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Induction of Early Oxidative Events in Soft Wheat Leaves Inoculated with *Septoria tritici* and their Relationship to Resistance of Moroccan Cultivars

ZINEB DIANI, EL MOSTAFA OUARRAQI, SALAMA AISSAM, DRISS HSISSOU AND CHERKAOUI EL MODAFAR[1]
Laboratory of Biotechnology Valorisation and Protection of Agro-Resources, Faculty of Sciences and Technology Gueliz, Cadi Ayyad University, 40 000 Marrakech, Morocco
[1]Corresponding author's e-mail: elmodafar@fstg-marrakech.ac.ma

ABSTRACT

The inoculation of soft wheat by *Septoria tritici* induces a foliar necrosis and an important modifications in the oxidative metabolism of which the time course clearly distinguishes the studied cultivars according to their behaviour to this disease. In the resistant cultivars (Mahdia, Achtar), the inoculation was accompanied by an early and localized necrosis and a fast and intense increase of the peroxidase (POD) and the superoxide dismutase (SOD) activities and of a fast, weak and transient accumulation of the malonydialdehyde (MDA). In the susceptible cultivars (Nasma & Marchouch), the necrotic symptoms appears tardily and preceded by a late increase of the activities of polyphenol oxidase (PPO), SOD and catalase (CAT) and an important accumulation of the MDA. These results suggest that the fast and localized necrosis reaction in resistant cultivars was related to the intervention of the POD, whereas the late and generalized necrosis in the susceptible cultivars was associated to the PPO. In addition, the differential responses of the SOD activity in the resistant and susceptible cultivars suggest a key role of the SOD in soft wheat defense.

Key Words: *Triticum aestivum*; *Septoria tritici*; Resistance; Early oxidative events

INTRODUCTION

Septoria *tritici* blotch of wheat constitutes one of the limiting factors of the wheat culture causing important damage of yield and can lead to the total destruction of the wheat culture (Eyal, 1999; Hardwick *et al.,* 2001; Bearchell *et al.,* 2005). The severity of the disease was related to several factors including the absence of an adequate resistance in the majority of wheat cultivars and the change of the cultivation methods and environmental conditions (Fraaije *et al.,* 2005; Zhan *et al.,* 2006; Fraaije *et al.,* 2007). The biology of the wheat infection by *S. tritici* has been largely studied (Cohen & Eyal, 1993; Kema *et al*., 1996a; Duncan & Howard, 2000; Rohel *et al*., 2001). No significant difference exists with regard to spore's germination capacity and the pathogen penetration between the susceptible and resistant cultivars. However, the speed of apparition and extension of the necrotic symptoms and the importance of the plant invasion by the parasite distinguish the resistant and susceptible cultivars (Eyal & Levy, 1987; Cohen & Eyal, 1993; Ballantyne & Thomson, 1995; Kema *et al*., 1996b; Shetty *et al*., 2003). In resistant cultivars, the mycelial penetration was accompanied by small necrotic lesions, whereas in the susceptible cultivars, the necrotic symptoms appear in the form of large necrotic spots then was generalized to all the leaf. In the same way, the mycelial growth and the pycnidial development were inhibited in the resistant cultivars, whereas the pathogen invades the leaves of the susceptible cultivars (Kema *et al*., 1996b; Shetty *et al*., 2003). Although the genetic determinism of the wheat resistance to *S. tritici* has been largely studied (Brown *et al.,* 2001; Simon *et al.,* 2001; Zhang *et al*., 2001; Brading *et al.,* 2002; Chartrain *et al.,* 2005), the defense mechanisms implied are not well-known. The factors responsible for the differential behaviours of the resistant and susceptible cultivars were induced after the pathogen penetration and must be required in the first stages of the infection.

In order to understand the difference of the invasion process of the plant host by the pathogen and the differential evolution of the necrotic symptoms in the resistant and susceptible cultivars, we sought in this work to characterize the early oxidative events accompanying the necrotic process in four Moroccan cultivars of soft wheat (*Triticum aestivum*) presenting differential behaviour to *S. tritici*. Indeed, the oxidative burst constitutes one of the early responses of the plant to the pathogen. The infection time course of five main components implied in the oxidative system was followed; peroxidase (POD), polyphenol oxydase (PPO), catalase (CAT), superoxide dismutase (SOD) and malonyldialdehyde (MDA), which translates the rate of lipids peroxidation.

