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Chitosan, antifungal product against *Fusarium oxysporum* f. sp. *albedinis* and elicitor of defence reactions in date palm roots

Majida El Hassni[1], Abdelbasset El Hadrami[2], Fouad Daayf[2], Essaid Ait Barka[3] and Ismail El Hadrami[1]

[1]Laboratoire de Physiologie Végétale, Equipe Biotechnologies et Physiologie Végétales, Département de Biologie, Faculté des Sciences Semlalia, BP. 2390, 40 001 Marrakesh, Morocco
[2]Department of Plant Science, 222 Agriculture Building, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada
[3]Université de Reims, Champagne-Ardenne, UFR Sciences, URVVC, Laboratoire de Physiologie Végétale, BP. 1039, 51687 Reims Cedex 2, France

Summary. The effect of chitosan on the growth and morphology of *Fusarium oxysporum* f. sp. *albedinis* (*Foa*), the causal agent of Bayoud disease, and its ability to elicit a defence reaction against this fungus in date palm roots were investigated. Chitosan at 1 mg ml^{-1} reduced the growth of *Foa* on potato dextrose agar medium by an average of 75%, while mycelial growth was totally inhibited in a liquid medium. When added to a solid medium, chitosan caused morphological changes in *Foa* mycelium. In addition, when injected into roots at three concentrations (0.1, 0.5 and 1 mg ml^{-1}), chitosan elicited peroxidase (PO) and polyphenoloxidase (PPO) activity and, particularly at the concentration of 1 mg ml^{-1}, increased the level of phenolic compounds. Concerning phenolics, chitosan led to an accumulation of non-constitutive hydroxycinnamic acid derivatives, known to be of great importance in date palm resistance to Bayoud. The accumulation of hydroxycinnamic acid derivatives was greater in cv. BSTN than in cv. JHL. Chitosan could be used to protect date palm against this vascular disease.

Key words: Bayoud, biocontrol, disease resistance, elicitors, phenolics, phytoalexins.

Introduction

Bayoud disease, a vascular disease of date palm (*Phoenix dactylifera* L.) is caused by a telluric fungus *Fusarium oxysporum* f. sp. *albedinis* (*Foa*). It represents the most serious problem for date palm cultivation particularly in North Africa. Bayoud has destroyed more than two thirds of date palm groves in Morocco and is now spreading through the west and center of Algeria. Although this disease is still advancing, no curative treatment yet exists. The propagation and use of resistant varieties is one possible way to reduce the impact of Bayoud. However, the quality of fruits in resistant varieties such as Bousthami Noire (BSTN), Iklane (IKL), Tadment (TDMT), Sair Layalet (SLY), Boufegous-Moussa (BFGM) and Bousthami Blanche (BSTB) is not as good as that in more susceptible cultivars such as Mejhoul (MJHL) and Boufegous (BFG). A viable alternative to the propagation and use of resistant cultivars may therefore be by induction of resistance in susceptible cultivars using biotic and/or abiotic elicitors.

Elicitors include macromolecules such as oligosaccharides (Shibuya and Minami, 2001), glycoproteins, and peptides or small molecules such as arachidonic, jasmonic or salicylic acid (Benhamou

Corresponding author: I. El Hadrami
Fax: +212 44 439997
E-mail: hadrami@ucam.ac.ma

and Nicole, 1999). Elicitors induce structural and/or biochemical responses in the host leading to the production of phytoalexins, the accumulation of antimicrobial proteins and the enhancement of the cell wall lignification.

One of the biotic elicitors is chitosan, a partially deacetylated derivative obtained from the chitin of crustacean shell wastes. Chitosan has been shown to have a potential role in the protection of plants against pathogens. It inhibits the growth of several fungi including *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou *et al.*, 1998), *Pythium aphanidermatum* (El Ghaouth *et al.*, 1994), *Botrytis cinerea*, *Monilinia laxa* and *Alternaria alternata* (Romanazzi *et al.*, 2002). Besides being an antifungal agent, chitosan also elicits defence mechanisms in the host plant. Treatment of wheat seeds with chitosan activates the phenylpropanoid pathway, leading to a higher accumulation of phenolic acids and lignin in the primary leaves (Reddy *et al.*, 1999). Dornenburg and Knorr (1997) reported that chitosan increased the activity of phenylalanine ammonia-lyase (PAL), the key enzyme involved in the synthesis of phenolic compounds and Rakwal *et al.* (2002) found that chitosan increased levels of 2-oxo-phytodieonic acid (OPDA) and endogenous jasmonic acid in rice (*Oryza sativa* L.) leading to the activation of the octadecanoid pathway. Chitosan also induces chitinase activity and ß-1,3 glucanase in oranges and strawberries (Romanazzi *et al.*, 2002) and increases lipoxygenase activity in wheat (*Triticum aestivum* L.) (Bohland *et al.*, 1997).

