RESISTANCE OF THE DATE PALM TO *Fusarium oxysporum* f. sp. *albedinis* ASSOCIATED TO ACCUMULATION OF CELL WALL-BOUND PHENOLIC COMPOUNDS, LIGNINS AND INCREASED DEFENSE ENZYME ACTIVITY

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Abstract. This paper investigates the components of resistance mechanisms of date palm (*Phoenix dactylifera* L.) to *Fusarium oxysporum* f. sp. *albedinis* (*F.o.a.*) using biochemical parameters of the root cell wall of two cultivars, one susceptible, Deglet Nour (DN), and one resistant, Takerbucht (TK). The spectrophotometric analysis of lignins, phenylalanine ammonia lyase (PAL) and guaiacol peroxidase (POX) activity strongly implicate phenylpropanoid metabolism in the resistance mechanisms of date palms against *F.o.a.* were assessed. Gas chromatography coupled with mass spectrometry (GC/MS) analysis revealed that *p*-hydroxybenzoic acid and benzenepropanoic acid, 3,5-bis (1,1 -dimethylethyl)-4-hydroxy-) octadecyl ester, also known as octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoate, were the main phenolic compounds detected. These results allow us to identify biochemical traits that characterise the cell wall structure of the resistant cultivar, thus contributing to a better understanding of the interaction processes in this pathogen system and revealing the essential role of the cell wall in *bayoud* resistance.

Key words: date palm; lignin; phenylalanine ammonia lyase; guaiacol peroxidase; cell wall-bound phenolic compounds.

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is the essential element of the oasis ecosystem. This fruit species is the backbone of Saharan agriculture and, thanks to the microclimate created by the shade of its palms, many underlying food crops are grown. From an ecological perspective, due to its resistance to arid conditions, the date palm is significant in the fight against desertification. In Algeria, the date palm finds a Saharan and pre-Saharan climate favourable to its development and the ripening of its fruit. Unfortunately, it is threatened by several diseases, the most dreaded being vascular fusariosis, known locally as "*bayoud*". This disease reduces the production of dates marketed worldwide and causes an imbalance in the oasis ecosystem (desertification, disappearance of underlying crops) [18], and the exodus of populations. *Bayoud* is caused by a telluric fungus, *Fusarium oxysporum* f. sp. *albedinis* [26], which invades the xylem vessels [38], causing rapid desiccation (Fig. 1A) and dieback by the death of susceptible cultivars (Fig. 1B).

The date palm has several varieties (cultivars) and more than 1,000 in Algeria, but only one is known to be resistant to *bayoud*, the Takerbucht cultivar. It produces lesser quality fruit than Deglet Nour, which is the most abundant and commercialised in Algeria, but it is unfortunately very susceptible to *bayoud*. Several researchers have conducted cytological, histological and biochemical studies to identify factors involved in vascular fusariosis *(bayoud)* resistance [4, 15, 37, 44]. Anatomical resistance, lignification of cortical tissue, cell wall thickening and induction of phenylpropanoid metabolism are aspects of polygenic resistance [4].

The characteristic component of the plant cell is the pectocellulosic cell wall, which constitutes a very effective natural physical barrier against aggressors. It is a dynamic structure whose importance is paramount given its position at the cell surface and thus at the plant-environment interface. When infected by a pathogen, the plant will defend itself by increasing the mechanical resistance of its cell walls through the deposition of polymers such as lignin, the accumulation of defence proteins, particularly the PR (Pathogenesis-Related) proteins, and the synthesis of phenolic compounds. These latest compounds, such as benzoic and cinnamic acids, play a significant role in the mechanisms of defense during parasite attack.

This study aims to identify the components of resistance mechanisms to *Fusarium oxysporum* f. sp. *albedinis* (*F.o.a.*) from the early stages of infection, based on the comparison of two cultivars, one susceptible (Deglet Nour) and the other resistant (Takerbucht) using biochemical approaches. In the present work, we are interested in the induction of cell wall-bound phenolic compounds**,** lignins and enzymes involved in phenylpropanoid metabolism, guaiacol peroxidase and phenylalanine ammonia-lyase, during the infection process.

Figure 1. Date palm tree, A: Dessication of the palms (symptoms of bayoud disease); B: Death of susceptible cultivars

MATERIALS AND METHODS

Plant material

The analyses were carried out on the roots of date palm seedlings of two cultivars Deglet-Nour and Takerbucht. Each cultivar is represented by many seedlings (n = 80) at the three-leaf juvenile stage of development. Seeds of the two cultivars were collected in Biskra (south-eastern Algeria) and Adrar (south-western Algeria), respectively.

The seeds were carefully sorted, soaked for 24 h, disinfected with 5% sodium hypochlorite for 20 min and rinsed several times with sterile distilled water. Germination was carried out at 30°C in the dark. After the radicle appears, the seeds were placed in pots containing a mixture of sand and potting soil (2/3, 1/3). Cultivation was carried out at a temperature of 25°C and a photoperiod of 16 h, until the juvenile stage with three leaves.

Fungal material

The fungal material used for the inoculation of date palm seedlings is a virulent strain of *Fusarium oxysporum* f. sp. *albedinis*, reference GH 56, isolated from the rachis of a palm tree affected by *bayoud*, from the Metlili region, Wilaya of Ghardaïa. It was made available to us by the regional station of the INPV (*Institut National de Protection des Végétaux*) of Ghardaia, Algeria.

Inoculum preparation and Seedling inoculation

The *Fusarium oxysporum* f. sp. *albedinis* strain was cultivated on potato dextrose agar (PDA) medium and incubated at 28°C. The inoculum was provided as a suspension of microconidia and macroconidia prepared from the solid medium and adjusted with sterile distilled water to the concentration of 10^6 conidia mL^{-1}.