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MATERIALS AND METHODS

Plant and fungal materials. Four Moroccan cultivars of soft wheat presenting differential behaviour to *S. tritici*; "Mahdia", "Achtar" (resistant), "Nasma" and "Marchouch" (susceptible) were selected for the present studies. The seeds of these cultivars were put to germinate on filter paper in the darkness at 25°C. After 24 h, the seeds were placed at 5°C for 48 h followed by incubation at 25°C for 24 h. The plants were then cultivated in a culture room at 19°C, a photoperiod of 16 h/8 h (day/night) and an illumination intensity of 240 µmol m^{-2} s^{-1}. The inoculation was carried out at the four leaves stage by pulverization of a suspension of *S. tritici* spores (10^7 spores mL^{-1}) prepared from a PDA (Potato Dextrose Agar) culture of strain 142 recognized by its virulence. The control plants were treated in the same way by replacing the suspension of spores by sterile distilled water. The plants were then placed at 19°C and the darkness for 48 h then under a photoperiod of 16 h/8 h (day/night) and an illumination intensity of 240 µmol m^{-2} s^{-1}. The burst oxidative responses were followed in the leaves during ten days according to time course of 0, 2, 4, 6, 8 and 10 days after inoculation. The results represent the means of ten repetitions.

Extraction and Determination of the Enzymatic Activities

Peroxydase, polyphenol oxidase and catalase activities. The leaves (100 mg) were crushed in 1 mL of phosphate buffer (20 mM, pH 7). The homogenate was centrifuged at 15000 g for 20 min at 4°C. The supernatant obtained was used for the determination of the enzymatic activities of POD, PPO and CAT (Zhang & Kirkham, 1994).

The POD activity was determined according to the technique previously described (Hori *et al.,* 1997). The reaction mixture consisted of 200 µL of H_2O_2 at 0.3%, 300 µL of guaiacol at 20 mM, 2 mL of phosphate buffer (0.1 M, pH 6), 1 mL of distilled water and 10 µL of enzymatic extract. After 2 min, the POD activity was determined at 470 nm against a control, where enzymatic extract was replaced by distilled water.

The PPO activity was determined according to the protocol described by Hori *et al*. (1997). The reaction mixture consisted of 500 µL catechol at 1.6% in phosphate buffer (0.1 M, pH 6), 250 µL of distilled water, 200 µL of phosphate buffer (0.1 M, pH 6) and 100 µL of enzymatic extract. After 2 min, the PPO activity was determined at 470 nm against a control, where enzymatic extract was replaced by distilled water.

The CAT activity was determined according to the method of Beers and Sizers (1952) modified by Blilou *et al*. (2000). The reaction mixture consisted 1 mL of H_2O_2 at 0.18% in phosphate buffer (0.1 M, pH 7) and 100 µL of the enzymatic extract. The CAT activity was determined, while following the decrease of the absorbance during 90 s at 240 nm.

Superoxide dismutase activity. The SOD activity was determined as described by Beauchamp and Fridovich (1971) and modified by Meloni *et al*. (2003). The leaves (100 mg) were crushed in 1 mL of phosphate buffer (50 mM, pH 7.8) and the insoluble PVP (polyvinylpyrrolidone) at 1%. The homogenate was centrifuged at 15000 g for 15 min at 4°C. The supernatant obtained (100 µL) representing the enzymatic extract was added to 500 µL of xanthine at 0.3 mM, 250 µL of EDTA at 0.6 mM and 250 µL of nitroblue tetrazolium at 0.15 mM. The SOD activity was determined at 560 nm after addition of the xanthine oxidase used to generate the superoxide anion during the conversion of the xanthine to uric acid.

In parallel, the quantity of proteins was determined by the method of Bradford (1976) and a standard curve was drawn out with the serum bovine albumin. The specific activities of POD, PPO, SOD and CAT were determined in the inoculated and the control plants (in enzymatic units mg^{-1} proteins min^{-1}) and the results were expressed in percent of specific activity compared to the control plants.