The effect of chitosan on the date palm-*Foa* pathosystem has not yet been studied. The aim of this work was to study the effect of chitosan *in vitro* on the growth of *Foa* in liquid and solid media, and to determine whether chitosan elicited a defence reaction in date palm seedlings. Constitutive and induced phenolics, peroxidases and polyphenoloxidases were also studied since these substances and enzymes have been found to play an important role in date palm resistance to *Foa* (El Hadrami *et al.*, 1996, 1997).

Materials and methods

Fungal and plant materials

Fusarium oxysporum f. sp. *albedinis* strain ZAG from Bayoud-infected date palm was isolated and after its aggressiveness was established in our laboratory it was grown on potato dextrose agar (PDA) medium at 25°C in darkness (Daayf *et al.*, 2003; Dihazi *et al.*, 2003; El Hassni *et al.*, 2004).

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°C.

Preparation of chitosan

Purified chitosan (Sigma Chemical Co., Louis, MO, USA) was prepared by dissolving the product in 0.25 N HCl, and centrifuging at 1000 *g* for 15 min to remove undissolved particles. To precipitate the chitosan, the viscous solution was neutralised with 2.5 N NaOH. Chitosan was recuperated by filtration, thoroughly washed with deionised water to remove salts, and lyophilised. Chitosan stock solution (10 mg ml^{-1}) was prepared by dissolving chitosan in 0.05 N HCl and adjusting the pH to 5.6 (Benhamou *et al.*, 1998).

Effect of chitosan on growth and morphology of *Foa*

Growth on solid medium

The *Foa* ZAG strain was grown on PDA without chitosan or with chitosan at three concentrations: 0.1, 0.5 or 1 mg ml^{-1}. Radial growth was measured every 2 days for 8 days and mycelium from the margin of the colonies was observed under the light microscope.

Growth in liquid medium

A hundred microliters of conidial suspension adjusted to 10^8 spores ml^{-1} was added to Erlenmeyer flasks containing 20 ml of Czapeck liquid medium (El Fakhouri *et al.*, 1996) unamended or amended with chitosan at 0.1, 0.5 or 1 mg ml^{-1}. After incubation on a rotary shaker (100 rpm) at 25°C for 8 days, the culture liquids were filtered and the biomass obtained with each concentration was weighed.

Chitosan treatment of date palm seedlings

Seedlings of cv. JHL and BSTN were surface-sterilized in 0.2% NaOCl, washed three times with sterile distilled water and the roots injected with 10 μl of chitosan solutions at three concentrations,

0.1, 0.5 and 1 mg ml^{-1}. Control seedlings were treated with sterile distilled water. For each concentration, at least 30 plants were used.

Enzyme activity assays

Peroxidases were extracted with Tris-maleate buffer (0.1 M, pH 6.5) containing Triton 100× (0.1 g l^{-1}) (El Hassni *et al.*, 2004). Peroxidase activity was assayed spectrometrically at 470 nm using guaiacol as a substrate. Twenty microlitres of enzyme extract (250 mg f wt 2 ml^{-1}) was added to 2 ml of a reaction mixture consisting of 0.1 M Tris maleate buffer (pH 6.5) and 25 mM guaiacol. Reactions were initiated with 20 μl of H_2O_2 (10%) and stopped after 3 min (El Hadrami and Baaziz, 1995). Polyphenoloxidase (PPO) activity was measured using the protocol described by Chen *et al*. (2000). 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 polyphenoloxidases. The rate of absorbance increase at 420 nm was measured for 3 min.

