MOBILIZATION OF TRANS-CINNAMIC ACID, PRECURSOR OF LIGNINS IN DATE PALM ROOTS OVER A COMPATIBLE INTERACTION WITH THE PATHOGENIC AGENT OF BAYOUD DISEASE, FUSARIUM OXYSPORUM F. SP. ALBEDINIS

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ABSTRACT

Bayoud disease of date palm Phoenix dactylifera L. is the result of specific interactions established between this plant and Fusarium oxysporum f. sp. albedinis (F.o.a.). Facing this aggression, the host plant uses its constitutive preventive system and also defense mechanisms triggered by the pathogen. Our work is based on the identification of secondary metabolites produced during this confrontation in date palm roots. Our results show that during infection, susceptible cultivar accumulates considerable proportions of para-hydroxybenzoic acid. In soils infested by F.o.a., a significant decrease of para-hydroxybenzoic acid and an accumulation of para-hydroxycinnamic acid were observed in the roots of resistant cultivar. The analysis we realized shows that preferential orientation of the precursor “trans-cinnamic acid” of phenolic metabolism is activated in the root in contact with the causative agent of Bayoud. It also shows the presence of a greatest amount of lignin in resistant cultivar roots when compared with the susceptible ones.

Keywords: date palm roots; bayoud disease; host-pathogen interaction; phenolic acids; lignin.

Abbreviations: ASL (acid soluble lignin) and AIL (acid insoluble lignin), CWR (cell wall residue), F.o.a. (Fusarium oxysporum f. sp. albedinis) TK (Takerbucht), TG (Tgaza).

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1. INTRODUCTION

The date palm Phoenix dactylifera L. is a plant of considerable socio-economic importance for the populations of the Algerian Sahara. Its fruit, the date, occupies a paramount place in the food and has significant agro-alimentary possibilities and can be preserved and consumed all over the year; its seeds can be consumed by animals.

Many diseases affect the date palm, the bayoud or vascular wilt is the most dangerous one; it threatens its culture and productivity [1,2]. The causative agent is Fusarium oxysporum f. sp. albedinis (F.o.a.), imperfect fungus of the soil mycoflora.

The resistance mechanisms of date palm against this infection are few known; they are generally based on secondary metabolism pathways, particularly leading to phenolic compounds [3,4]; [5-7], reinforcement of the cell walls by the intensification of lignifications [7-9] and steroidal saponins [10,11].

Phenolic compounds are represented by various classes in the date palm; their contents are often higher in resistant cultivars to bayoud disease [12]. Among these compounds, the phenolic acids of benzoic or cinnamic type are widely spread in plants, they are generally present in nature under various combinations of heterosides or esters [13] or as esters linked to cell wall polysaccharides [14]. These secondary metabolites (phenolic acids) take an important part in the resistance mechanisms of date palm against the pathogenic agent, F. oxysporum albedinis [4,12].

Phenolic acids deriving from benzoic (para-hydroxybenzoic, protocatechuic, vanillic, syringic and gentisic acids) and cinnamic acids (para-coumaric, caffeic, ferulic and sinapic acids) are soluble and insoluble phenolic parts of the leaflets and roots of date palms from Algeria [15]. The soluble phenolic radical component is principally constituted by caffeoylshikimic acid (or dactyliferic acid) [4]; ferulic, sinapic, para-coumaric and para-hydroxybenzoic acids are the main phenols associated with different cell wall constituents [16,17]. In order to assess the distribution of these two forms of phenolic acids, benzoic (C6-C1) and cinnamic (C6-C3), we chose to quantify them from root extracts of two Algerian cultivars, the first is resistant to F.o.a. and the second is sensitive. At the same time, we have checked lignin in roots. Lignin, is accumulated as response to mechanical damage or wounding. Therefore, the release of lignin and other wall-bound phenolic material constitutes the response of different plants to the microbial attack [18]. Our aim is to understand the interactions that may be installed in defense reactions between two antagonisms "resistant cultivar - F.o.a." and "susceptible cultivar - F.o.a.".
2. MATERIAL AND METHODS

2.1. Plant material
Our experimentation was carried out using roots collected in March 2008 from two date palm cultivars, Takerbucht (TK) is resistant to *F.o.a.* and Tgaza (TG) is susceptible. The roots of TK and TG were collected from two types of palm groves, the first (p1) one is uninfected by *F.o.a.* includes resistant (r) date palms encoded TKr-p1 and other healthy or strongones, susceptible (ss) encoded TGss-p1; in the second groove (p2), completely devastated by bayoud, are collected the samples TKr-p2 from resistant (r) palms and those from susceptible palms infected by *F.o.a.* and carrying the first symptoms of bayoud (sb) whose code is TGsb-p2.

These trees grow in the experimental station of the National Institute of Agronomic Research (INRA) of Adrar, in the South-west (27°54’ Northern, 0°17’1” West) of the Algerian Sahara. The samples are dried and then pulverized to a chemical analysis by a gas chromatograph, coupled to a mass spectrometer (GC-MS).