A quantity of 10 µL of the conidial suspension 10^6 conidia·mL^{-1} was injected at the main root at 1.5 cm from the crown. The control plants were treated in the same way by injecting 10 µL of sterile distilled water.The seedlings were watered regularly with sterile water and maintained at a temperature of 28°C and a photoperiod of 16 h. Determinations were made on the roots of seedlings of both cultivars during infection kinetics at 5, 9, 15 and 25 days.

Extraction and determination of cell wall-bound phenolic compounds

The roots were finely ground at 4°C in 80% methanol (500 µL per 100 mg of plant material), and kept under continuous stirring for 30 min at 4°C. The homogenate was centrifuged for 10 min at 10000 ×g. The pellet containing the cell walls was washed twice successively in 50% methanol, distilled water, 0.5% sodium dodecyl and 50% methanol. First, the homogenate was centrifuged for 10 min at 10000 ×g. Then, the pellet containing the cell wall was washed twice, successively in 50% methanol, distilled water, 0.5% sodium dodecyl sulphate (SDS), 1 M NaCl, 100% methanol, acetone and finally diethyl ether. The tube was shaken vigorously for each wash for 15 min at 4°C and then centrifuged for 10 min at 10000 ×g to precipitate the cell debris. At the last wash, the supernatant was removed, and the pellet was air-dried at room temperature. The dry residues obtained were subjected to alkaline hydrolysis with 2 M NaOH for 24 h to extract the cell wall-bound phenolic compounds. The saponified extracts were then neutralised with 2 N HCl. The extraction was performed three times with ethyl acetate. Finally, the ethyl acetate fraction was evaporated to dryness under pressure, resuspended in 0.5 mL aqueous methanol (2:1 v/v) and stored at -20°C for analysis of cell wall-bound phenolic compounds [37].

Quantitative determination

The quantitative determination of phenolic compounds was carried out with the Folin-Ciocalteu reagent. To 0.25 mL of each methanolic extract, 0.125 mL of Folin-Ciocalteu reagent and 0.25 mL of sodium carbonate were added and made up to a total volume of 3 mL with distilled water. Readings were taken against a control (distilled water plus reagents) at 765 nm, using a JENWAY 6305 UV/Vis spectrophotometer. Concentrations were calculated from the equation (Eq. 1) of a standard curve established with gallic acid: $Y = 6.5594x$ and $R^2 = 0.991$.

Qualitative determination

For the qualitative determination of phenolic compounds all samples are derived, derivatization was performed using N, O-bis [trimethylsilyl] acetamide (BSA). The cell wall bound phenolic extracts were evaporated and resuspended in 500 µL of aqueous dimethyl formamide (DMF) (silylation grade). Then, derivatization in DMF medium was performed by adding 20 µL of N, O-bis[trimethylsilyl] acetamide. The mixtures were stirred for 30 s, incubated for 30 min at 60°C and then analysed by GC-MS.

The derived samples were analysed by GC-MS on a Hewlett Packard 6890 chromatograph, coupled to an HP5973 electronic impact mass spectrometer. The chromatograph was equipped with a HP5MS capillary column (30 m ×0.25 mm ×0.25 µm). The carrier gas was helium with a flow rate of 1 mL/min. The injector and detector temperatures were 250 and 270°C, respectively. The column was programmed at 60°C for 2 min, and from 6°C/min to 270°C for 5 min. The volume of solution injected was 5 µL in split mode (1/50). The interface temperature was 280°C, and the source temperature was 230°C. The mass spectra were recorded by a quadrupole detector (150°C); ionisation was performed at a potential of 70Ev. Volatile compounds were detected according to their evolution order; their mass spectrum and retention time identified them. Only compounds with a recognition rate higher than 75% were considered; the others were considered unidentified substances (UIS). Each detected compound was characterised by its retention time,

distribution area and recognition rate according to the information provided by the mass spectra library (D: database \NIST 98.L).

Extraction and determination of lignin

Lignin was extracted according to the method of Bruce and West (1982) [11]. First, fresh roots were ground in 80% methanol to remove lipids and pigments. Next, the resulting homogenate was filtered through a Whatman No. 4 paper and rinsed with 80% methanol; the residue was dried in an oven at 60°C for 24 h. The dry residue obtained was used for the determination of the amount of lignin in date palm roots. To 50 mg of dry root residue, 1 mL of HCl (2 N) and 0.5 mL of thioglycolic acid (TGA) were added in stoppered glass vials; the vials were placed in a boiling water bath at 100°C for 4 h. The mixture was then centrifuged at 12000 ×g for 10 min; the resulting pellet was rinsed with 1 mL distilled H_2O and incubated in 1 mL 0.5 N NaOH solution. The mixture was shaken overnight at 25°C and centrifuged at 12000 ×g for 10 min. After centrifugation, the orange-brown pellet was dissolved in 10 mL of 0.5 N NaOH. It was centrifuged again at 12000 ×g for 10 min, and the absorbance of the TGA derivatives in the supernatant was measured using a JENWAY 6305 UV/Vis spectrophotometer at 280 nm. The results were expressed as the increase in absorbance, A280 nm $mL^{-1} \cdot mg^{-1}$ of dry cell wall residue.

Phenylalanine ammonia-lyase (PAL) activity

A 1 mg sample of root tissue was ground in 2 mL of potassium phosphate buffer pH 7.0 containing polyvinylpyrrolidone. The homogenate was centrifuged at 10000 ×g for 30 min; the supernatant constituted the enzyme extract. All these steps were carried out at 4°C. The protein content was determined as described by Bradford [10] using bovine serum albumin to produce the standard range.