Extraction and analysis of phenolic compounds

Frozen tissues (roots) from the different treatments were extracted three times with 80% aq MeOH at 4°C under continuous stirring. The homogenate was centrifuged at 7000 *g* × 3 min and supernatants were stored at -20°C until they were analysed by HPLC and spectrophotometry. To determine total phenolics, Folin Ciocalteu's reagent was used and the optical density was determined at 760 nm (El Hadrami *et al.*, 1997). The levels were expressed as μg equivalent of catechin per g of f wt. Phenolic extracts were also analysed by HPLC using a Waters 600E HPLC (Waters, Paris, France) equipped with a Waters 990 photodiode array detector and Millipore software for data analysis. The appropriate gradient of acetonitrile-*o*-phosphoric acidified bidistilled water (pH, 2.6) was used with an Interchrom C18 reversed phase analytical column (4.6×150 mm, 5 μm, Macherey-Nagel, Düren, Germany). Three wavelengths (280, 320 and 350 nm) were used during elution. Phenolics were identified on the basis of their retention times and their spectra as compared with standards. When necessary, co-injection and elution with standards were used to establish the identity of the compounds. For the quantitative measures of the hydroxycinnamic acid derivatives, the peak areas were determined as equivalent of chlorogenic acid.

Statistical analysis

Each treatment was carried out in triplicate and each experiment was repeated twice. Since differences between replicates were not significant, data were pooled and subjected to ANOVA (Statistica V. 5, USA) for caffeoylshikimic acid levels and total phenolic content. Means were compared between cultivars and between treatments, using the least significant differences (LSD) at a probability level of 0.05. The content of sinapic, *p*-coumaric and ferulic derivatives was compared between cultivars and between treatments when data were available according to the same test. For enzyme activity, the extractions and the activity kinetics were performed twice. Means were compared as above.

Results

Effect of chitosan on growth and morphology of *Foa*

The addition of chitosan to the PDA medium inhibited mycelial growth of *Foa*. The degree of inhibition depended on the concentration of chitosan. At a chitosan concentration of 1 mg ml^{-1}, radial growth of *Foa* was slower in the first 4 days than it was with 0.1 and 0.5 mg ml^{-1}. After 8 days, the fungus had completely overgrown the control Petri dishes, but inhibition was 10, 40 and 75% with chitosan at 0.1, 0.5 and 1 mg ml^{-1} respectively.

Under the light microscope, chitosan at 1 mg ml^{-1} induced marked changes in *Foa* morphology. Without chitosan, *Foa* mycelium showed a dense cytoplasm (Fig. 1A). With chitosan, there were large vesicles inside the cell walls (Fig. 1B), with vacuolation (Fig. 1C), and after 8 days mycelium often appeared empty (Fig. 1D).

When a liquid medium was used, chitosan after 8 days caused 100% inhibition of *Foa* at 1 mg ml^{-1}, 65% inhibition at 0.5 mg ml^{-1} and 5% inhibition at 0.1 mg ml^{-1}.

Chitosan treatment of date palm seedlings

Enzyme activity

Maximum PO and PPO activity was observed with 1 mg ml^{-1} of chitosan. In the roots of cv. BSTN and cv. JHL seedlings, chitosan at this concentration increased PO activity sixfold and PPO activi-

