



# Plant Pathology Journal

ISSN 1812-5387

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## An Hypoaggressive *Fusarium oxysporum* Isolate Induces Polyphenoloxidase Activity in the Date Palm Seedlings Allowing their Protection Against the Bayoud Disease

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**Abstract:** This study was conducted with the aim to determine the effect of an hypoaggressive isolate of *Fusarium oxysporum* on polyphenoloxidase activity in the interaction of date palm with *Fusarium oxysporum albedinis*, the agent causal of the Bayoud disease. Biochemical analyses indicated that treatment of resistant (BSTN) and susceptible (JHL) date palm seedlings with the hypoaggressive (AHD) or aggressive isolate (ZAG) of *Fusarium oxysporum* was correlated with the ability of roots to enhance their defense responses. The maximum of polyphenoloxidase activity was revealed to be 5 times higher than in control after 15 and 20 days for ZAG treatment and 3 times after 30 and 40 days for AHD treatment in BSTN and JHL cultivars, respectively. When seedlings were firstly treated by the hypoaggressive isolate and then by the aggressive one, the polyphenoloxidase activity was enhanced within 10 days and reached similar values as obtained by ZAG isolate alone. These results show that polyphenoloxidase induced by the hypoaggressive isolate of *Fusarium oxysporum* could have an important role in the establishment of date palm resistance against the Bayoud disease.

**Key words:** Date palm, *Fusarium* sp., polyphenoloxidase, biocontrol

### INTRODUCTION

Polyphenoloxidase is involved in the oxidation of phenolics into quinone using molecular oxygen as an electron acceptor<sup>[1]</sup>. These compounds are highly reactive and can tan proteins by covalent bonds or autooxidize producing brown pigments<sup>[2]</sup>. The occurrence of brown products has been recognized to be responsible for significant postharvest losses of fruits and vegetables<sup>[3]</sup>. In addition, polyphenoloxidases have been described to play an important role in food industry, in physiological functions in plant growth and development and in plant defense against pests and pathogens<sup>[4]</sup>. Indeed, increased polyphenoloxidase activities have been correlated with defense against pathogens in several plants, including cucumber, wheat, potato, cotton, tobacco and rice<sup>[5-7]</sup>. The active quinones produced by polyphenoloxidase may possess direct antibiotic and cytotoxic activities against pathogens<sup>[8,9]</sup>. In addition, polyphenoloxidase is involved in the lignification of plant cells that contribute to the formation of defense barriers against pathogens<sup>[6,7,10]</sup>.

*Fusarium* wilts are economically important soilborne diseases affecting many crops in the world. However, nonpathogenic *Fusarium oxysporum* isolates have been reported to have certain potential to control this disease in several plants such as carnation, cyclamen, flax and tomato<sup>[11]</sup>. Among mechanism of mode of actions of nonpathogenic *Fusarium oxysporum* is induction of resistance by stimulation of defense responses, including structural barriers and defense enzymes such as chitinases,  $\beta$ -1,3-glucanases, peroxidases and polyphenoloxidase<sup>[11-14]</sup>. Such induced disease resistance has been shown to occur in plants in response to a localized pretreatment with biotic or abiotic elicitors thus making them resistant to subsequent pathogen infection<sup>[15]</sup>.

In the case of *Fusarium* wilt in date palm (*Phoenix dactylifera* L.), the Bayoud disease, we have recently shown that pretreatment of date palm seedlings with an hypoaggressive *Fusarium oxysporum* isolate protected them partially from further infection by *Fusarium oxysporum* f. sp. *albedinis* (Foa), the Bayoud disease

pathogen. The hypoaggressive isolate induced the defense responses such as peroxidases and non constitutive phenolic compounds<sup>[16]</sup>.

This study was conducted in order to examine changes of polyphenoloxidase activity in date palm roots treated with the hypoaggressive isolate AHD and challenged with the aggressive ZAG isolate and to investigate the cytochemical localization of polyphenoloxidase into seedlings roots using DL-3,4-dihydroxyphenylalanine (DL-DOPA) as a substrate.