Phenylalanine ammonia-lyase is the enzyme that catalyses the first reaction in phenolic metabolism. It converts phenylalanine into cinnamic acid. PAL activity was assayed using a modified method of Mozzetti et al. (1995)[33]. The determination of PAL activity was performed at 290 nm by measuring the cinnamic acid produced from L-phenylalanine. The reaction mixture consisted of 0.5 M Tris-HCl buffer pH 8.0, 200 µL of 10 mM L-phenylalanine and 200 µL of the enzyme extract. The incubation was done at 37°C for 1 h, and the reaction was stopped by 0.05 mL of 5 N HCl. The cinnamic acid formed was extracted in 2 steps with 2 mL of diethyl ether. After evaporation of the ether, the residue was suspended in 500 µL of methanol, and the absorbance was determined at 290 nm. The cinnamic acid content was calculated using the standard curve performed under the same conditions with pure cinnamic acid. Thus, PAL activity was expressed as units of enzyme per mg of total protein.

Guaiacol peroxidase (POX) activity

To determine the Guaiacol peroxidase (POX) activity, a quantity of 0.25 g of roots were cold ground with a mortar in 1.5 mL of 5 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 1 mM $MgCl_2$. The homogenate was cold centrifuged at 9000 ×g for 7 min. The supernatant obtained corresponds to the soluble fraction of the enzyme. The pellet was washed 3 times in 1.5 mL of 5 mM Tris-HCl buffer (pH 7.2) containing 1% (v/v) Triton X-100. The residue was then incubated for 30 min in 1.5 mL of 5 mM Tris-HCl buffer (pH 7.2) containing 1 M NaCl. The supernatant obtained after centrifugation was the ionic fraction of the wall bound enzyme. It was used as an extract for the determination of guaiacol peroxidase (POX) activity, as well as for protein determination [1].

The reaction mixture with a final volume of 3 mL contained: 100 µL of enzyme extract, 50 µL of 0.03% H_2O_2, 50 µL of 8 mM guaiacol and 2850 µL of 0.1 M phosphate buffer (pH 7.2). Calibration of the apparatus was done in the absence of the enzyme extract. The reaction was triggered by the addition of hydrogen peroxide. The oxidation of guaiacol was determined by measuring the increase in absorbance at 470 nm (A 470) (Jenway 6305 UV/Vis spectrophotometer), for a linear molar extinction coefficient ε = 26.60 micro $M^{-1} \cdot cm^{-1}$. POX activity was expressed in IU. One POX unit was defined as the amount of enzyme that produces 1 µmol/min of oxidised guaiacol under the above conditions [21].

Statistical analysis

All measurements were performed in at least 5 replicates and the values were averaged. The results are given as means ± standard deviation (SD). ANOVA tests for comparing the different parameters studied in the different plants (roots of the susceptible and resistant cultivar), the separations of means were then performed with Tukey's HSD test at P= 0.05.

RESULTS

Quantitative analysis of wall-bound phenolic compounds

In the uninoculated DN (DNt) (Fig. 2A), the levels of cell wall-bound phenolic compounds varied from (2.84±0.89 $\mu g \cdot g^{-1}$ FW) on day 5 to 3.10±0.72 $\mu g \cdot g^{-1}$ dry matter on day 25. The highest level was measured on day 9 (3.91±1.58 $\mu g \cdot g^{-1}$ FW). After inoculation, an increase was observed in cell wall-bound phenolic compounds from 3.81±1.15 $\mu g \cdot g^{-1}$ FW on day 5, to 3.91±1.10 $\mu g \cdot g^{-1}$ FW on day 9, 5.94±2.51 $\mu g \cdot g^{-1}$ FW on day 15 and 6.20±1 $\mu g \cdot g^{-1}$ FW. Before inoculation, the resistant cultivar TK (Fig. 2B) showed levels very close to those of the susceptible cultivar DN until day 9, with higher levels on days 15 and 25 (4.52±0.54 and 7.87±1.41) $\mu g \cdot g^{-1}$ FW. After inoculation, there was a very high accumulation of cell wall-bound phenolic compounds in TK. On day 5 of inoculation, this increase was 2.5-fold ($P<0.05$) (8.63±0.88) in TKi

(inoculated TK) compared to 3.10±0.84 in TKt (control TK); it then increased 3-fold on day 9 ($P < 0.01$) and continued to increase until day 25 ($P < 0.05$). Thus, we observe a highly significant ($P < 0.001$) increase in cell wall-bound phenolic compounds in root cells of the resistant cultivar compared to the susceptible cultivar.

GC/MS analysis of wall-bound phenolic compounds

The experimental protocol adopted for the extraction of cell wall-bound phenolic compounds allowed to detect after derivatization with N, O-bis [trimethylsilyl] acetamide (BSA), and analysis by GC-MS a high percentage of phenolic compounds but also fatty acids and hydrocarbons (Table 1) The analysis of phenolic compounds associated with the root cell walls of date palm seedlings inoculated with *F.o.a*. allowed to detect 8 phenolic compounds (Table 2). Two molecules were particularly abundant in the extracts of both susceptible and resistant cultivars before and after their inoculation, namely *para-hydroxybenzoic* acid (benzoic acid, 4-hydroxy-) and benzenepropanoic acid, 3,5-bis (1,1 dimethylethyl)-4-hydroxy-, octadecyl ester (octadecyl 3-(3,5-di-ter-butyl-4-hydroxyphenyl)propanoate).