Determination of malonyldialdehyde contents. The lipid peroxidation was evaluated by the contents of MDA being major product generally used for the evaluation of the lipoperoxydation rate (Esterbauer *et al*., 1990; Hodges *et al*., 1999; Al-Ghamdi, 2009). The MDA contents were determined according to the method previously described (Dhindsa *et al*., 1981). The leaves (100 mg) were crushed in 1.5 mL of thiobarbituric acid at 0.1%. The homogenate was then centrifuged at 10,000×*g* for 10 min. The supernatant obtained (1 mL) was added with 1 mL of trichloracetic acid at 20% containing 0.5% of thiobarbituric acid. The mixture was heated at 95°C for 30 min. The reaction was stopped by a bath of ice followed by a centrifugation at 10000 g for 10 min. The absorbance of the supernatant obtained was determined at 532 nm. The MDA contents were calculated by using the molar extinction co-efficient of the MDA (1.55 x 10^5) (Heath & Packer, 1968). The results were expressed in percentage compared to the control plants.

RESULTS

Foliar symptoms. The infection of soft wheat by *S. tritici* induces foliar necrotic symptoms of which the speed of apparition and extension clearly distinguishes the cultivars according to their behaviour to the pathogen. In the resistant cultivars (Mahdia, Achtar), the first symptomatic event consists of small necrotic lesions visible macroscopically on the 2nd day following the inoculation. These necrotic lesions were visible on both the sides of the leaf and number of lesions becomes constant on the 6th day after inoculation. In the susceptible cultivars (Nasma & Marchouch), the necrotic symptoms appear only on the 6th day in the form of large necrotic spots then were generalized to all the leaf.

Response of oxidative metabolism to inoculation. The inoculation of soft wheat by *S. tritici* was accompanied by the important modifications of the oxidative metabolism, which clearly distinguishes the various cultivars studied according to their behaviour to *S. tritici*.

Fig. 1. Time course induction of peroxidase activity in resistant (Mahdia, Achtar) and susceptible (Nasma, Marchouch) cultivars of soft wheat inoculated by *S. tritici*, The values represent the means of 10 replicates

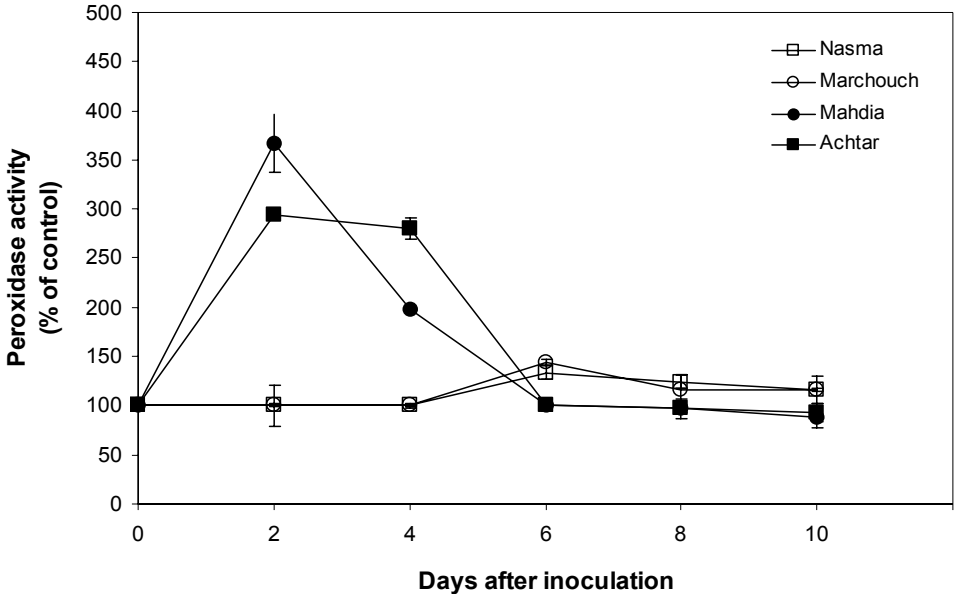
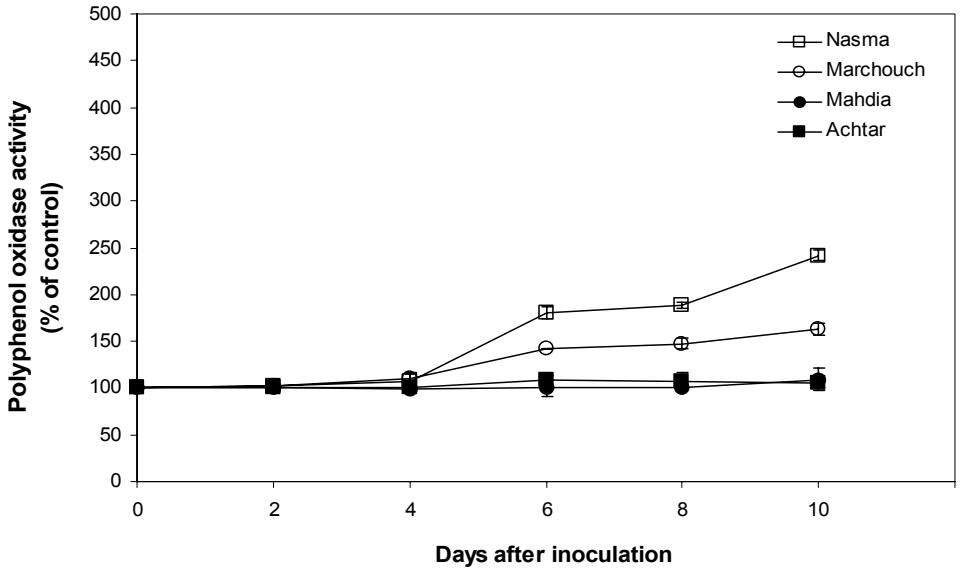