## MATERIALS AND METHODS

**Plant and fungal materials:** Hypoaggressive (AHD) and aggressive (ZAG) isolates of *Fusarium oxysporum* were isolated and screened in our laboratory from date palm rachis affected by the Bayoud disease<sup>[17]</sup>. Fungal cultures were conducted on Potato Dextrose Agar (PDA) and petri dishes were incubated at 25±2°C.

This study is being conducted during the period 2002-2005 at the Faculty of Sciences Semlalia, Marrakech-Morocco. The date palm seedlings (3 to 4 months old) of two cultivars, Jihel (JHL, susceptible) and Bousthami noire (BSTN, resistant) were used in this study. They were grown in pots containing a mixture of sterile sand and peat in greenhouse under 16 h light regimes at 25±2°C.

The seedlings were artificially inoculated using 10 µL of conidial suspensions (10<sup>6</sup> spores/mL) of either hypoaggressive or aggressive *Fusarium* isolates. Control plants were treated with sterile distilled water. The infected seedlings were incubated in the same conditions as for their culture.

Forty seedlings were used for each treatment and the experiment was carried out in triplicates.

**Polyphenoloxidase activity assay:** Fresh date palm seedling roots were extracted with Tris-maleate buffer (0.1 M, pH 6.5) containing Triton X-100 (0.1 g L<sup>-1</sup>) at 4°C. The tissue extracts were centrifuged at 7000 g for 3 min and the supernatants were used for the enzymatic activity assay.

Polyphenoloxidase activity was measured using the modified protocol described by Chen *et al.*<sup>[6]</sup>. The reaction mixture contained 1550 µL of phosphate buffer (0.1 M, pH 6), 50 µL of enzyme extract and 400 µL of catechol (0.2 M) as a substrate of the polyphenoloxidase. The enzymatic activity was evaluated by measuring the increase of optical density at 420 nm.

Protein concentrations were measured by the method of Bradford<sup>[18]</sup> using bovine serum albumin as a standard.

Experiments were performed with a minimum of three replicates per treatment and per time point and each result was expressed as mean ±SE.

**Histolocalization of polyphenoloxidase:** The protocol used for the histolocalization of polyphenoloxidase was described by Gahan<sup>[19]</sup>. Samples of roots near the infection sites (0.5 cm) were cut under a fixing solution into 1 to 3 mm<sup>2</sup> pieces, which then were fixed for 30 min in 4% paraformaldehyde in 0.2 M potassium phosphate buffer at pH 7.2. Following fixation, samples were washed twice for 15 min in 0.2 M sodium cacodylate buffer at pH 7.4. Following washing, samples were transferred into sodium cacodylate buffer containing 25x10<sup>-3</sup> M of DL-DOPA as a substrate of polyphenoloxidase and 2x10<sup>-3</sup> M of sodium pyruvate to suppress endogenous H<sub>2</sub>O<sub>2</sub> production. The optimal time for staining was previously determined as 60 min.

## RESULTS AND DISCUSSION

Pretreatment of date palm seedlings of both cultivars BSTN and JHL with the hypoaggressive isolate AHD is translated by the development of necrotic lesion around the sites of infection (Fig. 1) in comparison with susceptible roots inoculated directly with aggressive ZAG isolate which showed generally a diffused wet necrosis leading to root tissue softening and foliar witherings as symptoms of the Bayoud disease<sup>[16]</sup>.

