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Effect of exogenous application of jasmonic acid on date palm defense reaction against *Fusarium oxysporum* f. sp. *albedinis*

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Summary. The effect of jasmonic acid in the date palm defence reaction against *Fusarium oxysporum* f. sp. *albedinis* was investigated taking into account changes in H_2O_2 and malonyldialdehyde (MDA) levels and peroxidase activity. Treatment of seedlings of two cultivars with jasmonic acid increased levels of H_2O_2 and enhanced lipid peroxidation as indicated by MDA accumulation and an increase in peroxidase activity. Similar changes occurred with date palm seedlings infected with *Foa* and showing necrotic hypersensitive-reaction like lesions. In general, both *Foa* and jasmonic acid increased H_2O_2 to a level 2 to 7 times that of the control, depending on the treatment and the time of analysis. Peroxidase activity was 2 to 3 times greater and MDA levels were increased 2 to 8 times. In contrast, seedlings presenting disease symptoms did not show any such reactions. It is suggested that oxidative burst (H_2O_2 generation) and its consequences (lipid peroxidation) and the change in peroxidase activity are used by date palm to resist *Foa* and that jasmonic acid is a signal for the expression of these defence reactions.

Key words: Bayoud, hydrogen peroxide, *Phoenix dactylifera*, peroxidases.

Introduction

Fusarium oxysporum f. sp. *albedinis* (*Foa*) is the causal agent of *Fusarium* wilt of date palm (*Phoenix dactylifera* L.), called bayoud, which is the most important disease of this crop. Over the past 100 years, this pathogen has killed more than 10 million palm trees in Morocco (Saaidi, 1992) and 3 million in Algeria (Djerbi, 1988), and now threatens other date palm growing Maghreb countries. Consequently, the principal goal of research must be to find a means to control *Foa*. Planting resistant cultivars is the most effective method to control bayoud in date palm (Louvet, 1991). Moroccan palm groves contain a diversified and interesting genetic material that must be saved and used in conventional date palm-genetic improvement (Sedra *et al*., 1996). Characterization of the biochemical mechanisms that underlie the resistance of date palm to *Foa* is crucial for the understanding and control of bayoud disease epidemics.

In most plants, when a pathogen is detected, a number of signal transduction pathways are activated leading to the expression of numerous genes and the synthesis of various defence proteins. Reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide are the earliest responses to the pathogen infection (Lamb and Dixon, 1997). ROS has direct antimicrobial activity and

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therefore reduces pathogen viability (Mellersh *et al*., 2002). H_2O_2, the most stable ROS, contributes to the construction of barriers against pathogens, since it is required by peroxidases for cross-linking plant cell walls using structural proteins and phenolic compounds that strengthen the cell walls (Bestwick *et al*., 1995). H_2O_2 may also act as a diffusible signal for the induction of defence genes and the development of systemic acquired resistance (Orozco-Cardenas *et al*., 2001). Peroxidase enzymes play an important role in the plant defence reaction, regulating the level of H_2O_2, since they are active in H_2O_2-dependent reactions associated with cross-linking mechanisms and with the deposition of lignin and suberin in cell walls (Espelie *et al*., 1986; Monties, 1989). Jasmonic acid has been recognized as important in plant stress signalling, being responsible for induced systemic resistance (Pieterse *et al*., 1998). It modulates the expression of numerous genes and influences specific aspects of plant growth and the response to biotic and abiotic stresses (Creelman and Mullet, 1997). It also activates genes involved in phytoalexin biosynthesis (Tebayashi *et al.,* 2001) and affects gene transcription of secondary metabolites, such as phenylpropanoids and alkaloid biosynthesis, which are involved in plant defence (Memelink *et al.,* 2001; Tebayashi *et al*., 2001). In addition, jasmonic acid activates genes encoding antifungal proteins (Peninckx *et al*., 1998). In date palm, our preliminary results showed that jasmonic acid treatment causes the synthesis of phenolic compounds similar to those induced in plants resistant to *Foa* (El Hadrami *et al*., 2000; Jaiti *et al*., 2002)

The aim of the present study was to determine the relationship between jasmonic acid, H_2O_2 contents, lipid peroxidation and peroxidase activity in the establishment of date palm resistance against *Foa*.

Materials and methods

Plant material, fungal inoculation and jasmonic acid treatment.