Chromatographic profiles of control root extracts of the susceptible variety DN (DNt) reveal a simple phenolic profile with the presence of only four wall-associated phenolic compounds representing 42.59% of the total compounds detected (Table 1). Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-,octadecyl ester representing 21.01% and *para*-hydroxybenzoic acid, representing 17.53% of the total phenolic compounds (Table 2, Fig. 3) are the most represented. The other two compounds, 2,2'-Methylenebis(6-tert-butyl-4-ethylphenol) and 2,5-Cyclohexadien-1-one, 2,6-bis (1,1-dimethylethyl)-4-ethylidene, represented only a small percentage, 3.38% and 0.62% respectively (Table 2, Fig. 3). In the inoculated DN seedlings (DN9j), an increase in the proportion of *para*-hydroxybenzoic acid (34.24%) and benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-,octadecyl ester (31.26%) of the total compounds identified was noted. In addition, a diversification of the phenolic compounds profile is also observed with the appearance of 3 compounds that were not detectable in the control seedlings. These are 2-methoxy-4-vinylphenol, Propylparaben and 1-Isopropoxy-2-di-tert-butyl-silyloxybenzene (Table 2, Fig. 4).

The root cell walls of the control seedlings of the resistant variety TK are characterised by a particular abundance of phenolic compounds (68.89%) (Table 1) and a more diverse phenolic profile (6 compounds identified) compared to the profile of the susceptible cultivar (DNt). Indeed, 2 new compounds were detected in the resistant cultivar, 2-methoxy-4-vinylphenol, Phenol, 2,4-bis(1,1-dimethylethyl). The majority compound, benzopropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-, octadecyl ester, accounted for 52.99% of the total phenolic compounds associated with the cell wall (Table 2, Fig. 5). In the resistant cultivar Takerboucht, the proportion of *para-hydroxybenzoic* acid increased significantly from 4.25% in control roots to 59.61% in roots inoculated with *F.o.a.,* i.e., a14-fold increase. There was also a strong decrease (10-fold) in benzopropanoic acid, 3,5-bis (1,1 -dimethylethyl)-4-hydroxy-, octadecyl ester in the presence of the pathogen (Table 2, Fig. 6).

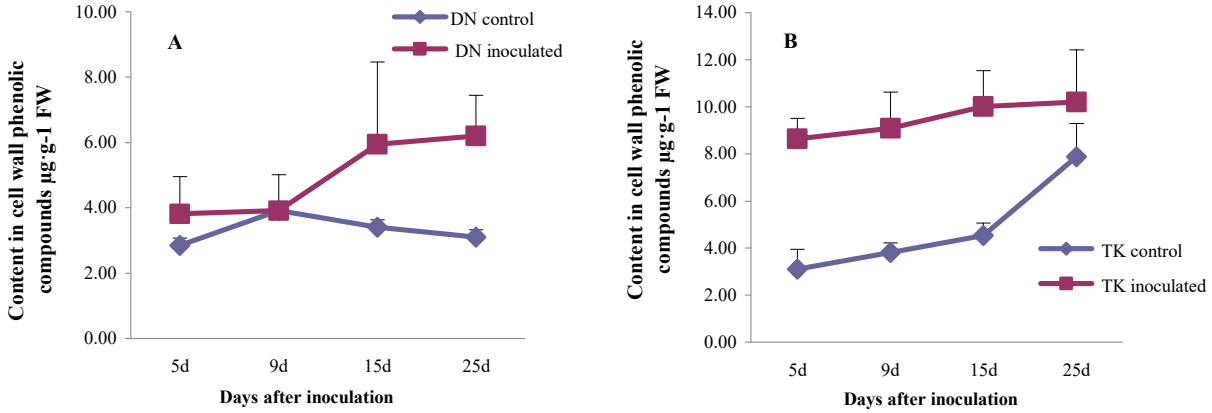

Figure 2. Time course evolution of cell wall phenolic compounds by *F. oxysporum* f. sp. *albedinis* inoculation in resistant (TK) and susceptible (DN) date palm cultivars. Data are means ± s.d. of five independent experiments; FW: fresh weight

Table 1. Total components (%) detected by GC-MS from root extracts of control and inoculated seedlings of resistant and susceptible cultivars date palm

compounds	Relative area %			
	DNc	DN9d	TKc	TK9d
Phenolic compounds	42.59	71.23	68.89	72.72
Fatty acids and aliphatic hydrocarbons	49.48	18.12	28.56	22.41
unidentified	5.23	2.9	1.39	1.5
Total components	97.3	9225	98.84	96.63

DNc : Deglet Nour control, DN9d : Deglet Nour 9 days post inoculation, TKc : Takerbucht control, TK9d : Takerbucht 9 days post inoculation.

Table 2. Phenolic compounds (%) detected by GC-MS from root extracts of control and inoculated seedlings of resistant and susceptible cultivars date palm

N°	Compounds identified	RT (min)	Relative area (%)			
			DNc	DN9d	TKc	TK9d
1	2-Methoxy-4-vinylphenol	15.72	-	1.35	1.79	-
2	Phenol, 2,4-bis(1,1-dimethylethyl)	19.90	-	-	4.40	-
3	**Benzoic acid, 4-hydroxy-**	**19.98**	**17.53**	**34.24**	**4.25**	**59.61**
4	Propylparaben	20.99	-	1.98	-	-
5	1-Isopropoxy-2-di-tert-butyl-silyloxybenzene	21.53	-	0.67	-	-
6	2,5-Cyclohexadien-1-one, 2,6-bis(1 ,1-dimethylethyl)-4-ethylidene	27.32	0.67	0.88	2.73	1.40
7	2,2'-Methylenebis(6-tert-butyl-4-ethylphenol)	36.10	3.38	0.85	0.98	-
8	**Benzenepropanoic acid, 3,5-bis(1,1 -dimethylethyl)-4-hydroxy-**	**58.03**	**21.01**	**31.26**	**52.99**	**5.16**

RT (min): retention time, DNc : Deglet Nour control, DN9d : Deglet Nour 9 days post inoculation, TKc : Takerbucht control, TK9d : Takerbucht9 days post inoculation.

