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Full Length Article

Role of Phenolic Metabolism in the Defense of the Olive-tree against Leaf-spot Disease Caused by *Spilocaea oleagina*

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Abstract

In order to study the role of phenolic metabolism in the defense of the olive-tree against *Spilocaea oleagina*, three defense components: soluble phenols, parietal phenols, lignin and phenylalanine ammonia-lyase (PAL), were studied in two different varieties: a resistant (Picholine du Languedoc) and a susceptible (Moroccan Picholine). The inoculation of olive-tree leaves by *Spilocaea oleagina* induces a foliar necrosis whose speed of onset and expansion distinguishes clearly the two studied varieties according to their behaviour to the leaf-spot disease. For the resistant variety, these symptoms are composed of small necrotic lesions, whereas for the susceptible variety, they appear as extended necrotic spots. These symptoms are accompanied by an increase in the accumulation of the contents of soluble and parietal phenols, the intensification of the lignification and the induction of the PAL activity of which the speed and intensity plainly distinguish both varieties under study. These results reveal that the response of phenolic metabolism to the resistance of the olive tree to the leaf-spot disease appears to occur in the early stages of infection leading to an increase in the biosynthesis of the contents of three defense components (soluble phenols, parietal phenols and lignin).

Keywords: Olive; *Spilocaea oleagina;* Phenylalanine ammonia-lyase; Phenols; Lignin

Introduction

The leaf-spot disease caused by *Spilocaea oleagina* represents the most widespread fungal disease of olive-tree (*Olea europaea*) in the world (Anton and Laborda, 1989). This disease appears as circular spots on leaves and fruits leading to their fall and the general weakening of the olive-tree (Sanchez *et al*., 1998; Trapero and Blanco, 2001). The resistance of the olive-tree varieties to the leaf-spot disease is largely studied (Mekuria *et al*., 2001; Bellini *et al*., 2008), but the defense mechanisms of olive-tree are not well-known (Uccella, 2000; Benitez *et al*., 2005). The phenolic compounds are the molecules often implied in plant defence to pathogens and associated with the plant host resistance (El Modafar and El Boustani, 2005). This preliminary study attempts to explore the role of phenolic compounds metabolism in defense of the olive tree against fungus.

Material and Methods

Biologic Material and Inoculation

This work is undertaken on a resistant variety (Picholine du Languedoc) and a susceptible variety (Moroccan Picholine) to the leaf-spot disease caused by *S. oleagina*. These varieties were planted in the station of National Institute of Agronomic Research of Marrakech. The inoculum consists of a spore suspension prepared from the leaves with typical symptoms of the disease including the spring spots especially rich in conidia. The inoculation was carried out by deposit of droplets of conidial suspension of *S. oleagina* (titrated to 10^7 conidia. mL^{-1}) on the upper surface of the leaves maintained in survival in glass Petri dishes containing Whatman filter paper. The leaves were incubated in a cold room at 15°C and in darkness for 48 h, they are afterwards transferred to a culture room at 19°C under a light intensity of 240 $\mu mol.m^{-2}.s^{-1}$ (16 h/8 h; day/night period). The response of leaves to the inoculation was followed for two months according to kinetics of 0, 2, 15, 30, 45 and 60 days after inoculation. For each point of the kinetics, ten repetitions were performed in inoculated plants as in control plants.

Extraction and Dosage of Phenols and Lignin

Extraction of soluble phenols: A 500 mg of leaves are

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crushed in the presence of liquid nitrogen and then suspended in cold methanol (80%). The extracts are then stirred for 20 min at a temperature of 4°C then filtered. After vacuum evaporation, the aqueous phase was depigmented by petroleum ether and then resuspended 2 times by ethyl acetate (V/V), evaporated under vacuum at 35°C. The dried residue is redissolved in 2 mL of methanol.

Extraction of parietal phenols: Parietal phenols are extracted from the residue of cell walls after depletion of soluble compounds. The esterified phenols in the wall are released by alkaline hydrolysis. The residue of cell walls (250 mg) is incubated with 4 mL of 2 M NaOH for 24 h at room temperature. The mixture is then acidified with 2M HCl solution. After vacuum filtration, the phenols are extracted three times with diethyl ether which was evaporated to dryness and then diluted in 1 mL of methanol.