The specific polyphenoloxidase activity increased faster into roots following the inoculation with aggressive isolate ZAG in comparison with the hypoaggressive AHD isolate. Inoculation with ZAG induced an increase of polyphenoloxidase activity within the first 5 days and



Fig. 1: Localized necrosis developed when roots are firstly treated with AHD and then by aggressive ZAG isolate of Foa



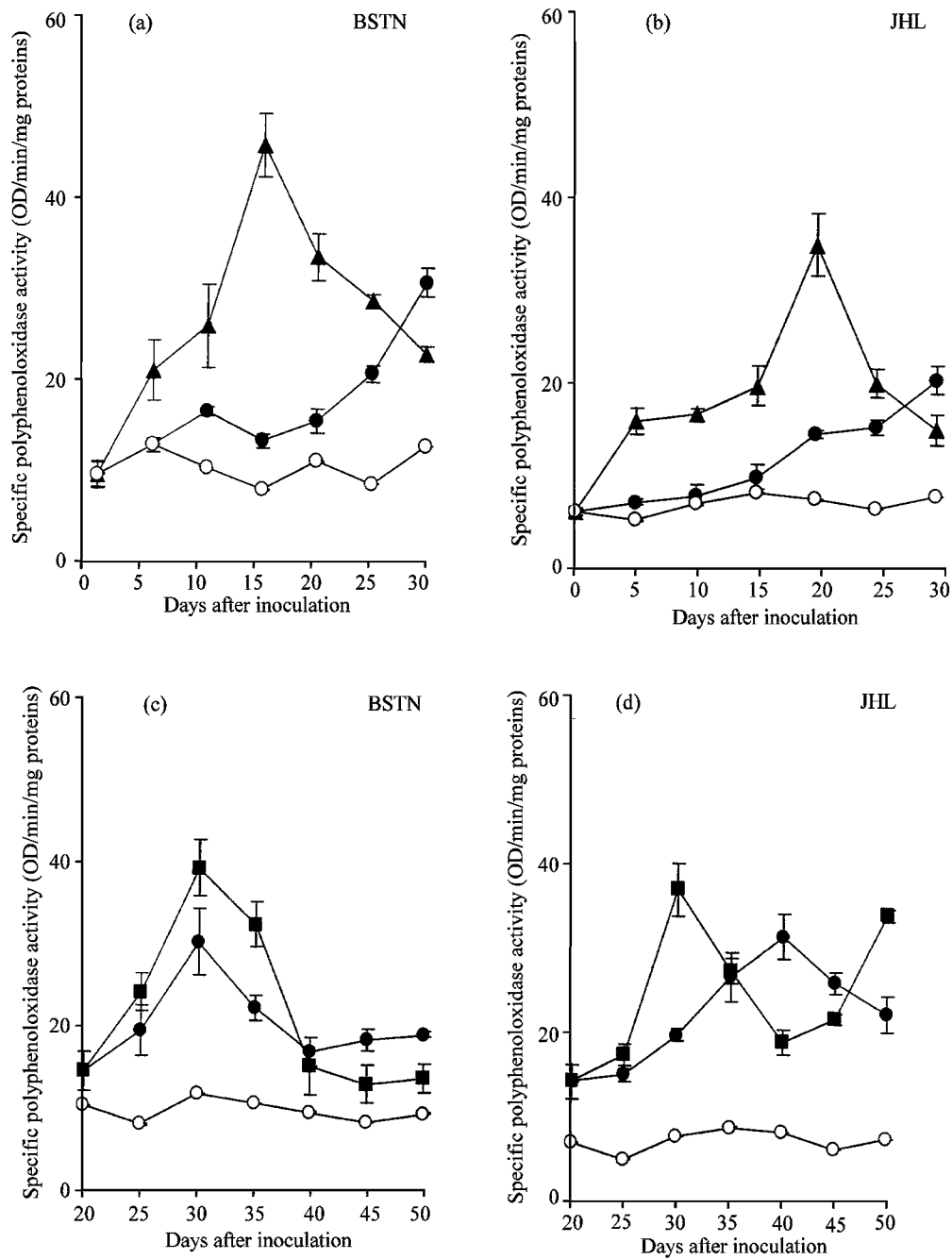


Fig. 2: Time course of specific polyphenoloxidase activity in date palm roots of resistant BSTN (a and c) and susceptible JHL (b and d) cultivars exposed to four treatments (  $\blacktriangle$  ZAG;  $\bullet$  AHD;  $\blacksquare$  challenge (AHD 20 days-ZAG) and  $\circ$  water control)

the maximum of activity was recorded after 15 and 20 days (5 times than in control) in BSTN and JHL cultivars, respectively and then decreased (Fig. 2a and b).