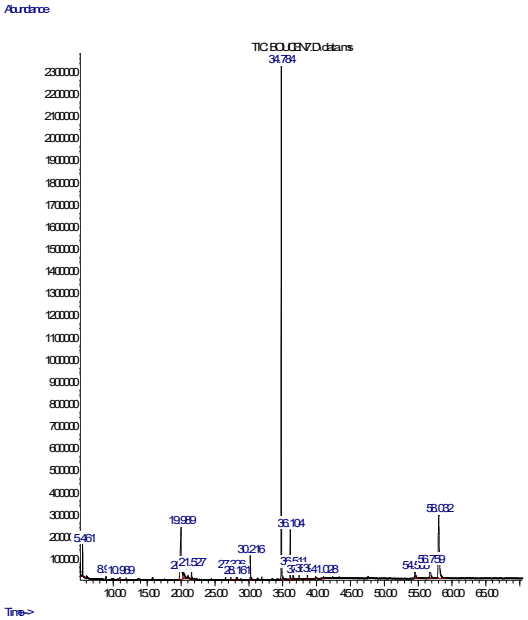

Figure 3. Total ion chromatogram obtained by GC-MS analysis of cell wall-bound phenolic compounds extracted from control date palm roots of susceptible cultivar DNc (Deglet Nour control)

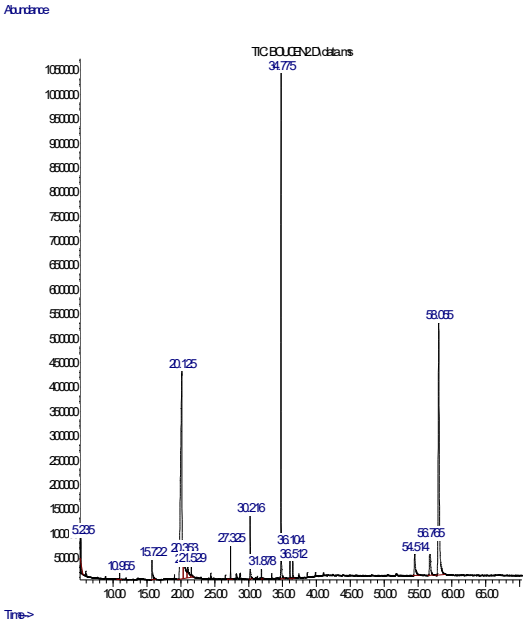

Figure 4. Total ion chromatogram obtained by GC-MS analysis of cell wall-bound phenolic compounds extracted from inoculated date palm roots of susceptible cultivar DN9d (9 days post inoculation)

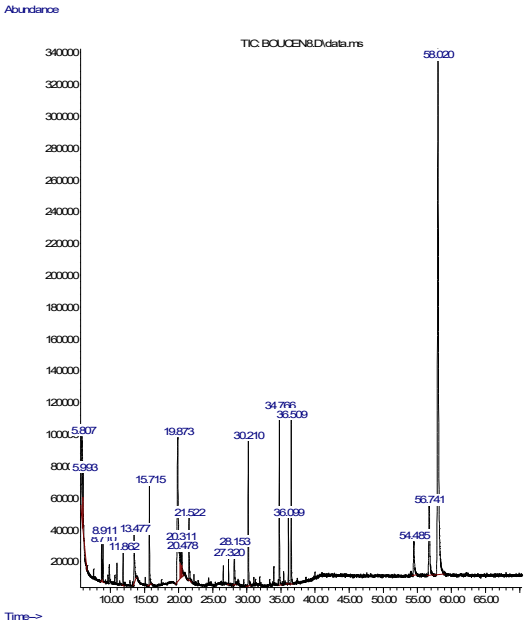

Figure 5. Total ion chromatogram obtained by GC-MS analysis of cell wall-bound phenolic compounds extracted from control date palm roots of resistant cultivar TKc (Takerbucht control)

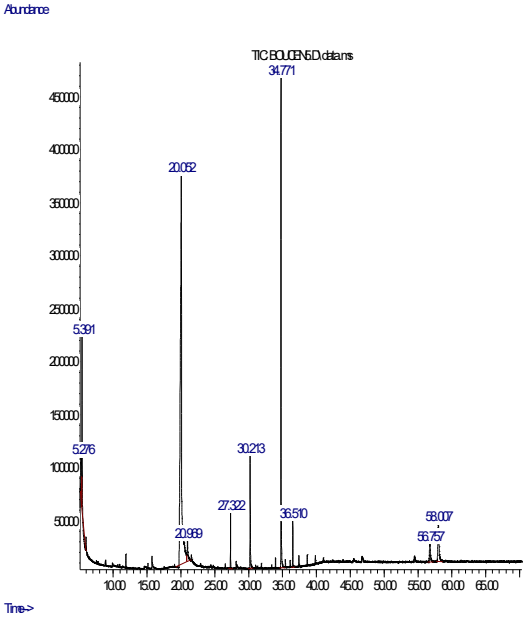

Figure 6. Total ion chromatogram obtained by GC-MS analysis of cell wall-bound phenolic compounds extracted from inoculated date palm roots of resistant cultivar TK9d (9 days post inoculation)

Quantification of lignins

The results obtained show very similar values in the amounts of root cell wall lignin of the two non-inoculated cultivars DN and TK (Fig. 7A, B); these values vary between 0.030±6.45 and 0.036±6.32 (A 280 nm $mL^{-1}{\cdot}mg^{-1}$ of cell walls) for the susceptible cultivar. For the resistant cultivar, the values are almost similar to those of DN and vary between 0.033±9.40 and 0.040±3.56 (A 280 nm $mL^{-1}{\cdot}mg^{-1}$ of cell walls). After inoculation of the seedlings, an increase in the quantities of lignin in the cell walls of the roots of both cultivars was noted. This increase is not significant in the walls of the roots of the susceptible cultivar, on the other hand a rather important accumulation of lignin was noted in the roots of the resistant cultivar. In the latter cultivar an increase of 0.003, 0.001, 0.008 and 0.011 was noted on day 5, 9, 15 and 25 respectively (A 280 nm $mL^{-1}{\cdot}mg^{-1}$ of cell walls) respectively.