Seedlings obtained from seeds produced by two mother cultivars of date palm Bousthami noir (BSTN, a bayoud-resistant cultivar) and Jihel (JHL, a susceptible cultivar) were inoculated at the two-leaf stage by injecting 10 μl of a conidial suspension (10^6 spores ml^{-1}) of *Foa* into the roots. The *Foa* isolate used (ZAG) was isolated from naturally diseased date palm tissues originating in Zagora, Morocco. The aggressiveness of this isolate was regularly tested on seedlings of resistant and susceptible cultivars as described by El Idrissi-Tourane *et al*. (1995). Fungal culture was routinely conducted in darkness on malt extract medium at 25±2°C. For the elicitor, seedling roots were injected with 10 μl of jasmonic acid solution (50 μM). Control plants were inoculated with distilled water. The seedlings were incubated in the same conditions (25°C with a 16 h day) and sampled at 0, 1, 3, 6, 12, 24 and 48 days after *Foa* inoculation or jasmonic acid treatment.

H_2O_2 contents

Hydrogen peroxide levels were determined according to Velikova *et al*. (2000). Briefly, plant material (500 mg f wt) was homogenized in 2 ml trichloroacetic acid (TCA) solution (1 g l^{-1}). After centrifugation at 12,000 *g* for 15 min, 0.5 ml of the supernatant was added to the reaction mixture containing 0.5 ml 10 mM of potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. Absorbance was determined at 390 nm and the amounts of H_2O_2 were evaluated using a standard curve conducted under the same conditions.

Peroxidase extraction and activity assays

Plant material (200 mg f wt) was homogenized in 1 ml of tris maleate buffer pH 6.5 (0.1 M) containing Triton X-100 (0.1 g l^{-1}). After centrifugation at 12,000 *g* for 15 min, the supernatant was used for enzymatic activity determination.

Peroxidase activity was assayed by measuring the oxidation of guaiacol at 470 nm. Twenty microlitres of enzyme extract was added to 2 ml of reaction mixture consisting of a solution of 0.1 M tris maleate buffer (pH 6.5), 25 mM guaiacol and 25 mM H_2O_2. Peroxidase activity was expressed as enzymatic unit g^{-1} f wt.

Lipid peroxidation

The level of lipid peroxidation was measured in terms of malonyldialdehyde (MDA) contents determined by the thiobarbituric acid (TBA) test (Zhang and Kirham, 1996). Plant material (500 mg f wt) was homogenized in 2 ml of TCA (1 g l^{-1}). The homogenate was centrifuged at 12,000 *g* for 15 min. The supernatant (0.5 ml) was added to a TBA solution (5 g l^{-1}). The mixture was heated to 95°C for 30

min and cooled in an ice bath to stop the reaction. After centrifugation at 10,000 *g* for 5 min the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient (155 mM^{-1} cm^{-1}).

All the experiments were done with Sigma products (Paris, France) and performed with a minimum of three replicates per treatment and per time point, and data were expressed as the means ± SE.

Results

Fusarium oxysporum f. sp. *albedinis* inoculation of date palm seedlings produced disease symptoms that appeared 8 days after infection. Seedlings of the susceptible cultivar showed diffused wet necrosis at root level earlier than seedlings of the resistant cultivar. However, most resistant seedlings developed a limited necrotic lesion around the inoculation point, whereas the susceptible seedlings did not. Two to 3 weeks after inoculation, the symptomatic seedlings showed root tissue softening and foliar wilting leading to the death of the seedling. After one month, 70% of seedlings of the susceptible cultivar and 30% of seedlings of the resistant cultivar had died. In both cultivars, seedlings treated with jasmonic acid also developed limited necrotic lesion around the injection point after the first week of treatment. These lesions were similar to those in *Foa*-resistant seedlings. Only a few seedlings (2%) did not react to the jasmonic acid concentration used.

H_2O_2 contents

When seedlings without disease symptoms were infected with *Foa*, H_2O_2 levels in the roots of both cultivars remained unchanged for the first 12 days. After that period the H_2O_2 level increased considerably, and differences between BSTN and JHL started to appear. After 48 days, H_2O_2 levels were 2.4 times higher than the control in BSTN, and 4.4 times higher in JHL (Fig. 1a and b). In seedlings treated with jasmonic acid, BSTN and JHL seedlings differed in their H_2O_2 levels from the start: in BSTN they remained constant for the first 12 days, then increased to 4 times that of the control by day 24, and thereafter decreased again slightly to 3 times that of the control by day 48 (Fig. 1a). In JHL seedlings on the other hand, H_2O_2 levels peaked 6 days after inoculation, with a 10-fold higher H_2O_2 content than the control. This H_2O_2 burst was followed by a lowering of the H_2O_2 level between 6 and 24 days, and a second increase between 24 and 48 days, when maximal values rose to 6.9 times that of the control (Fig. 1b).