Characterization of parietal phenols: The parietal phenols are characterized by various chromatographic techniques as previously described (El Modafar *et al.*, 2001). They are identified by their retention time in HPLC, retention factor (Rf) in the Thin Layer Chromatography (TLC) - using the different solvents - and by their fluorescence (Wood's light at 254 and 366 nm) in presence or absence of Benedikt reagent that distinguishes between the orthodiphenols of monophenols (El Modafar and El Boustani, 2001). The isolation of each spot in semi-preparative TLC is used to establish its absorption spectrum by HPLC which is compared to standard phenols.

Phenolic Compounds Assay

Phenolic compounds are determined according to the technique previously described by Macheix (1974). 5 µL of the phenolic extract was added to 250 µL of Folin-Ciocalteu reagent diluted 3 times in distilled water to which we add 0.5 mL sodium carbonate (20%). Optical density was determined at 760 nm against a control, where the phenolic extract was pretreated with the AT Polyclar (insoluble PVP 0.5%).

Extraction and Lignin Assay

The residue of the cell walls (10 mg) was incubated in the presence of 250 µL of acetyl bromide in 25% acetic acid (v/v). After one hour incubation at 70°C, 250 µL of NaOH are added to the mixture, which was then centrifuged at 10000 g for 10 min. A quantity of 100 µL of 7.5 M hydroxylamine was added to the supernatant. The lignin contents are determined by reading the optical density at 280 nm and are expressed in A_{280} nm.g^{-1} wall.

Phenylalanine Ammonia Lyase (PAL) Activity

PAL activity was determined using the technique previously described (El Modafar *et al.*, 2001; 2006). 100 mg of leaves are crushed in the presence of 2 mL of a sodium borate buffer (100 mM, pH 8.8) containing 5% of insoluble PVP and 14 mM ß-mercaptoethanol. The homogenate is then centrifuged for 30 min at 20000 g. The reaction mixture consists of 100 µL of supernatant containing the enzyme extract, 1 mL of sodium borate buffer and 100 µL of L-phenylalanine at 10 mM. After incubation for 60 min at 40°C, the trans-cinnamic acid formed is extracted using diethyl ether. This latter is evaporated to dryness and the residue is resuspended in 500 µL of methanol. The test is then compared to the methanol blank at 290 nm.

At the same time, proteins content was determined by Bradford method (1976) and a standard curve was drawn out with the serum bovine albumin. The results were expressed in percentage of specific activity and compared to the control.

Results

Foliar Symptoms

The inoculation of olive-tree leaves by *S. oleagina* induces foliar necrotic symptoms. The speed of apparition and extension of these symptoms clearly distinguishes both varieties according to their behaviour to the pathogen. In the resistant variety (Picholine du Languedoc), the first symptoms were small necrotic lesions. In the susceptible variety (Moroccan Picholine), the necrotic symptoms appear as large necrotic spots then were generalized to all the leaf.

Characterization of Parietal Phenols of Olive-tree Leaves

The analysis of parietal phenols by TLC (Fig. 1) and HPLC (Fig. 2) shows the presence of three compounds of which two have a blue fluorescence under UV (compounds 1 and 2) and the third is dark with no fluorescence (compound 3). After pulverization of the ammonia vapor, these compounds change their fluorescence (compound 1 becomes blue-green, compound 2 becomes intensely blue and compound 3 changes to fluorescent purple) indicating the presence of a free phenolic function. In the presence of the Benedikt reagent, the fluorescence of compound 1 is extinct, indicating the presence of a function ortho-diphenol. The comparison of the chromatographic (time of retention, R_f values, fluorescence) and spectral characteristics as well as the co-chromatography with the phenolic standards allows to identify the caffeic acid (compound 1), p-coumaric acid (compound 2) and ferulic acid (compound 3). These phenolic compounds are highlighted in both varieties, no qualitative difference is observed.

Phenolic Compounds and Lignin Constitutive Contents

The resistant variety “Picholine du Languedoc” is relatively richer than the susceptible variety “Picholine Marocaine” according to soluble phenols and especially in relation to