Phenylalanine ammonia lyase (PAL) activity

Fig. 8A and 8B show that this enzyme activity varies in the two cultivars (DN and TK). Initially, the roots of the susceptible cultivar DN showed a higher PAL activity than that found in the roots of the cultivar TK, 0.77±0.10 IU·mg^{-1} protein compared to 0.22±0.06 IU·mg^{-1} protein. A similar progressive increase was observed in both cultivars at the different test times with a higher activity for DN except on day 25 where we noted a higher PAL activity in the roots of the resistant cultivar, 3.99±0.12 IU·mg^{-1} protein compared to 2.49±0.06 IU·mg^{-1} protein.

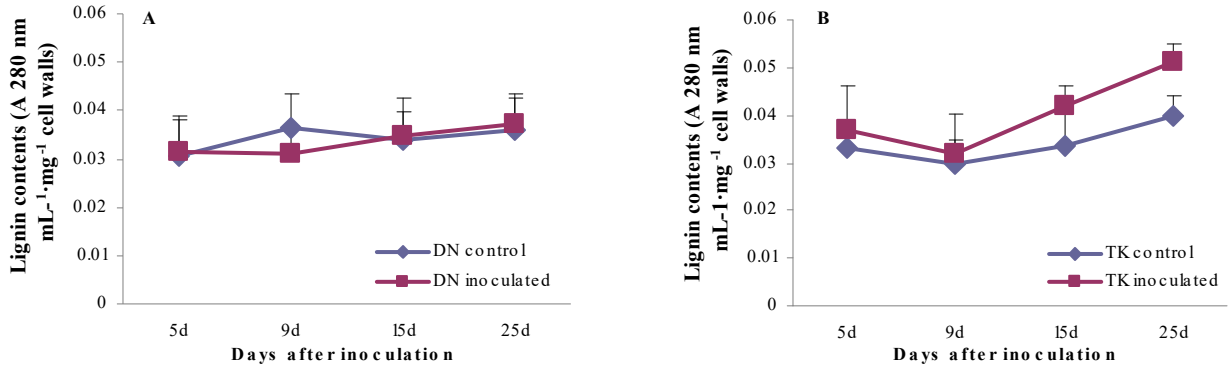

Figure 7. Time course evolution of lignin contents by *F. oxysporum* f. sp. *albedinis* inoculation in resistant (TK) and susceptible (DN) date palm cultivars. Data are means ± s.d. of five independent experiments

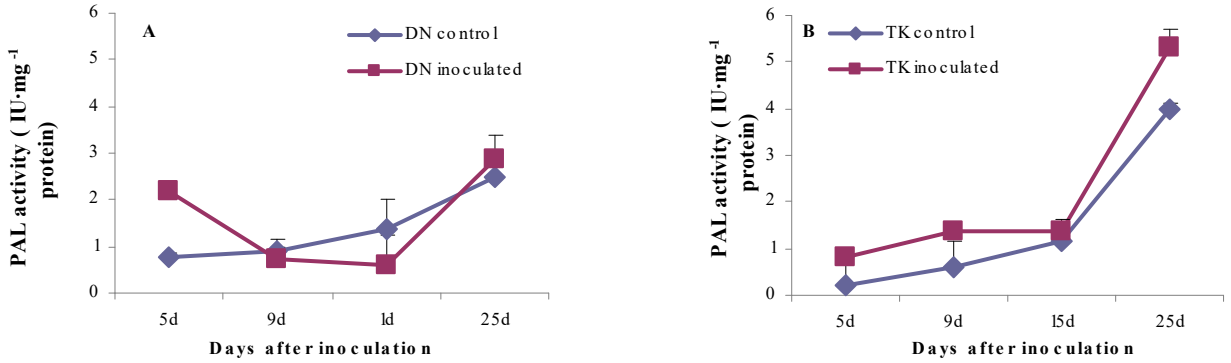

Figure 8. Time course changes of PAL activity by *F. oxysporum* f. sp. *albedinis* inoculation in resistant (TK) and susceptible (DN) date palm cultivars. Data are means ± s.d. of five independent experiments

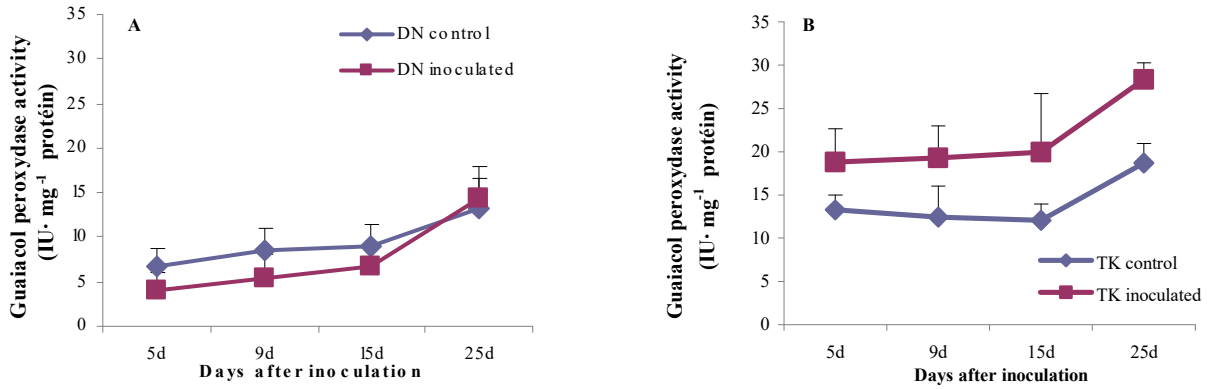

Figure 9. Time course changes of guaiacol peroxidase activity by *F. oxysporum* f. sp. *albedinis* inoculation in resistant (TK) and susceptible (DN) date palm cultivars. Data are means ± s.d. of five independent experiments