In the opposite, inoculation with the hypoaggressive isolate leads to a low stimulation of polyphenoloxidase

activity after 20 days of incubation for BSTN and 15 days for JHL in comparison with the controls. This stimulation becomes more significant after 30 and 40 days of incubation in BSTN and JHL cultivars, respectively to reach equal levels obtained following inoculation with ZAG isolate.

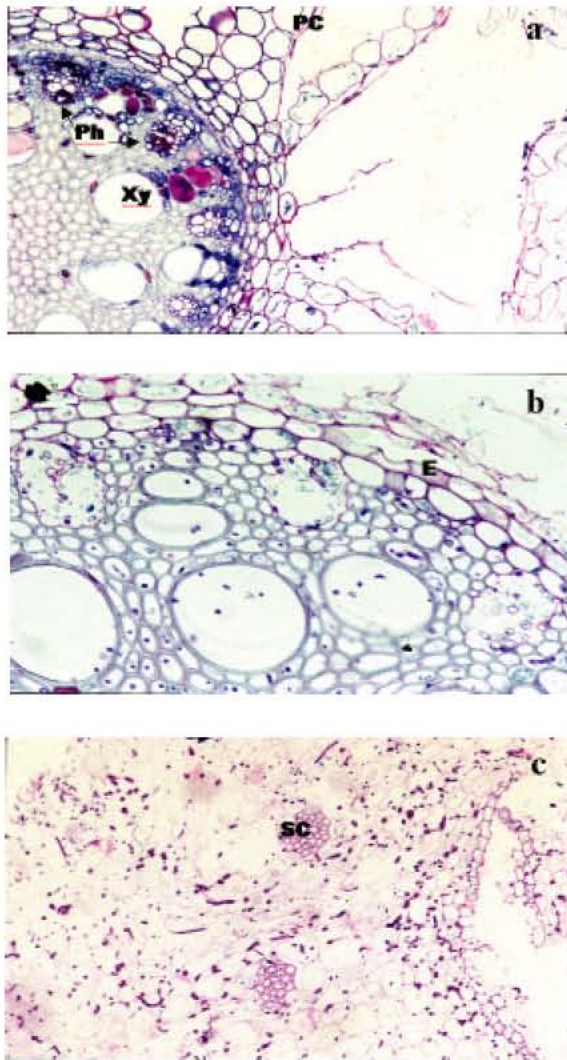


Fig. 3: Histology of date palm roots (cv. JHL) inoculated by aggressive ZAG isolate of *Foa* showing (a) invasion of *Foa* in the parenchymatous cells, (b) concentration of *Foa* in the stele and (c) digestion of endodermic cells and disappearance of the phloem and xylem tubes  
SC: Sclerenchymatous cells; PC: Parenchymatous cells; E: Endodermic cells; Ph: Phloem tubes; Xy: Xylem vessels

Pretreatment of date palm seedlings with the hypoaggressive AHD isolate during 20 days and then their challenge with ZAG isolate revealed a maximum of activity within 10 days after co-inoculation to reach equal levels in BSTN and a greater levels in JHL cultivar as compared with ZAG isolate (Fig. 2c and d).

Polyphenoloxidase activity has been usually reported in defense reaction against pathogens. Mohammadi and

Kazemi<sup>[7]</sup> revealed an induction of the specific polyphenoloxidase activity in resistant wheat against Fusarium Head Blight (FHB) caused by *Fusarium graminearum*. Localized inoculation of tomato leaflets with *Pseudomonas syringae* induces a significant increase in polyphenoloxidase activity and leads to a systemic resistance to the subsequent infection by *P. syringae*<sup>[20]</sup>. Constabel *et al.*<sup>[21]</sup> showed that in the transgenic tomato plants overexpressing prosystemin, polyphenoloxidase activities are co-activated with two constitutively induced defensive proteinase inhibitors. Further, Li and Steffens<sup>[4]</sup> reported that tomato plants overexpressing polyphenoloxidase exhibited a great increase in resistance to the *Pseudomonas syringae* pv. *tomato*. Moreover, tomato plants grown in suppressive soil were protected against subsequent infection by *F. oxysporum* f. sp. *lycopersici*. The protected plants express higher activity of peroxidases, polyphenoloxidase and  $\beta$ -1,3-glucanases as well as higher levels of phenolic compounds<sup>[22]</sup>.