2.2. Extraction and separation of organic phases
Two complementary and specific extractive processes of phenolic compounds are applied [28] and [29]. According to the first process, 5g of dry roots material are comminuted and macerated in distilled water for 24 hours. After filtration, the organic solution is mixed with NaOH 6% and then concentrated under nitrogen for two hours. Pure hydrochloric acid is added to the extract recovered to obtain a solution to 2N. Five other grams are hydrolyzed for the second protocol during 40 min by hydrochloric acid (2N) in boiling “bain-marie” with oxygen insufflation all 10min.

The organic phases separation were permitted by three consecutive extractions with diethyl ether, the aqueous phases are excluded. The organic phases are evaporated; the first (R1) and the second (R2) dry residues are taken again by absolute methanol and directly analyzed by GC-MS. Three chromatographic analyses are carried out for each sample.

2.3. GC-MS analyses
Analyses of the two methanol roots extracts R1 and R2 was performed using a Hewlett Packard gas chromatograph Model 6890 coupled to a mass spectrometer, Electron Impact HP 5973 type. Determination and recognition of compounds was tackled by comparing the attained mass spectra with those of the National Institute of Standards and Technology Library (Nist98). Each compound detected is characterized by its retention time (RT), its distribution area and its recognition percentage (%) defined by Nist98. The chromatograms are traced with Origin Pro 8.0 software.
2.4. Lignin analysis

Three biological replicates of roots of each cultivar are realized. Dry roots material is relied on consecutive extractions with water, ethanol, toluene: ethanol (1:1,v/v), and acetone. The acquired cell wall residue (CWR) was a one gram of CWR was diluted into 72% sulfuric acid, then incubated for 1 h at 30°C. Sulfuric acid was mitigated to 4% by adding distilled water. The admixture was autoclaved for 1 h at 120 °C and filtered through a pre-weighed filter paper, the filtrate was applied in the detection of acid soluble lignin (ASL). Filter paper was then dried overnight at 105 °C and weighted, the weight increase was considered as the acid-insoluble lignin (AIL). Acid soluble lignin was detected spectrophotometrically by measuring the absorption at 205 nm following formula ASL (mg/g) = [(A× D × V)/(a × b × M)] × 1000, A is the absorbed measure at 205 nm, D is the dilution factor, V is the volume of the filtrate, a is the extinction coefficient of lignin in g/l cm, b is the cuvette path length and M is the sample weight (as 100% dry matter) before acid hydrolysis/suspension g⁻¹. The entire lignin content was revealed as the sum of ASL (acid soluble lignin) and AIL (acid insoluble lignin).

2.5. Statistical analysis

The analyses were done in triplicate. Means and standard deviations of data were calculated. All figures were represented with error bars corresponding to the ratio of standard deviation (RSD).

3. RESULTS AND DISCUSSION

3.1. GC-MS analysis of phenolics

This study shows that the chemical composition of root extracts of date palm is diverse. We note within two types of extracts, an abundance of fatty acids and aliphatic hydrocarbons and a significant accumulation of phenolic compounds (Table 1). The percentage of presence which varies according to the resistance or the susceptibility cultivars. Table 1 shows the general chromatographic profile of one test among the three tests realized for each sample. Two series of phenolic acids characterize these polyphenols, phenylpropanoids found in R₁ extracts; whose carbon skeleton is C₃-C₆ and benzoic acids (C₁-C₆) in R₂ extracts (Table 2).
The chromatographic profiles obtained by GC-MS (Table 2) show that: cinnamic derivatives (C₆-C₃) abundance is lower in the resistant cultivar (5.79%) (Fig. 1a₁) as well as in the healthy sensitive cultivar (11.17%) (Fig. 1b₁). Benzoic acids (C₆-C₁) abundance in the roots of two cultivars from unscathed palm groves, is more important, in the resistant (47.77%) (Fig. 2a₁) than in the susceptible (16.17%) (Fig. 2b₁).
We note a significant diversion of trans-cinnamic acid, common precursor to both cultivars, in the roots originating from infested palm groves by F.o.a. Concerning the resistant cultivar, the diversion is expressed in both an accumulation of para-hydroxycinnamic acid derivatives (20.74%) (Fig. 1a2) and by a significant decrease in the para-hydroxybenzoic acid (3.02%) (Fig. 2a2).

So, we observed that the cinnamic pool in the susceptible cultivar is slightly affected by fusariosis wilt, the abundance of these composites in infected roots (8.89%) (Fig. 1b2) is almost similar to that obtained in healthy roots (11.17%) (Fig. 1b1). However, a significant accumulation of para-hydroxybenzoic acid was observed in this cultivar during its infection by F.o.a. (Fig. 2b2), these compounds represent more than half (65.8%) of all volatiles substances detected in R2 extracts.