Inoculating seedlings of both resistant and susceptible cultivars with *Fusarium oxysporum* f. sp. albedinis induced PAL activity. The PAL activity response of the roots of the resistant cultivar was faster and higher than that of the roots of the susceptible cultivar. TK seedlings showed a significant increase in PAL activity ($P < 0.001$), at 5 days of inoculation it was 0.61±0.01 IU·mg^{-1}protein compared to 0.22±0.57 IU·mg^{-1}protein in the control, this increase is noted during all experimental times. In the roots of the susceptible cultivar, a strong stimulation of PAL activity was noted on the 5th day after inoculation, followed by a decrease of this activity during the next 2 periods of the experiment, i.e., 9 and 15 days after inoculation. On the 25th day of inoculation PAL activity was restored; it was similar between the control and the inoculated susceptible cultivar but clearly lower than the resistant cultivar.

Guaiacol peroxidase activity [POX: E.C. 1.11.1.7]

Guaiacol peroxidase activity was monitored by spectrophotometric assay in the roots of both cultivars during infection kinetics. Fig. 9A and Fig. 9B show that the activity of this enzyme varies in both cultivars, DN and TK. This difference was noted even before their inoculation, after inoculation a different reaction is also observed in the two cultivars. In uninoculated DN, the peroxidase activity in the root walls was 6.74±1.93 IU·mg^{-1} protein on day 5 of inoculation, and on days 9, 15 and 25 it was 8.51±2.59, 9.02±2.44 and 13.22±3.45 IU·mg^{-1} protein, respectively. A decrease in POX activity was noted in inoculated roots compared to control roots, with a slight recovery on day 25. POX activity in the root walls of the resistant cultivar is very high even before inoculation, it is 13.28±1.72 IU·mg^{-1} protein on day 5 of inoculation, it is 12.41±3.62; 12.03±1.91; 18.72±2.26 IU·mg^{-1} protein on days 9, 15 and 25 respectively. These values are much higher than those obtained in the susceptible cultivar ($P<0.003$). In the resistant cultivar, the POX activity increases significantly from the 5th day of inoculation, it is 18.80±3.85 IU·mg^{-1} of protein, it is stable at the two following times to increase almost to double at the 25th day, which represents a 50% increase over the susceptible cultivar ($P < 0.001$).

DISCUSSION

The inoculation of roots of resistant (TK) and susceptible (DN) cultivars of date palm seedlings by *F.o.a.* induced an increase in the amount of lignin in the root cell walls of both cultivars. These same results are found in our previous work [8, 9]. Further work has shown that the experimental inoculation of date palm roots with *F.o.a.* induces the accumulation of this polymer in the cell walls of the resistant Moroccan cultivar Bousthami Noir [14].

Lignin plays a multiple role in plant defence, with lignin-like phenolic polymers often deposited rapidly in response to biotic and abiotic stresses [40]. Lignin accumulation in response to infection has been described in several species [6]. For example, after infection of wheat leaves, increased lignin synthesis is observed at the penetration site of *Botrytis cinerea* and *Mycosphaerella pinodes* fungi, making the wall more resistant to maceration and stopping the growth of the fungus [39]. Similarly, inoculation of the leaves of the *Solanum tuberosum* plant with the pathogen *Phytophthora infestans* induces a significant increase in lignin in resistant varieties compared to susceptible varieties [2]. Lignin is a phenolic polymer, insoluble and highly resistant to pathogen-secreted wall-degrading enzymes, and it makes cell walls more resistant to mechanical pressure forces. It is generally accepted that lignin increases the resistance of walls to compressive forces, limits the diffusion of toxins and enzymes from the parasite to the host, and prevents water and nutrients from the host to the parasite [7, 32, 42]. The phenylpropanoid pathway also leads to the synthesis of many other phenolic compounds in addition to monolignols, including phenolic phytoalexins, stilbenes, coumarins and flavonoids. A number of these compounds have also been implicated in plant defence [30], for example, the defence signalling hormone salicylic acid (SA) is derived from the phenylpropanoid pathway [36].

The results obtained in this study indicate that inoculation of seedlings of both resistant and susceptible cultivars with *Fusarium oxysporum* f. sp. *albedinis* induce a faster and greater increase in PAL activity (key enzyme of phenylpropanoid metabolism) in the resistant cultivar than in the susceptible cultivar. Stimulation of PAL activity has often been correlated with plant resistance to pathogens [43]. Increased PAL and POX activities in Asparagus plants infected with *F. oxysporum* f. sp *asparagi* with strengthening of the cell wall and restriction of penetration and infection has been shown [22].

Moreover, the results obtained are in agreement with previous work which reported the difference in the induction of defense reactions between sensitive and resistant date palm seedlings after inoculation with *F.o.a.* linked to a difference in PAL activation [17]. In addition, we noted that the accumulation of lignin following inoculation is greater in seedlings of the resistant cultivar than in those of the susceptible cultivar. PAL is the main enzyme involved in synthesising phenolic compounds that can be incorporated into cell wall compounds; PAL participates in the synthesis of lignin precursors that can contribute to the strengthening of cell walls during pathogen attack.Our results show that the peroxidase activity varies between the two cultivars. This difference is noted even before their inoculation. The resistant cultivar shows a very high activity compared to the susceptible cultivar, which confirms the association of *bayoud* resistance with high levels of constitutive peroxidases [1].