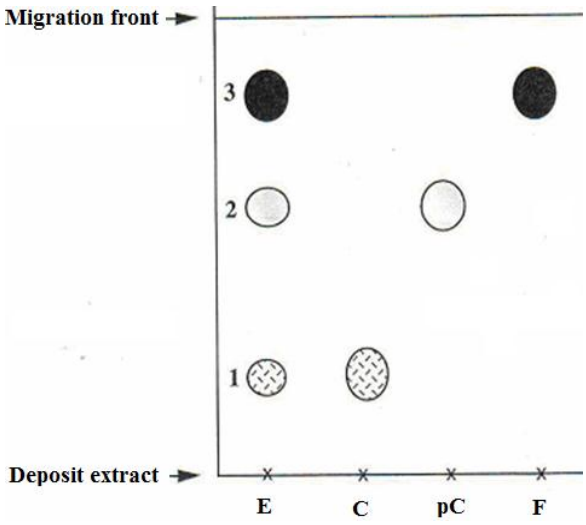

Fig. 1: TLC of the purified extract of parietal phenols of olive-tree leaves (E)
Standard phenols: caffeic acid (C), p-coumaric acid (pC) and ferulic acid (F)

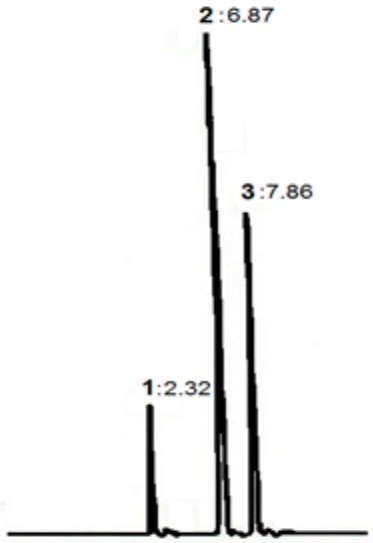

Fig. 2: Chromatogram of parietal phenols of olive-tree leaves (purified extract)
caffeic acid (1), p-coumaric acid (2) and ferulic acid (3)

parietal phenols. The contents of parietal phenols of the resistant variety are more than twice higher than those of the susceptible variety. As for the lignin content, they are not significantly different according to the two varieties (Fig. 3).

Effect of Inoculation on the Phenolic Compounds and Lignin Contents

The inoculation of olive-tree by *Spilocaea oleagina* in the resistant variety induces a fast increase in soluble phenolic contents reaching a maximum accumulation on the 2nd day, followed by a reduction to find similar contents to those of

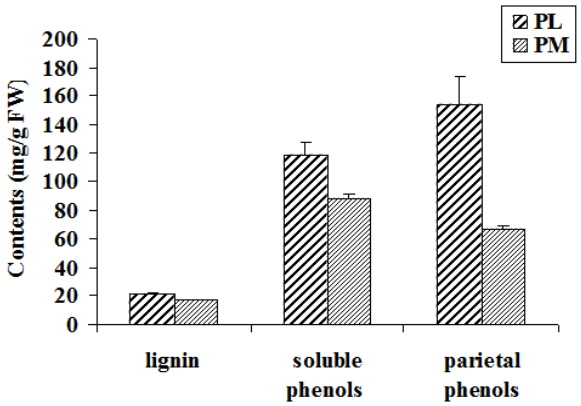

Fig. 3: Phenolic compounds and lignin constitutive contents in the "Picholine du Languedoc" (PL) and the "Moroccan Picholine" (PM)
The values represent the means of 10 replicates

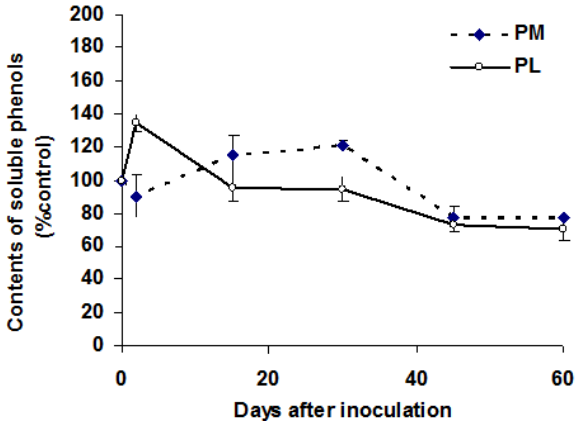

Fig. 4: Kinetic of content accumulation of soluble phenols in resistant (Picholine du Languedoc: PL) and susceptible (Moroccan Picholine: PM) varieties of the olive-tree inoculated by *S. oleagina*

the control up till the 15th day following the inoculation (Fig. 4). In the susceptible variety, the increase in soluble phenolic contents was late with a maximum on the 30th day followed by a reduction to the level up till the 45th day.

The contents of parietal phenols of the resistant variety undergo a rapid increase twice the control with a peak around the second day. While in the susceptible variety, this increase is later and lower (approximately 1,5 times higher than the control with a maximum in the fifteenth day). In both cases, the maximum accumulation is followed by a decrease reaching levels close to control (Fig. 5).