Fig. 2. Time course induction of polyphenol oxidase activity in resistant (Mahdia, Achtar) and susceptible (Nasma, Marchouch) cultivars of soft wheat inoculated by *S. tritici*, The values represent the means of 10 replicates



Peroxydase and polyphenol oxidase activities. The POD activity increases after inoculation in the resistant cultivars (Mahdia, Achtar) reaching its maximum (approximately 3 to 3.5 times) on the 2nd day, then decreases to a level of that of the control plants (Fig. 1). In the susceptible cultivars (Nasma & Marchouch), the inoculation does not express any effect on the PO activity (Fig. 1), but rather on the PPO activity (Fig. 2). The PPO activity increases in the susceptible cultivars on the 4th day following the inoculation reaching to approximately 1.5 times and 2.5 than that in the control plants respectively in Nasma cutivar and Marchouch cutivar (Fig. 2). In the resistant cultivars, contrary to the effect of the inoculation on the POD activity (Fig. 1), the post-infection time course of the PPO activity was identical to that of the control plants (Fig. 2).

Superoxide dismutase and catalase activities. The inoculation of soft wheat by *S. tritici* induces an increase in SOD activity in the resistant and the susceptible cultivars (Fig. 3). But, the time course of SOD activity induction clearly distinguishes the various cultivars according to their behaviour to *S. tritici*. Thus, in the resistant cultivars, the evolution of SOD activity presents two induction phases. The first phase consists of a fast increase with a maximum of activity approximately 3 to 3.6 times on the 2nd day

Fig. 3. Time course induction of superoxide dismutase activity in resistant (Mahdia, Achtar) and susceptible (Nasma, Marchouch) cultivars of soft wheat inoculated by *S. tritici*, The values represent the means of 10 replicates