In the leaves, both *Foa* and jasmonic acid increased H_2O_2 levels. In the BSTN cultivar after 48 days, H_2O_2 content was 65.03 $\mu g\ g^{-1}$ f wt in jasmonic acid-treated seedlings, 56.06 $\mu g\ g^{-1}$ fwt in *Foa* infected seedlings and 36.59 $\mu g\ g^{-1}$ fwt in control seedlings (Fig. 1c). In the JHL cultivar, similar levels of H_2O_2 occurred only after *Foa* infection or after jasmonic acid treatment. From the 12th day, H_2O_2 levels were twice as high as in the control (Fig. 1d). In seedlings showing disease symptoms, levels of H_2O_2 in the roots and leaves were lower than in resistant seedlings (Table 1).

Peroxidase activity

In roots without symptoms, peroxidases were activated earlier in the BSTN cultivar than in the JHL cultivar. BSTN seedlings had a peroxidase activity two times higher than the control 6 and 24 days after *Foa* infection (Fig. 2a). In JHL seedlings, peroxidase activity increased only after 12 days of inoculation (Fig. 2b). Enzymatic activity increased considerably with jasmonic acid treatment in both cultivars, particularly after 12 days. In the leaves, enzyme activity was not significantly different from that in the control (results not shown). In symptomatic seedlings, peroxidase activity decreased in the roots and leaves after expression of the first symptoms (Table 1).

Lipid peroxidation

In roots without symptoms, MDA levels rose following *Foa* infection and jasmonic acid treatment. Figure 3a and b shows that jasmonic acid treatment hastened the rise in MDA levels in both cultivars from the 3rd day. In *Foa*- infected seedling roots, MDA levels rose from day 6 in BSTN seedlings, and from day 12 in JHL seedlings. In the leaves, MDA levels rose more strongly in BSTN treated seedlings than in JHL seedlings. This increase started earlier (day 6) in the BSTN seedlings, while in treated and infected JHL seedlings it did not start until day 12 and day 24, respectively (Fig. 3c and d)

The roots and leaves of symptomatic seedlings had lower levels of MDA than roots and leaves of asymptomatic seedlings (Table 1).

Discussion

Jasmonic acid produced hypersensitive-reaction like lesions in the roots (site of treatment) similar to those caused by *Foa* in resistant seedlings. Study of the defence reactions at these lesion sites showed that both *Foa* and jasmonic acid increased levels of H_2O_2 and MDA and intensified peroxidase activity as compared with seedlings showing disease symptoms and control seedlings. However, the response of tissues to jasmonic acid treatment was greater than the response to *Foa*, suggesting that jasmonic acid has an important role in these metabolic pathways. These results

Table 1. Changes in guaiacol oxidase activity and H_2O_2 and MDA contents in date palm seedlings cv. BSTN one month after *Foa* inoculation.

Date palm seedlings	H_2O_2 level (μg g^{-1} f wt)		Guaiacol oxidase activity (U g^{-1} f wt)		MDA content (μM g^{-1} f wt)	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
Asymptomatic	45.56±0.183	90.97±2.213	1537.50±32.5	1735±245.05	14.96±0.912	20.14±1.258
Symptomatic	33.04±0.67	56.06±3.033	386.25±22.98	950±98.99	7.50±0.169	16.12±1.258