The histological study revealed that the date palm roots contained a discontinuous cortical parenchyma delimited outside by a suberoid and inside by an endodermic cells suberified in U on the border of the central stele. This one contains a pericycle, xylem, phloem and a medullar sclerenchyma.

Inoculation with ZAG or AHD isolates shows, in the first days, a certain development of tissue browning on the infected part of roots by micro-injection protocol<sup>[22]</sup> thus revealing the progression of the pathogen. After 10 to 15 days and following inoculation with ZAG isolate, histological structure of roots was totally desorganized in comparison with hypoaggressive AHD isolate which shows very low cell damage. ZAG isolate colonises all tissues and became concentrated in the stele rather than in the cortex. Indeed, roots showed softening and a disconnection of the stele from the cortex. Moreover, certain endodermic cells showed digested suberified walls and the phloem tubes completely disappeared (Fig. 3)<sup>[22]</sup>.

Cytochemical studies of polyphenoloxidase activity on infected roots sections reveal a certain development of tissue browning on the infected part of roots in comparison with intact ones was conducted. No enzymatic reaction has been revealed in intact tissue (Fig. 4a). In part without tissue browning of infected roots, polyphenoloxidase activity was revealed as black vesicles, which could be assimilated to plastids (Fig. 4b and c). However, in the other part showing tissue browning, polyphenoloxidase activity was revealed as brown deposit in cell walls and in cytoplasm (Fig. 4d and e). Such an activity was localized in the parenchyma cells and particularly near the central stele.