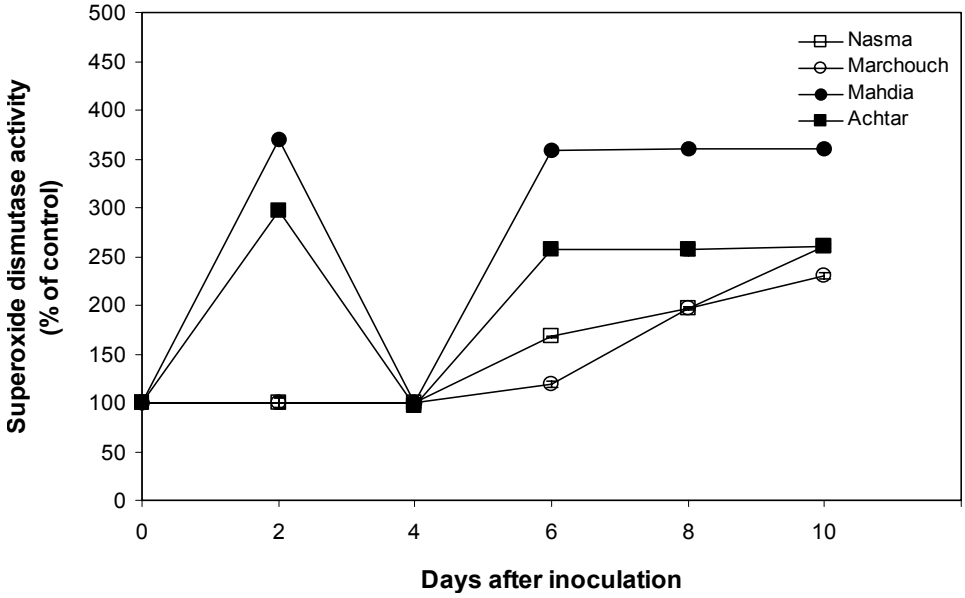
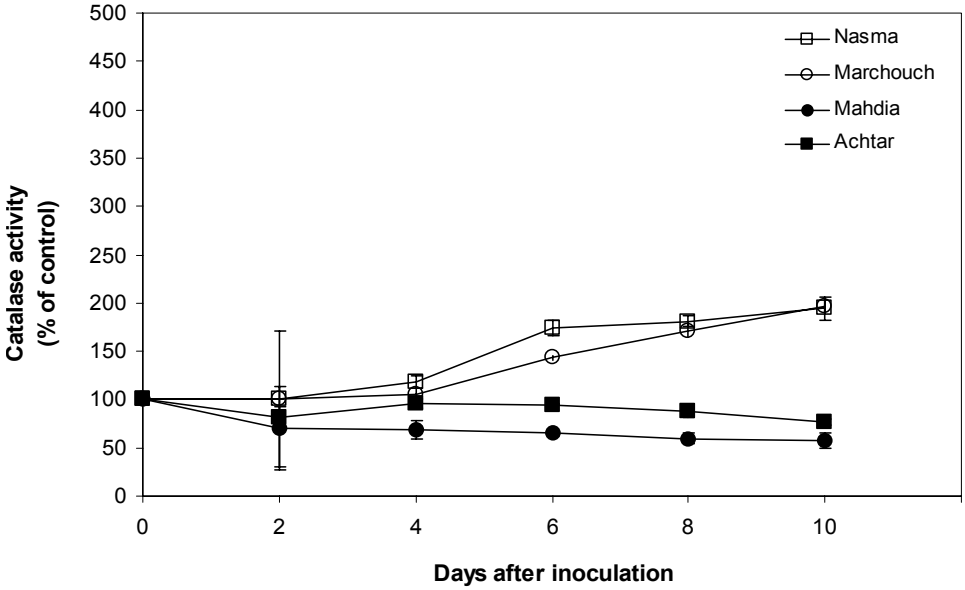


Fig. 4. Time course induction of catalase activity in resistant (Mahdia, Achtar) and susceptible (Nasma, Marchouch) cultivars of soft wheat inoculated by *S. tritici*, The values represent the means of 10 replicates