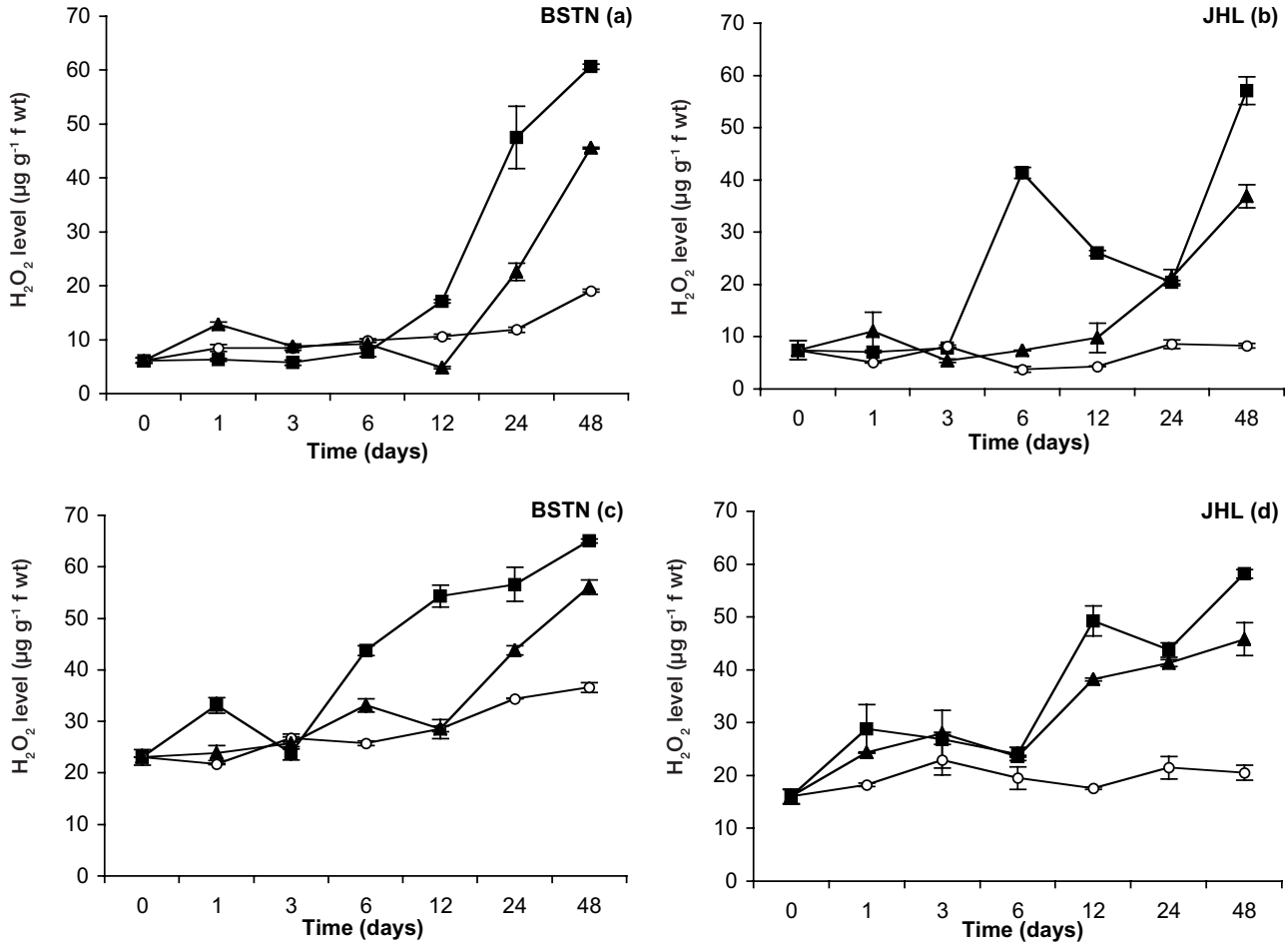

Fig 1. Changes in H_2O_2 level in seedling roots (a, b) and leaves (c, d) of date palm cultivars BSTN and JHL after inoculation with *Fusarium oxysporum* f. sp. *albedinis* (*Foa*) (▲); treatment with jasmonic acid (■); distilled water control (O).