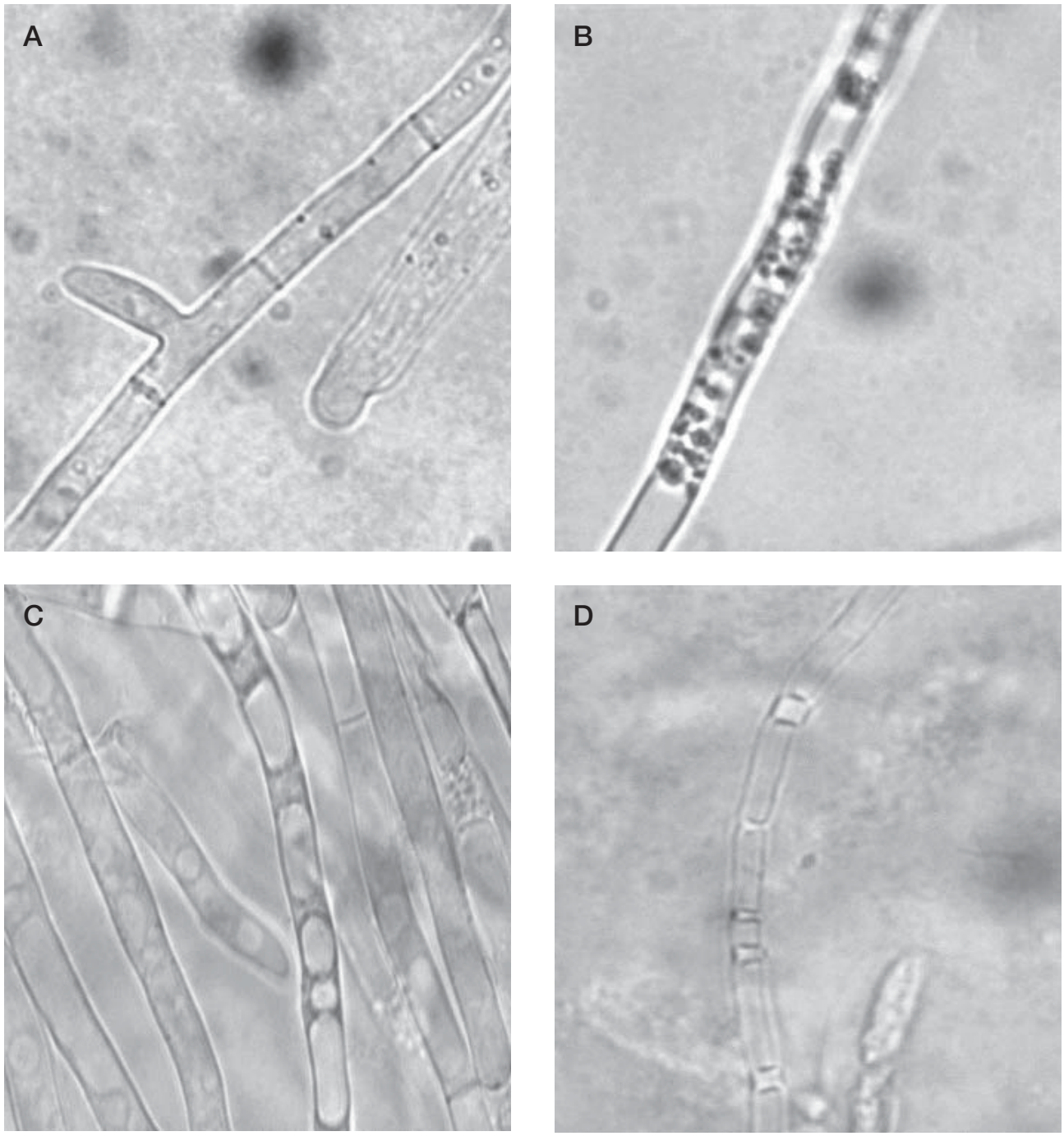

Fig. 1. Microscopic study of mycelium of *Fusarium oxysporum* f. sp. *albedinis* treated with chitosan. A; control treated with 1 mg ml^{-1} chitosan; B, presence of vesicles in mycelium; C, vacuolation; D, emptied mycelium. Bar=60 μm (A-C), 80 μm (D).

ty threefold. The highest PO activity was recorded after 10 days of incubation in the resistant cv. BSTN roots (Fig. 2A) and after 20 days in the susceptible cv. JHL roots (Fig. 2C). PPO activity was greatest after 10 days of treatment in both cultivars. There was no difference in PPO activity between seedling roots treated with 0.1 mg ml^{-1} and the control roots (Fig. 2B and D).

Elicitation of phenolic compounds

Untreated roots of cv. BSTN, generally had higher levels of total phenolics than did untreated cv. JHL ones. Chitosan increased total phenolics in both cultivars. The highest phenolic levels were recorded at a chitosan concentration of 1 mg ml^{-1} 30 days after incubation, when they were about 3 times higher than in the control roots. At 0.5 mg ml^{-1} of chitosan, phenolic compounds were also higher, with maximum levels being reached after 30 days. At 0.1 mg ml^{-1} the concentration of phenolics 20 days after injection was about 2 times higher than in the control (Table 1).