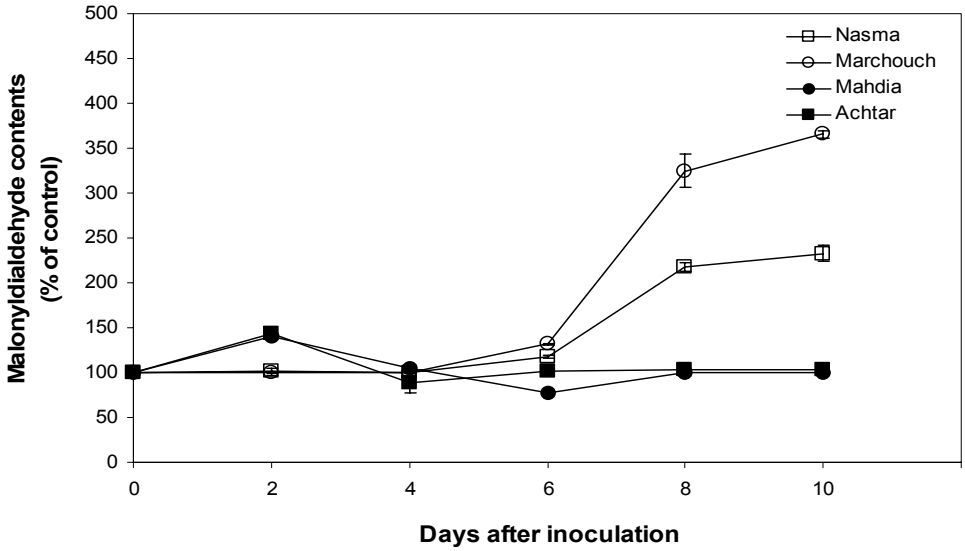


followed by a reduction to the level of 0 day being reached at on the 4th day in the identical values to those of the control plants. The second induction phase was also characterized by a fast increase to reaching on the 6th day an activity 2.5 times to 3.5 times than that of the control plants, respectively in Achtar cultivar and Mahdia cultivar. In the susceptible cultivars, only the second phase of induction of the SOD was observed and whose activity expresses a weak and late increase reaching an activity only 2.5 times than that of the control plants on the 10th day following the inoculation.

The CAT activity increased progressively in the susceptible cultivars up till the 4th day to reaching a level of two times more than that of the control (Fig. 4). However, in the resistant cultivars, the CAT activity is generally lower than that of control plants.

Malonyldialdehyde contents. The peroxidation rate of the lipids was estimated by the MDA contents. The inoculation of the resistant cultivars of soft wheat by S. *tiritici* induced a fast, weak and transient increase of MDA contents reaching the maximum accumulation, approximately 1.4 times on the 2nd day followed by a reduction to find similar contents to

Fig. 5. Time course induction of malonyldialdehyde accumulation in resistant (Mahdia, Achtar) and susceptible (Nasma, Marchouch) cultivars of soft wheat inoculated by *S. tritici*, The values represent the means of 10 replicates



those of the control plants up till the 4th day following the inoculation (Fig. 5). However, in the susceptible cultivars, post-infection accumulation of the MDA was slower (6th day) and reached the level of 2.3 times (Nasma cultivar) to 3.7 times (Marchouch cultivar) of the control plants on 10th day.

DISCUSSION

The inoculation of soft wheat by a strain of *S. tritici* recognized by its virulence (strain 142) was accompanied by foliar necrotic symptoms and important modifications in the oxidative metabolism of which the time course of induction clearly distinguishes the studied cultivars according to their behaviour to *Septoria tritici* blotch. Thus, in the resistant cultivars (Mahdia, Achtar), the inoculation induced small necrotic lesions, which appeared macroscopically on the 2nd day and their number became stable on the 6th day after inoculation. This early and localised necrotic process was preceded by a fast and intense increase of the POD activity and the SOD activity and by a fast, weak and transient accumulation of the MDA, with a maximum of the enzymatic activities and MDA contents on the 2nd day following the inoculation. The SOD activity expressed a second increase as from the 4th day in the resistant cultivars. In the susceptible cultivars (Nasma, Marchouch), the necrotic symptoms appeared tardily on the 6th day in the form of large necrotic spots then was generalized to all the leaf. These necrotic symptoms were preceded by a late increase (4th day) of the activities of PPO, SOD and CAT and of an important accumulation of MDA.