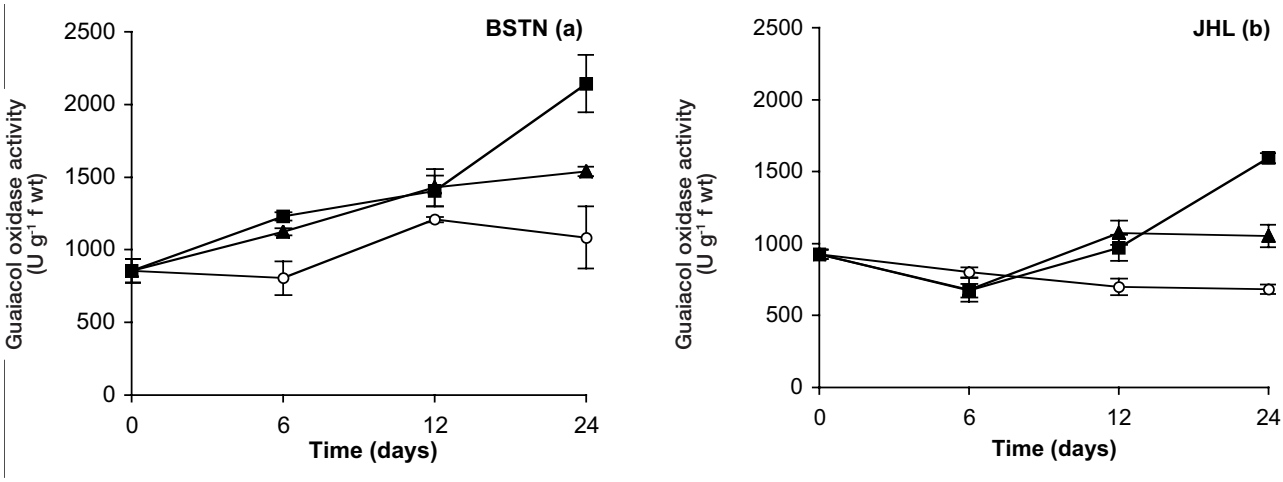

Fig. 2. Guaiacol oxidase activity in date palm seedling roots cv. BSTN (a) and JHL (b) after inoculation with *Fusarium oxysporum* f. sp. *albedinis* (*Foa*) (▲); treatment with jasmonic acid (■); distilled water control (O).