In both varieties under study, the lignin contents decrease to a level of that of the control (Fig. 6) followed by a rapid increase in the resistant variety (up till the 15th day) and a late increase in the susceptible variety (up till the 30th day). Then, the lignin accumulation finds similar contents to those of the control plants up till the 45th day following the inoculation.

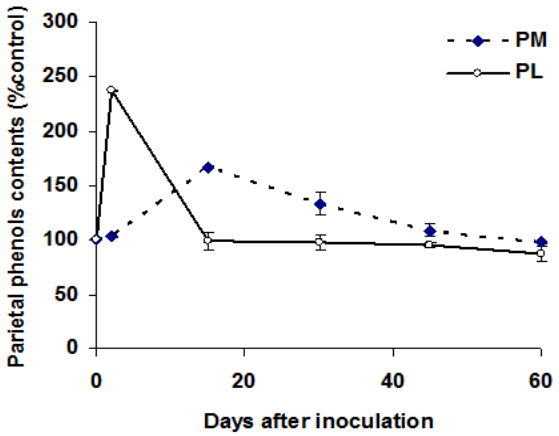

Fig. 5: Kinetic of content accumulation of parietal phenols in resistant (Picholine du Languedoc: PL) and susceptible (Moroccan Picholine: PM) varieties of olive-tree inoculated by *Spilocaea oleagina*

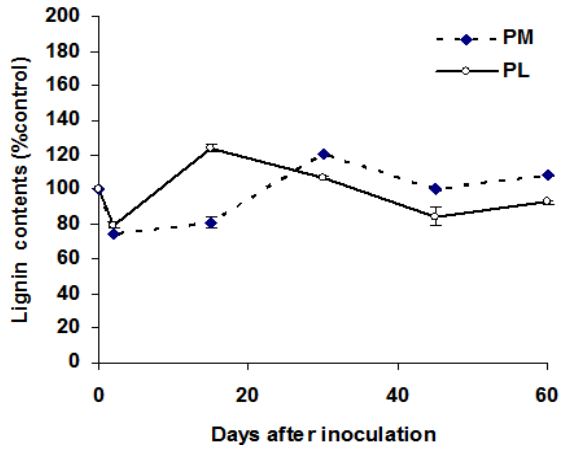

Fig. 6: Kinetic of content accumulation of lignin in resistant (Picholine du Languedoc: PL) and susceptible (Moroccan Picholine: PM) varieties of olive-tree inoculated by *Spilocaea oleagina*

Effect of Inoculation on the PAL Activity

The *S. oleagina* inoculation of olive leaves causes an increase in PAL activity (Fig. 7). However, the response to the resistant variety is faster and more intense than that of the susceptible variety. Thus, the PAL post-inflectional activity of resistant variety leaves increases rapidly to reach a maximal activity of about 4.6 times greater that the control on the second day.

In the susceptible variety, the PAL activity remains low during the first two weeks and reaches a maximum (about 3 times higher than the control) at the 30th day; however, it remains lower than the activity observed in the resistant variety. In both cases, and starting from the 45th day after inoculation, the PAL activity decreases to an activity close to that of the control.

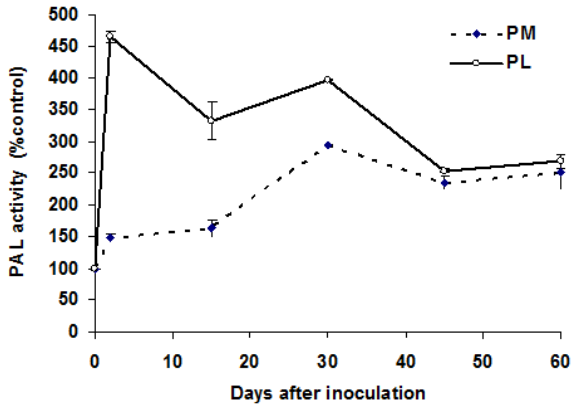

Fig. 7: Kinetic of induction of phenylalanine ammonia-lyase activity in resistant (Picholine du Languedoc: PL) and susceptible (Moroccan Picholine: PM) varieties of olive-tree inoculated by *Spilocaea oleagina*

Discussion

The resistance of the olive tree varieties to the leaf-spot disease is largely studied (Mekuria *et al*., 2001; Bellini *et al*., 2008; Rahioui *et al*., 2009), but the defense mechanisms of olive-tree are not well-known (Uccella, 2000; Benitez *et al*., 2005). The phenolic compounds are often implied in defense to the plant pathogens and associated with the plant host resistance (Nicholson and Hammerschmidt, 1992; El Modafar and El Boustani, 2005; Zine El Aabidine *et al*., 2010).