Analysis of the phenolic extracts of the roots by HPLC revealed a preferential accumulation of iso-

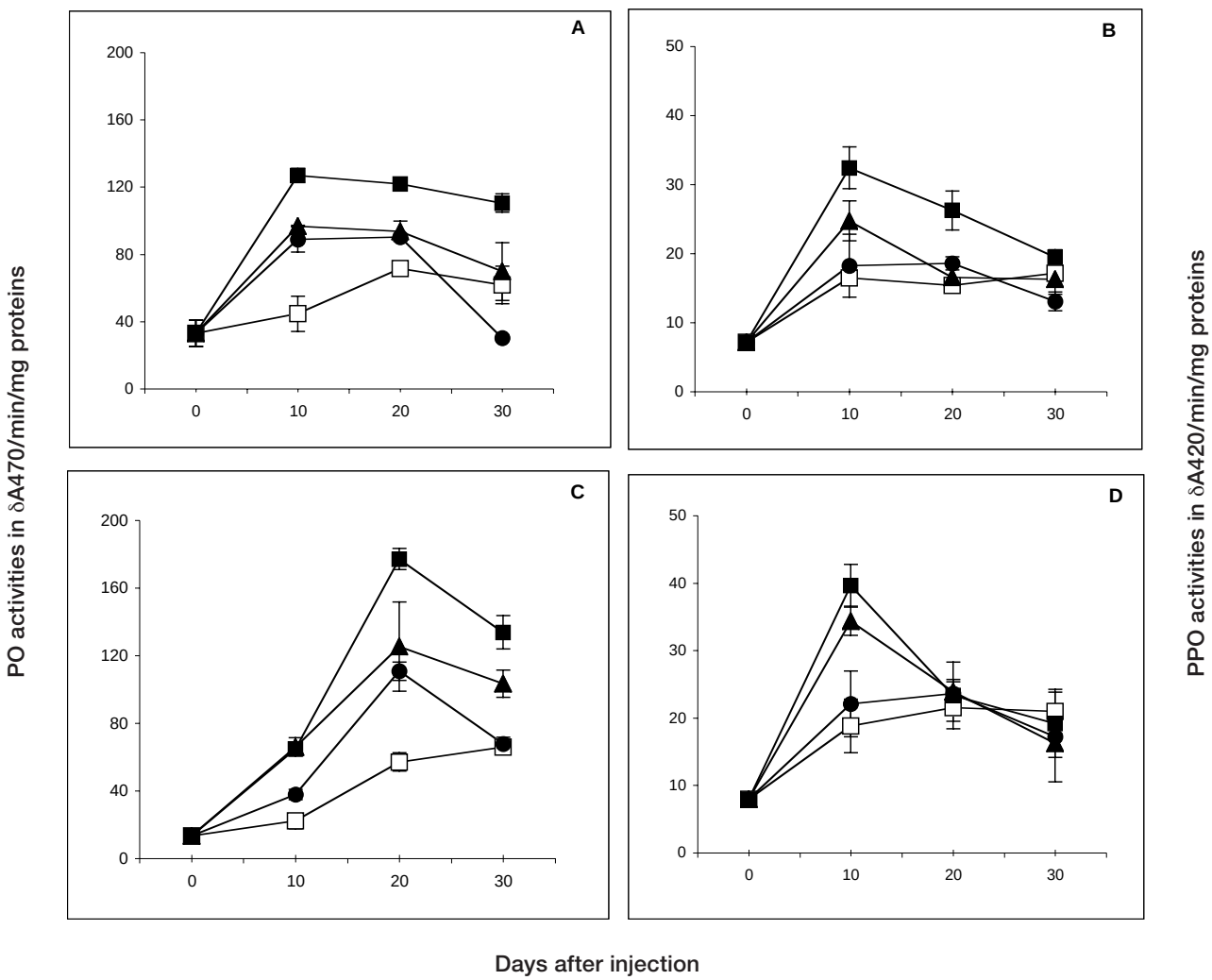

Fig. 2. Effect of various concentrations of chitosan injected into date palm roots on peroxidase (PO) and polyphenoloxidase (PPO) activity. A, B, cv. BSTN; C, D, cv. JHL. -■- 1 mg ml^{-1}; -▲- 0.5 mg ml^{-1}; -●- 0.1 mg ml^{-1}; -□- 0 mg ml^{-1}

Table 1. Hydroxycinnamic acid derivatives and total phenolic compounds ($\mu g\ g^{-1}$ FW) in root's date palm seedlings after 30 days of incubation following treatment with chitosan.