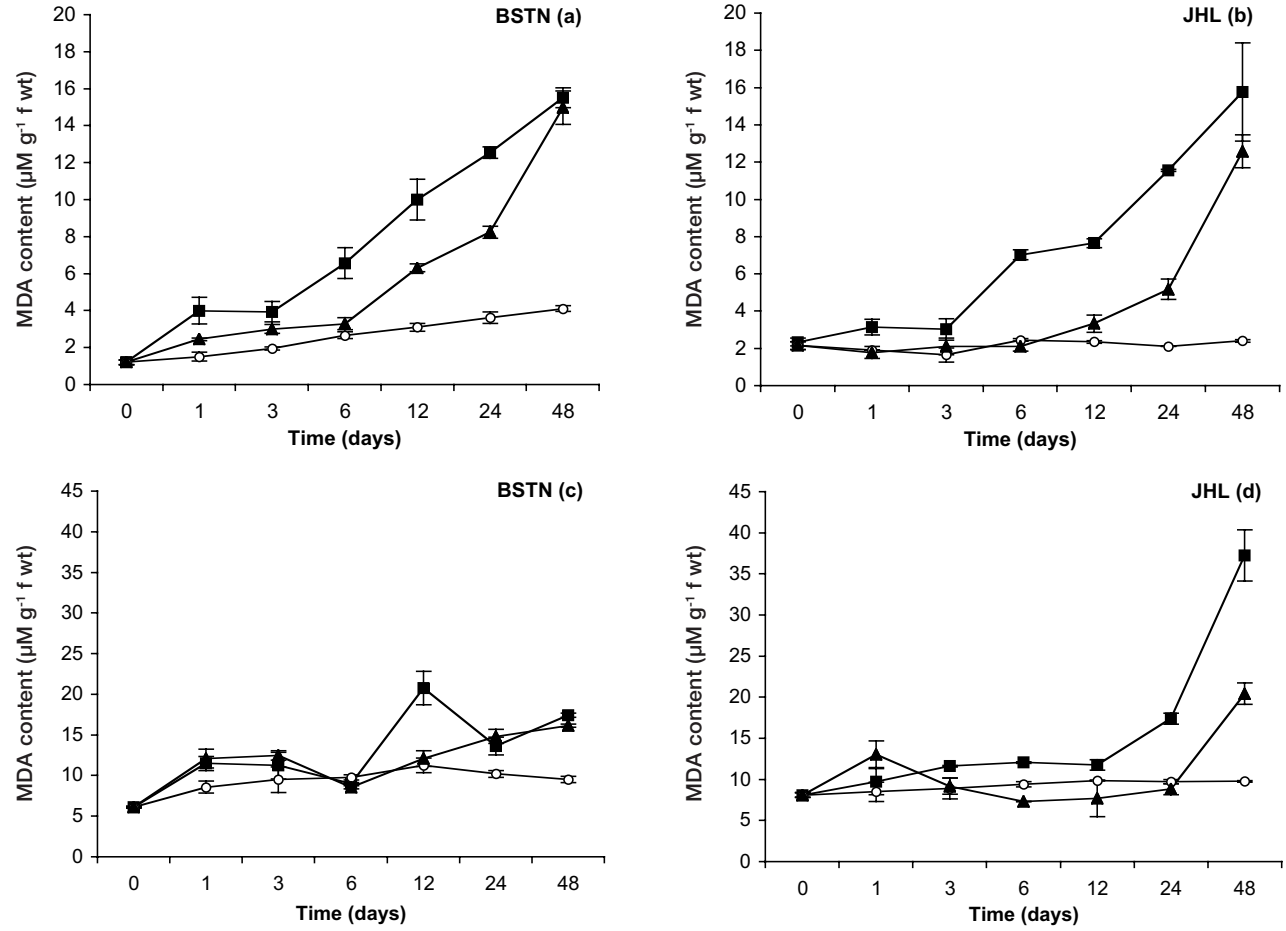

Fig 3. Changes in MDA content in date palm seedling roots (a, b) and leaves (c, d) of cultivars BSTN and JHL after inoculation with *Fusarium oxysporum* f. sp. *albedinis* (*Foa*) (▲); treatment with jasmonic acid (■); distilled water control (O).

are consistent with those of Orozco-Cardenas *et al*. (2001) who showed a relationship in tomato plants between jasmonic acid, systemin, oligogalacturonides and H_2O_2 signals for systemic signalling in responses to wounding. In the same way, Rakwal *et al*. (2002) reported a rapid increase in endogenous jasmonic acid level after fungal elicitor treatment of rice seedling leaves. In addition, Mueller *et al*. (1993) found that in several plant species, the induction of phytoalexins such as alkaloids by the fungal wall in plant cell suspensions was strictly correlated with the synthesis of jasmonic acid. In date palm, the new hydroxicinnamic acid derivatives produced in roots infected with *Foa* also occurred in jasmonic acid-treated seedlings (El Hadrami *et al*., 2000). Jasmonic acid may be considered a signal for a defence reaction such as an oxidative burst, and a signal for the synthesis of phenolic phytoalexins in date palm as a defence against bayoud disease.

H_2O_2 has been stated to play an important role in the development of resistance in many species (Grant and Look, 2000). In date palm up to now no study has examined the increase in H_2O_2 levels after *Foa* infection. Higher levels of H_2O_2 may have a role in causing limited necrotic lesions (HR-like lesions). Lipid peroxidation, which coincided with H_2O_2 accumulation in the two date palm cultivars studied supports this hypothesis. In numerous systems, lipid peroxidation has been described as a consequence of oxidative burst (Rusterucci *et al*., 1996). Foyer *et al*. (1997) found that H_2O_2 initiated localized oxidative damage leading to disruption of metabolic function and the loss of cellular integrity at the accumulation site. Mudd (1997) reported that ozone, which mimics the oxidative burst, caused alterations in the lipid composition of the plasma membrane and increased the production of linoleic acid, a precursor of jasmonic acid. Thus oxidative burst, which caused lipid peroxidation in the date palm/*Foa* pathosystem, may have increased endogenous levels of jasmonic acid, stimulating the defence reaction. H_2O_2 may also have acted as a signal for defence reactions in the date palm-*Foa* interaction since it accumulated in the uninfected leaves of resistant plant. Similarly, Vanacker *et al*. (2000) found that in the barley-powdery mildew interaction, healthy mesophyll cells exhibited transient H_2O_2 production, which induced a defence reaction.

The present work found a positive correlation between H_2O_2 level and peroxidase activity. Thus, increases in peroxidase activity have been found in seedlings showing a defensive reaction (HR-like lesions). Root peroxidases started to rise earlier in BSTN seedlings than in JHL seedlings. These results are consistent with Baaziz *et al*. (1996) who found a limited mortality of date palm seedlings with high levels of soluble peroxidases. Similarly, Kristensen *et al*. (1999) purified and characterized a barley-coleoptile peroxidase that had accumulated after powdery mildew infection or epidermal cell wounding. They suggested that this peroxidase (Prx 7) was responsible for the biosynthesis of antifungal compounds know as hordatines. In the same pathosystem, Scott-Graig *et al*. (1995) earlier stated that an extracellular peroxidase was involved in the deposition of phenolic compounds in papillae to prevent further pathogen attacks. This work shows that in the date palm-*Foa* interaction peroxidases are related to the establishment of resistance. These enzymes may have a role in the biosynthesis of phytoalexins and diverse molecules with antifungal properties. This role is related to the fact that these enzymes reinforce the cell walls. Jasmonic acid may have an important role in the establishment of these defense reactions in date palm.

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