Furthermore, the results show that after inoculation, POX activity increases significantly in TK, whereas in

DN a decrease is noted during the first 9 days but a tendency to resume higher activity was noted on day 15**.** Indeed, elicitor perception by plants involves the expression of a coordinated series of biochemical changes leading to the induction of defence responses. These include the accumulation of reactive oxygen species (ROS), phytoalexins, and the activation of various enzymes [34]. Among these enzymes, peroxidases are oxidoreductases that participate in reinforcing the cell wall and thus block fungal penetration [29].The peroxidases can also oxidise a large number of phenolic compounds that acquire antimicrobial properties in response to a pathogen [27]. Increased POX activity has been observed in many plant-fungus interactions [41]and also in plant-microbe-elicite interactions [3]. In date palm, peroxidases are abundant and diverse [11]. High levels of peroxidases may be responsible for enhanced lignification in *bayoud*-resistant plants and wall rigidity through the formation of diphenyl bridges. Studies showed that resistance enhancement by treating seedlings with a hypoaggressive isolate of *F. oxysporum* induced an increase in POX activity [16]. Moreover, it has been shown, in inoculated date palm seedlings, that the stimulation of POX activity is observed in both resistant and susceptible plants. However, the difference is related to the earliness and extent of the response of these plants to the infection [25].

In the present work, the phenolic fraction of the root wall of healthy and inoculated palms of the susceptible cultivar was analysed quantitatively and qualitatively and compared to that of the resistant cultivar. Comparing the phenolic profiles of the susceptible cultivar DN with those of the resistant TK before inoculation with *F.o.a.* reveals very similar contents. After inoculation with *F.o.a.,* a very significant increase was observed in the cell wall-bound phenolic compounds of the roots of the resistant cultivar compared to those of the susceptible cultivar, which would contribute to the resistance of this cultivar. Indeed, it is known that phenolic acids play a very important role in reinforcing cell walls. The insolubilisation of phenolic compounds in cell walls can modify its mechanical properties by decreasing its extensibility [24] and thus the wall is less easily degradable. The abundance of phenols in cell walls makes polysaccharides less sensitive to degradative enzymes secreted by pathogens [31].

GCMS analysis of cell wall-bound phenolic compounds in the root of susceptible and resistant cultivars revealed the presence of two compounds that were particularly abundant in both cultivars: 3,5-bis (1,1-dimethylethyl)-4-hydroxybenzoic acid octadecyl ester and *para*-hydroxybenzoi*c* acid. Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-, octadecyl ester, whose common name is octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoate, is very abundant in extracts of the resistant cultivar even before inoculation. Recently, studies identified this molecule in extracts of *Alcaligenes faecalis* cultures and reported its antifungal activity against *Candida albicans* and *Aspergillus niger* [43]. Benzenepropanoic acid octadecyl ester, 3,5-bis (1,1 -dimethylethyl)-4-hydroxy-, contains tert-butylphenol and can be considered a natural analogue of 2,4 di tert-butylphenol (2,4-DTBP) [20]. 2,4-DTBP is produced by many soil bacteria such as *Pseudomonas monteilii* and *Streptomyces* spp. Several authors have reported it as molecules with antioxidant, antibacterial and antifungal activities against *C. albicans* and some filamentous fungi such as *Fusarium* and *Aspergillus* [5, 35].

In the presence of *F.o.a.* the *para*-hydroxybenzoic acid content increased remarkably in TK extracts. It thus seems that *F.o.a.* triggers an overproduction of this molecule, to the detriment of benzenepropanoic acid, 3,5-bis (1,1 -dimethylethyl)-4-hydroxy-, octadecyl ester. Several works have highlighted the primordial role of *para*-hydroxybenzoic acid, in defence mechanisms [8]. It was reported that experimental inoculation of date palm roots with *F.o.a.* induces the accumulation of this molecule in the walls of the resistant cultivar Bousthami Noir [19]. *Para*-hydroxybenzoic acid is synthesised in the cytosol and accumulated in the wall during a pathogen attack [23]. Its accumulation is associated with antimicrobial [12] and antifungal [13] activity. *Para*-hydroxybenzoic acid aldehyde derivatives show, *in-vitro*, antifungal action on *Botrytis cinerea, Penicillium digitatum, Sclerotinia sclerotiorum, Fusarium oxysporum* and *Alternaria* spp. [28].

Chromatographic profiles of phenolic extracts from roots revealed a clear difference between the cell wall of susceptible and resistant cultivars even before inoculation. After inoculation, benzopropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-, octadecyl ester and *para*-hydroxybenzoic acid increased in the walls of the susceptible cultivar. For the latter molecule, the increase is much smaller than for the resistant cultivar. Moreover the resistant cultivar has a richness of constituent molecules such as benzopropanoic acid, 3,5-bis (1,1 -dimethylethyl)-4-hydroxy, which seems to be involved in root protection, through its antifungal activity. This molecule is described for the first time in plants, would it rather come from the rhizospheric microflora of the resistant cultivar, another path to be explored in future work. This resistance is also correlated with a strong induction of the defence enzymes, phenylalanine ammonia lyase PAL and guaiacol peroxidase POX, and increased lignin levels. The elevated PAL and POX enzyme activities, increased lignin and cell wall bound phenolic compounds are the response to the causal agent in inoculated plants. This reaction is earlier and more intense in plants of the resistant cultivar compared to the susceptible. Further work is underway and the results will be presented later.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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Received: December 21, 2021
Accepted: July 19, 2022
Published Online: July 21, 2022
Analele Universităţii din Oradea, Fascicula Biologie
https://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House