Hydroxycinnamic derivatives	Chitosan concentration (mg ml^{-1})	Days of incubation							
		BSTN cv				JHL cv			
		0	10	20	30	0	10	20	30
Caffeoylshikimic acids isomers	0	45.17±1.92	48.06±1.86	50.83±2.53	44..66±3.27	30.95±1.54	32.40±0.86	34.87±1.73	27.30±1.36
	0.1	45.17±1.92	33.7±1.28	98.6±4.06	63.73±3.17	30.95±1.54	42.84±2.44	56.14±7.33	54.3±2.25
	0.5	45.17±1.92	39.6±2.4	68.68±3.42	77.85±6.37	30.95±1.54	37.75±1.88	33.62±1.76	39.45±1.96
	1	45.17±1.92	58.53±2.9	57.70±3.09	71.36±2.15	30.95±1.54	27.76±1.38	49.12±2.44	40.36±2.34
Sinapic derivative	0	-	-	-	-	-	-	-	-
	0.1		-	-	1.47±0.08		-	-	-
	0.5		-	75.17±4.17	130.26±7.23		-	-	27.36±1.51
	1		-	7.39±0.41	167.47±9.29		-	14.76±0.81	97.67±5.42
p-coumaric derivative	0	-	-	-	-	-	-	-	-
	0.1		-	-	5.10±0.28		-	-	-
	0.5		-	1.41±0.07	326.32±18.11		-	-	-
	1		-	23.24±1.29	163.69±9.08		-	15.78±0.87	-
Ferulic derivative	0	-	-	-	-	-	-	-	-
	0.1		-	-	3.43±0.19		-	-	-
	0.5		-	62.26±3.45	12.02±0.66		-	-	-
	1		-	-	35.32±1.96		-	-	69.07±3.83
Total phenolic compounds*	0	643.15±32.03	631.42±31.44	600±29.88	780±38.84	440.23±21.92	531.42±26.46	476.19±23.71	325.71±26.18
	0.1	643.15±32.03	477.14±23.76	1236.73±61.59	954.28±47.53	440.23±21.92	754.28±37.56	953.57±47.49	685.71±34.15
	0.5	643.15±32.03	468.57±23.33	1163.26±56.61	1285.71±64.03	440.23±21.92	382.85±19.06	425.71±21.20	542.85±27.03
	1	643.15±32.03	560±27.89	857.14±42.69	1962.85±97.76	440.23±21.92	540±26.89	1011.42±50.37	1182.85±58.91

Total phenolic compounds were determined by using Folin Ciocalteu's reagent.

mers of the caffeoylshikimic acids and an accumulation of flavan-3-ols: (+)-catechin and (-)-epicatechin (results not shown). After treatment with chitosan, we here found for the first time an accumulation of non-constitutive hydroxycinnamic acid derivatives including sinapic, *p*-coumaric and ferulic derivatives (Fig. 3). This accumulation was greater in cv. BSTN than in cv. JHL after 20 days of treatment with 0.5 and 1 mg ml^{-1} of chitosan (Table 1).

As regards the constitutive phenolic compounds, treatment with the various concentrations of chitosan generally led to an increase in the caffeoylshikimic acids. The maximum level which was as twice its level at the start of the experiment, was recorded after 20 days of treatment with chitosan at 0.1 mg ml^{-1} in both cultivars. In addition, a follow-up of the non-constitutive hydroxycinnamic acid derivatives revealed that the highest level of the sinapic derivative was recorded with a chitosan concentration of 1 mg ml^{-1} after 30 days of incubation. The amount recorded was about 167.47 μg g^{-1} f wt in BSTN and 97.67 μg g^{-1} f wt in JHL (Table 1). The *p*-coumaric derivative accumulated