The inoculation of olive-tree by *S. oleagina* was accompanied by foliar necrotic symptoms and important modifications in the phenolic metabolism of which the induction rate clearly distinguishes the studied varieties according to their behaviour to *S. oleagina* blotch. Thus, in the resistant variety (Picholine du Languedoc), the inoculation induced small necrotic lesions. This early and localized necrotic process was preceded by a fast and intense increase of the phenolic compounds and lignin contents and by a fast increase of the PAL activity, with a maximum of the enzymatic activities on the 2nd day following the inoculation. In the susceptible variety (Moroccan Picholine), the necrotic symptoms appeared late in the form of large necrotic spots and then generalized to all the leaf. These necrotic symptoms were preceded by a late increase of the phenolic compounds contents and of the PAL activity.

Stimulation of phenolic metabolism, particularly the PAL activity, represents one of the earliest reactions in plant in response to pathogen infection. This response is often associated to the plant resistance (Mozzetti *et al.*, 1995; Sharan *et al*., 1998; El Modafar *et al.*, 2006). In fact, the PAL is the key enzyme of phenolic metabolism leading to the biosynthesis of fungitoxic phenolic compounds, phytoalexins, and salicylic acid precursors of lignin (Hahlbrock and Scheel, 1989; Weisshaar and Jenkins, 1998;

El Modafar and El Boustani, 2005). In the interaction Olive tree/*S. oleagina*, the infection is accompanied by an increased accumulation of soluble and parietal phenols and an intensification of lignification. In all cases, the response of the resistant variety was faster and more intense than the susceptible variety.

Since these components of defense induced in the olive tree are all dependent on the level of the PAL activity, their post infectional response is presumably related to an increased activity of this enzyme. The differential response of the PAL activity induction in the resistant variety and in the susceptible variety can explain the difference in the accumulation intensity and in the speed of soluble and cellular wall phenols as well as in the lignification process.

Soluble phenolic compounds have direct action against phytopathogenic microorganisms (Hassan *et al*., 2004; Gunen *et al*., 2005). They can play a role in the defense of the olive-tree to *S. oleagina* and seem to constitute potential markers of the resistance of the olive-tree to this disease (Rahioui *et al*., 2009). Several studies have shown that the soluble phenols express a fungitoxic effect (Pereira *et al*., 2007; Rahioui *et al*., 2009; Zine El Aabidine *et al*., 2010). They seem to be implied in the defense of the olive-tree by inhibiting the pectinases of *S. oleagina* and by constituting a precursor of phytoalexins (Uccella, 2000), extremely toxic molecules induced in response to pathogen infections (El Modafar *et al*., 1999; Rahioui *et al*., 2009; Zine el Aabidine *et al*., 2010).

In this study we have identified the parietal phenols related to the olive tree. These phenols are represented by the caffeic acid, p-coumaric acid and ferulic acid. No qualitative difference was observed in both varieties studied. However, the content components of parietal phenols of the resistant variety are twice more important than the susceptible variety. Moreover, the inoculation is expressed through an increase in the insolubilization of the parietal phenols, which is the highest in the cell wall of the resistant variety.

A high accumulation of phenols in the cell walls is reported in various host-parasite interactions which reflects a defense reaction (Friend, 1981; Ikegawa *et al*, 1996). A non-solubility of phenols in the wall may change its mechanical properties by making it less extensible (Fry, 1986; Ikegawa *et al*., 1996) and therefore less-biodegradable (Eraso and Harteley, 1990). The walls rich in phenols, then, become more resistant to the action of parasitic hydrolytic enzymes (Eraso and Harteley, 1990).

On the other hand, if the constitutive contents of the lignin are not significantly different between the resistant and the sensitive variety, the post-infectional contents undergo a more rapid and a more intense increase in the resistant variety. The acceleration of the lignification process is a reaction, which is often detected during host-parasite interactions (Bell, 1981; Nicholson and Hammerschmidt, 1992). The implication of lignin in plant resistance to pathogenic agents is thoroughly demonstrated (Rioux and Biggs, 1994). The role of the lignin in the resistance resides in the fact that it is not degraded by most of the pathogens, except for lignolytic fungi (Blanchette, 1994).

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