The precocity of necrosis apparition and its restriction in the leaves of the resistant cultivars has been observed in several host-parasite interactions (Obukowicz & Kennedy, 1981; Goodman & Novacky, 1996; Wang *et al*., 2007). It is currently allowed that the speed of apparition and extension of the necrotic reaction was related to the speed and the intensity of the early oxidative events induced precociously in plant in response to pathogen (Croft *et al.,* 1990; Goodman & Novacky, 1996; Shetty *et al.,* 2008). Indeed, the necrotic process results from a cellular decompartmentalization related to the membrane disorganization and represents one of the early events of plant attacked by the pathogen ones (Keppler *et al*., 1987; Keppler & Novacky, 1987; Bestwick *et al.,* 1997; Shetty *et al.,* 2008). The membrane disorganization was associated to the effect of the reactive oxygen species, particularly the free radicals, leading to the massive production of hydroxyperoxyl of polyunsaturated fatty-acids (Rustérucci *et al.,* 1996; Bestwick *et al.,* 1997; Van Ginkel & Sevanian, 1997; Shetty *et al.,* 2008). Thus, generation of the reactive oxygen species represents one of the earliest reactions in plant in response to pathogen infection (Sutherland, 1991; Baker & Orlandi, 1995; Low & Merida, 1996; Lamb & Dixon, 1997; Wang *et al.,* 1997; Rustérucci *et al.,* 1999; Lam *et al.,* 2001; Babitha *et al*., 2006). The cellular decompartmentalization has as a consequence the putting in contact of the enzymes of oxidation in particular POD and PPO, with their phenolic substrates (Mayer & Harel, 1979; Yemenicioglu *et al.,* 1999; Demeke & Morris, 2002; Takahama, 2004; Melo *et al*., 2006). The oxidation of the phenolic compounds, particularly *o*-diphenols, leads to *o*-quinones and melanins (Takahama, 2004; El Modafar & El Boustani, 2005; Tada & Ishimaru, 2006), the very toxic products to the pathogen micro-organisms (Harborne, 1989; Nicholson & Hammerschmidt, 1992; El Modafar *et al.,* 2000) and which appear in the form of brown pigments during the necrotic reactions (Goodman & Novacky, 1996; Richard-Forget & Gauillard, 1997; Dehon *et al.,* 2002; El Modafar & El Boustani, 2005). The necrotic process was considered as a defense mechanism associated with the hypersensitive reaction, when it was fast and localised,

whereas its generalization to important foliar surfaces rather represents a consequence of the pathogen development in tissues of the plant host (Goodman & Novacky, 1996; Wang *et al.,* 1997; Rustérucci *et al.*, 1999). The SOD, catalysing the dismutation reaction of the free radicals to the hydrogen peroxide H_2O_2, seems to play a key role in the apparition and extension of the necrotic reaction. The induction of SOD activity was frequently reported in the plants in response to pathogen invasion and constitutes a reaction often associated to the plant resistance (Buonaurio *et al.,* 1987; Edreva *et al.,* 1991; Daza *et al.,* 1993; Adam *et al.,* 1995; Kwon & Anderson, 2001; Babitha *et al.,* 2002; Garcia-Limones *et al.,* 2002; Zhou *et al*., 2005; Jetiyanon, 2007). In soft wheat, the reactive oxygen species were produced by oxalate oxidases, which increase during the host-pathogen interactions (Berna & Bernier, 1999; Yarullina *et al.,* 2005; Troshina *et al.,* 2007) and the restriction of *S. tritici* in the incompatible interactions were associated with the production of hydrogen peroxide by SOD (Shetty *et al*., 2003; Shetty *et al.,* 2007).