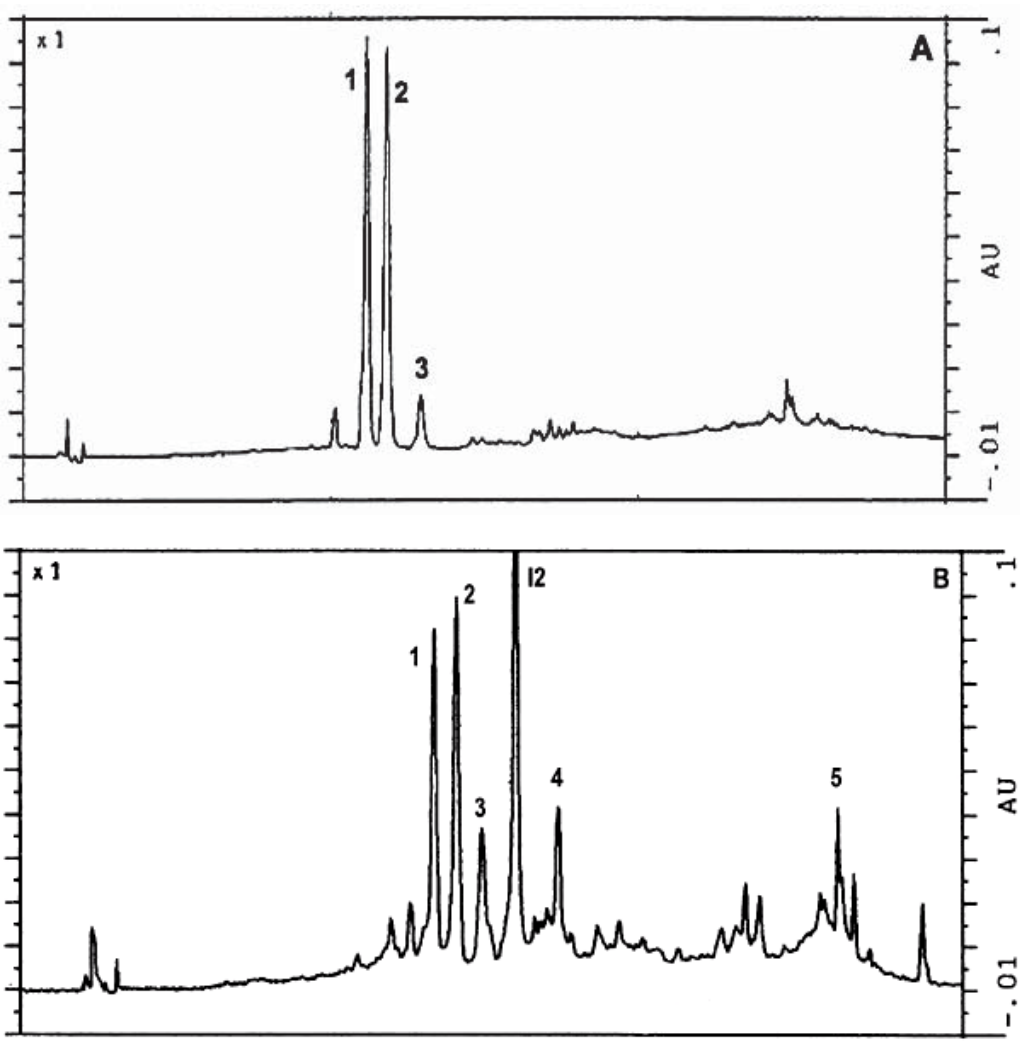

Fig. 3. HPLC profiles of phenolics extracts at 320 nm from cv. BSTN date palm roots: A, control; B, roots treated with 1 mg ml^{-1} of chitosan. 1, 2 and 3, caffeoyshikimic acids isomers; I2, sinapic derivative; 4, ferulic derivative; 5, *p*-coumaric derivative.

more strongly in BSTN than in JHL, reaching a peak after treatment with chitosan at 0.5 mg ml^{-1} (326.32 $\mu g\ g^{-1}$ f wt after 30 days of incubation), compared with 15.78 $\mu g\ g^{-1}$ f wt in JHL with a chitosan concentration of 1 mg ml^{-1} after 20 days. By contrast, the ferulic derivative accumulated more strongly in cv. JHL (max. 69.07 $\mu g\ g^{-1}$ f wt), than in BSTN (35.32 $\mu g\ g^{-1}$ f wt) after 30 days of chitosan treatment at 1 mg ml^{-1} (Table 1).

Discussion

The addition of chitosan to the culture medium inhibited mycelium growth of *Foa* by up to 75%. By contrast, in Czapeck liquid medium, where the fungus was entirely in contact with the added chitosan, mycelial growth was totally inhibited.