**Fig.1.** Chromatograms of R1 root extracts of resistant and susceptible cultivars from palm groves unininfested and infested by F.o.a. p1: palm groves unininfested; p2: palm groves infested by F.o.a.; TKr: resistant cultivar; TGs: susceptible cultivar; ss: strong sensitive date palm, sb: sensitive date palms carrying the first symptoms of bayoud, 2: Hydrocinnamic acid, 3,5-di-tert-butyl-para-hydroxy-, methyl ester and 3: Hydrocinnamic acid, 3,5-di-tert-butyl-para-hydroxy-
Fig. 2. Chromatograms of R2 root extracts of resistant and susceptible cultivars from palm groves uninfested and infested by F.o.a.
p1: palm groves uninfested; p2: palm groves infested by F.o.a.; TKr: resistant cultivar; TGs: susceptible cultivar; ss: strong susceptible date palm, sb: sensitive date palms carrying the first symptoms of bayoud; 7: Methylparaben; 9: Vanillic acid, ethyl ester; 10: para-Hydroxybenzoic acid; 12: 3-Hydroxy-para-anisic acid; 13: Vanillic acid, diethyl amide

3.2. Lignin content
Lignification can be defined as the process of producing the phenylpropanoid macromolecules termed lignin. Lignin is a polymeric product that is constituted of phenylpropanoid units sorted out from three cinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohols. Lignification requires monolignol biosynthesis, moved to the cell wall and polymerize. Polymerization is then initiated by an enzymatic oxidation mechanism involving peroxidases and / or laccases which generate radicals that couple spontaneously [19,20].
Lignin and other wall-bound phenolic material are released as a response to mechanical damage or wounding and to microbial attack [18]. Hence, we have tested the lignin substance in roots of date palm trees, picked from two types of palm groves; uninfested and infested by F.o.a. (Fig. 3). Our results show that the contents of lignin are approximately equal for both cultivars...
in palm groves uninfested. In the infested ones, the lignin contents rise in both cultivars, its more important in the resistant cultivar roots.

**Fig.3.** Lignin contents in date palm roots of susceptible (TG) and resistant (TK) cultivars from palm groves uninfected and infested by *F.o.a.* Means± SE, n= 3

Phenylalanine ammonia-lyase (PAL), is the key enzyme in synthesis of main precursors leading to phytoalexins, lignin monomers and antifungal phenolics [21,22]. The synthesis of phenylpropanoids from the active form of *para*-coumaric acid is conducted by its precursor *trans*-cinnamic acid [23]. Three phenylpropane units, syringyl, coumaryl and hydroxyphenyl are derived from cinnamic acid, they take an active part in the lignin structure, which reinforces the cell walls and provide rigidity and mechanical strength [24]. Lignin has a protective effect on cellulose and hemicelluloses by preventing the action of pathogens lytic enzymes [25].

Unlike the resistant cultivar, the susceptible one reacts differently. We notice a significant stock of *para*-hydroxybenzoic acid in infected roots of this cultivar, these composites are characterized by their antifungal activity [26]; they produce salicylic acid, which plays crucially role in the defense mechanisms of plants to pathogens [27]. Our results corroborate with those of [26]; these researchers demonstrated that *Fusarium oxysporum* f. sp. *elaeidis* in the roots of oil palm induces the synthesis of cinnamic and benzoic acids.

Depending on the analysis, the preferential orientation of the precursor *trans*-cinnamic acid of phenolic metabolism is stimulated in the root when it is connected with the causative agent of bayoud. In soil infested with *F.o.a.*, susceptible cultivar used for its defense phenolic compounds of the benzoic series; the resistant cultivar used the cinnamic series. Similarly, lignifications should also occur more intensively in resistant than susceptible cultivar. The lignin pathway resulted in the synthesis of phenolic monomers that were eventually esterified
and blended into the cell wall fraction as lignin. Lignins resist strongly to the attack by microorganisms, the inducive release of lignin in cell walls prevents pathogen entrance and spread.

4. CONCLUSION
Our study shows that during an attack by the pathogen (F.o.a.), date palm will first use its defense preexisting barriers. We suggest the following hypothesis: during an interaction between the plant and F.o.a., the resistant cultivar initially rich in para-hydroxybenzoic acid, produced phenylpropanes from cinnamic acid. These compounds would strengthen the lignified (sclerenchyma) or suberized (exoderm) peripheral tissues walls to prevent any penetration of pathogen in its root. Moreover, the sensitive cultivar, not previously containing in its roots a higher rate of para-hydroxybenzoic acid, accumulates in infected roots a large quantity of this antifungal phenolic acid. These results cannot be compared with the reactions of other pathosystems, because few is known about the metabolic diversion induced by Fusarium sp.

In this regard, it will be interesting to undertake a comprehensive transcriptome analysis to better identify the genes involved in defense. Thus, we will be able to confirm the differential expression of the resistant and susceptible cultivars against F. oxysporum albedinis, causal agent of date palm vascular wilt.

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6. REFERENCES


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