In our study, two "waves of dismutation", representing the conversion of the free radicals to hydrogen peroxide by the SOD are highlighted in the resistant cultivars and could explain the restriction of the necrotic reaction. The first "wave of dismutation" constitutes an early reaction, which could explain the fast, weak and transient accumulation of the MDA and consequently the restriction of foliar necrosis. The hydrogen peroxide resulting from the intervention of the SOD would be used by the POD, which expresses in a concomitant way a fast and intense increase in the resistant cultivars and seems to be responsible for the browning phenomenon in the incompatible interaction of soft wheat-*S. tritici*. The second "wave of dismutation" was induced at the 4th day following the inoculation and would be at the origin of the protection of the healthy cells against the action of the free radicals as reported in other plant-pathogen interactions (Levine *et al.*, 1994). It is often observed in the incompatible interactions that the free radicals, particularly the superoxide anion and the hydroxyperoxyl radical (very reactive radicals presenting one very short lifespan), are quickly transformed by the SOD to hydrogen peroxide, which is more stable and permeate and able to penetrate the plasmic and nuclear membranes (Wojtaszek, 1997). The hydrogen peroxide can be eliminated by various enzymes of which the catalase. In addition to their role of substrate of the POD and the CAT, the hydrogen peroxide produced by the SOD intervenes the lignin formation (Kobayashi *et al.,* 1995; Low & Merida, 1996; Sticher *et al.,* 1997) and express an antifungal effect to *S. tritici* (Shetty *et al*., 2003 & 2007) and various other fungal pathogens (Sutherland, 1991; Peng & Kuc, 1992; Baker & Orlandi, 1995; Low & Merida, 1996; Lamb & Dixon, 1997; Huogen & Higgins, 1999). In addition, because of its diffusible character, the hydrogen peroxide induces the expression of defense genes like those of phenylalanine ammonia-lyase, chalcone synthase, endochitinases and enzymes of phytoalexins biosynthesis (Mehdy, 1994; Tenhaken *et al.,* 1995) and would be a key signal of the necrotic reaction associated with hypersensitivity (Brisson *et al.,* 1994; Bolwell, 1999; Shetty *et al.,* 2008). The hydrogen peroxide also induces the genes expression of cellular protection generally coding for antioxidant proteins like the glutathion-S-transferase, the glutathion peroxidase and the polyubiquitins, which prevent the death of the healthy cells (Levine *et al.*, 1994).

In the susceptible cultivars, the increase of SOD activity was induced slightly and tardily on the 4th day following the inoculation. This could explain, on the one hand the absence of an early and intense POD activity like that in the resistant cultivars probably for lack of hydrogen peroxide and on the other hand the important accumulation of MDA testifying to the necrosis generalization because of a weak SOD activity not making it possible to protect sufficiently the plant cells against the attack by the free radicals responsible for the membrane lipoperoxydation. In the susceptible cultivars, the necrosis generalization could be a consequence of the pathogen development rather than a defence reaction as reported in various plant-pathogen interactions (Hammerschmidt *et al*., 1982; Smith & Hammerschmidt, 1988; Svalheim & Robertsen, 1990; Georgieva *et al*., 1999; Mohammadi & Kazemi, 2002). The apparition of the necrotic symptoms in the susceptible cultivars was preceded by the increase of the CAT activity generating oxygen from hydrogen peroxide. This could explain the concomitant increase in the PPO activity using oxygen to oxidize the phenolic compounds. Indeed, the PPO activity was correlated to the increase in MDA contents and the evolution of the necrotic symptoms. The late and generalized necrosis in the susceptible cultivars seems to be related to the intervention of the PPO, contrary to the resistant cultivars in which the fast and localised induction of necrosis was associated to the POD. The increase in the POD activity was frequently observed in the plants in response to pathogen infections and constitutes a reaction associated to resistance of wheat (Patykowski *et al.,* 1988; Flott *et al*., 1989; Shetty *et al.,* 2003; Yusupova *et al.,* 2006; Adhikari *et al.,* 2007) and of other cereals (Kerby & Somerville, 1989; Reimers *et al*., 1992; Young *et al*., 1995; Thordal-Christensen *et al*., 1997; Nafie, 2003).

In conclusion, the inoculation of soft wheat by *S. tritici* was accompanied by foliar necrosis and early but important modifications in the oxidative metabolism. The time course of induction clearly distinguished the cultivars for their behaviour to *S. tritici*. The foliar necrosis seemed to be different in resistant and susceptible cultivars, and was related to the intervention of POD in the tolerant but PPO in the susceptible cultivars. Also, a differential induction of the SOD activity in the resistant and susceptible cultivars suggests its key role in the necrotic and defense of soft wheat. The necrosis was associated to the defense reaction when it is fast and localised, whereas its generalization on important leaf areas rather represented a consequence of the pathogen development in the host tissues.

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