Light microscopy examination showed that chitosan at 1 mg ml^{-1} led to considerable morphological alterations in the mycelium which showed cellular disorganisation with vesicles, and strong vacuolation. Many mycelial cells appeared emptied of their contents after one week of culture. Very similar changes were reported by Benhamou *et al.* (1992) and Benhamou and Nicole (1999) for *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) treated with chitosan. The electron microscope revealed that chitosan caused plasmalemma retraction and cytoplasm aggregations, with chitin deposition between the retracted plasmalemma and the cell wall.

Injection of chitosan into date palm roots increased PO and PPO activity particularly at 1 mg ml^{-1}. This activity was accompanied by necrosis around the sites of injection. In the resistant cv. BSTN, peroxidase activity was stimulated after 10 days of treatment, whereas in the susceptible cv. JHL it appeared only after 20 days. In contrast, maximum polyphenoloxidase activity occurred in both cultivars after 10 days. The various concentrations of chitosan also increased the contents of the caffeoylshikimic acids, the major phenolic constitutive compounds in date palm roots; and chitosan at 0.5 and 1 mg ml^{-1} increased non-constitutive hydroxycinnamic acid derivatives such as the sinapic derivative (I2) and the *p*-coumaric and ferulic derivatives. This increase was particularly evident in the resistant cv. BSTN. The sinapic derivative in the date palm-*Foa* pathosystem has been described by El Hadrami *et al.* (1997). Higher levels of this derivative were found in the roots and rachis of palms infected with *Foa*. This compound greatly inhibits conidial germination and mycelial growth of *Foa* (El Hadrami *et al.*, 1997; Ramos *et al.*, 1997). *P*-coumaric and ferulic derivatives are also implicated in enhancing resistance of date palm to *Foa* (Daayf *et al.*, 2003; Dihazi *et al.*, 2003; El Hassni *et al.*, 2004). In the same way, Reddy *et al.* (1999) reported that treatment of wheat seeds with chitosan against *Fusarium graminearum* increased caffeic acid, *p*-coumaric acid and ferulic acid as well as the benzoic acid derivatives (benzoic, protocatechuic and gallic acids), and chlorogenic acid, which all have an antimicrobial effect. Chitosan also increased the lignin content.

In date palm, there was a positive relation between higher levels of phenolic compounds and greater PO and PPO activity after treatment with chitosan. Peroxidase and polyphenoloxidase play a role in the oxidation of substrates leading to the accumulation of toxic compounds and catalyse the formation of lignin that contributes to the reinforcement of the cell wall structure preventing the penetration of pathogen (Chen *et al.*, 2000). In addition, the sinapic, *p*-coumaric and ferulic derivatives are well known to have antifungal activity (Walker, 1994) and are precursors of lignin (Vance *et al.*, 1980).

In other pathosystems, chitosan elicits biochemical and physical changes in the host plant that limit pathogen penetration and development in the host tissues. Lafontaine and Benhamou (1996) reported that treatment of tomato seedlings with chitosan protected them against *Forl*. Host cells exhibited marked changes in response to the increase of deposits enriched with callose and others with large amounts of phenol-like compounds, which constitute a barrier preventing penetration of the pathogen which was restricted to the epidermis and the outermost cortical cell layers (Benhamou and Nicole, 1999). The mode of action of chitosan was also discussed. Its activity seems to be mediated by the interaction between positively charged chitosan molecules and negatively charged residues on the fungal cell surface, as well as by its regulatory effect on plants, thus causing important changes in the membrane composition (Benhamou and Nicole, 1999; Shibuya and Minami, 2001).

In the date palm-*Foa* pathosystem, when the

seedling roots of both cv. BSTN (resistant) and cv. JHL (susceptible) were treated with chitosan and then inoculated with *Foa*, seedling mortality was reduced (results not shown). Without chitosan treatment, diseased control plants showed a diffused wet necrosis 2 to 3 weeks after inoculation leading to root softening and foliar withering. Chitosan could protect the seedlings by causing a localised necrosis (hypersensitive-like reaction) around the Foa inoculation site, surrounding the point of inoculation with *Foa* as well as by eliciting a host defence reaction.

The results first described in this paper show that chitosan inhibits and strongly affects *Foa* in addition to eliciting mechanisms of defence against *Foa*, especially by the accumulation of non-constitutive hydroxycinnamic acid derivatives (phytoalexins) known for their antifungal properties. It thus appears that chitosan can protect date palm against vascular Bayoud disease, at least during the first stages of its development in the greenhouse. Further studies are needed to examine the durability of the resistance.

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