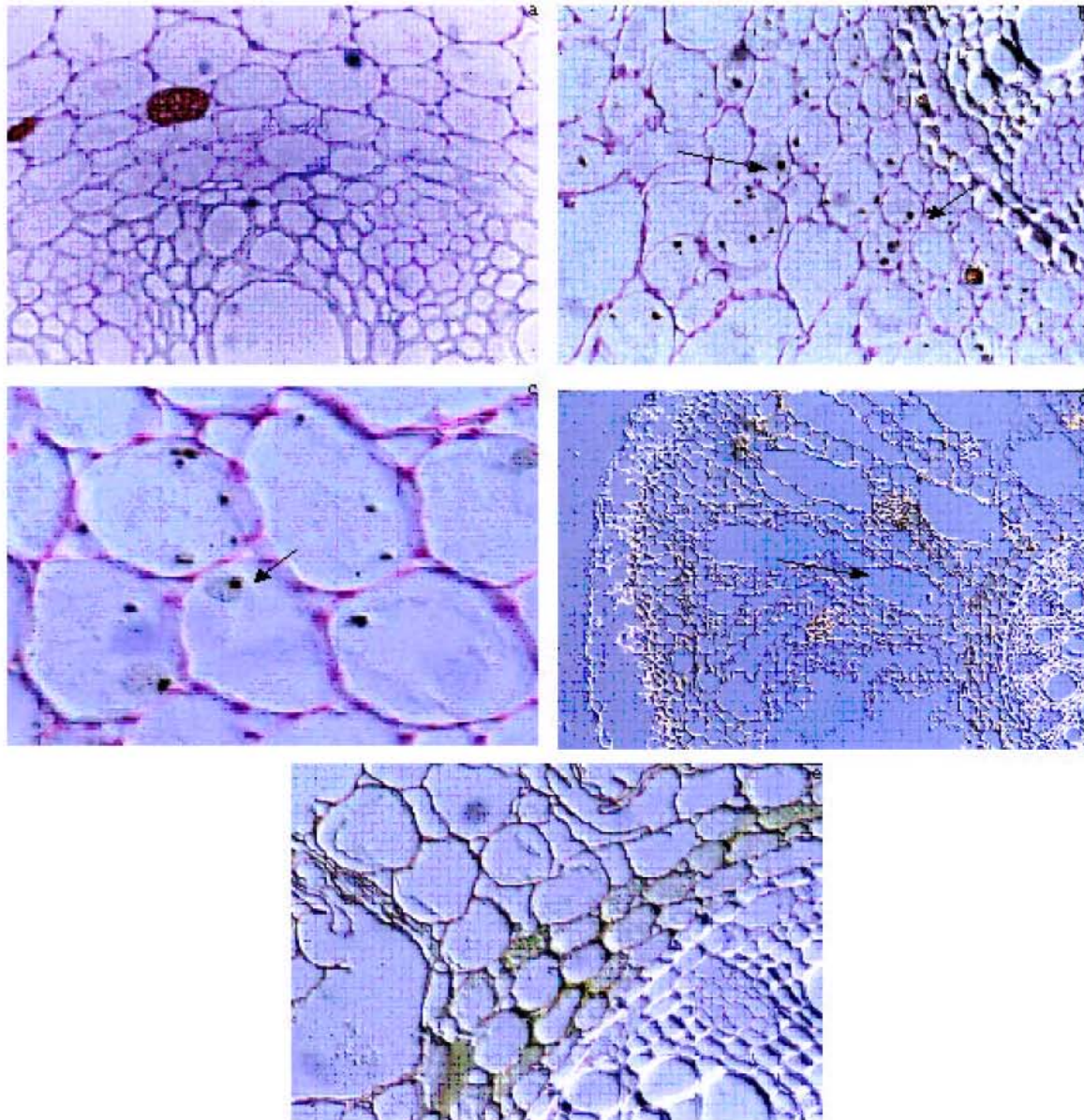


Fig. 4: Cytochemical localization of polyphenoloxidase activity in date palm roots of susceptible JHL cultivar; (a): Absence of polyphenoloxidase activity in control roots, (b and c): Tissues revealed induction of polyphenoloxidase activity (black vesicles which could be assimilated to plastids) localized in the parenchymatous cells near the central stele, (d and e): Tissues invaded by pathogen revealing brown deposit in cell walls and in the cytoplasm

For other systems, subcellular localization of polyphenoloxidase was revealed in plastidial membranes: chloroplasts for leaves, amyloplasts for tubercules and leucoplasts for carrot<sup>[23]</sup>. In addition, ultrastructural microscopic studies of chloroplasts in *Sorghum bicolor* L. showed that polyphenoloxidase activity appeared to be associated with the thylakoids of mesophyll plastids, as it has been described for

mesophyll plastids of other spices<sup>[24]</sup>. The brown deposit revealed in date palm tissues invaded by the pathogen could be due to the oxidation of phenols to quinones. Indeed, Li and Steffens<sup>[4]</sup> revealed that polyphenoloxidase overexpressing transgenic plants have an ability to oxidize phenolic compounds to quinones upon disruption of the plastidic and vacuolar compartments by the pathogen.

Different studies have shown that inducibility of polyphenoloxidase activity could be reached by numerous biotic and abiotic factors including mechanical wounding, fungal and bacterial infection and treatment with the signaling molecules such as system in, oligosaccharide and chitosan<sup>[21,25,26]</sup>. The results reported here showed that hypoaggressive *Fusarium oxysporum* AHD isolate could enhance the control of date palm seedlings against *Fusarium oxysporum albedinis* (Foa), the agent causal of Bayoud disease. The mode of action is being in relation with the ability of this fungus to induce defense responses such as polyphenoloxidase activities revealed into root's plasts. These results show that polyphenoloxidase could have an important role in the establishment of date palm resistance against the Bayoud disease.

#### ACKNOWLEDGMENTS

Financial support for this study was provided by PRAD 02-02, Protars II N° P51/14, AI (France-Maroc) 01/23 and AI (Tunisie-Maroc) 24/02.

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