fusarium oxysporum* f. sp. *albedinis* and can be used as chemical markers of the fungus.

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