European Journal of Plant Pathology (2005) 113:241–254
DOI 10.1007/s10658-005-8675-y

Effect of juglone on active oxygen species and antioxidant enzymes in susceptible and partially resistant banana cultivars to Black Leaf Streak Disease

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Accepted 13 June 2005

Key words: Antioxidant systems, AOS, Banana, Black Leaf Streak Disease, juglone, *Mycosphaerella fijiensis*

Abstract

The black leaf streak disease (BLSD), caused by *Mycosphaerella fijiensis*, is the most destructive disease of bananas and plantains around the world. Breeding for resistance is the most promising strategy to fight this disease especially in small farmer plantations. *Mycosphaerella fijiensis* produces many phytotoxins such as juglone, which can be used, jointly with field and inoculations under controlled conditions, for screening banana cultivars for BLSD-resistance. This non-host specific phytotoxin has been shown to act on chloroplasts and disturbs the proton electrochemical gradient across the plasmalemma membrane. Moreover, an involvement of the oxidative burst during the interaction has been suggested. The present study was carried out using two cultivars that differed for either their juglone-responses or their resistance to BLSD (cv. Grande Naine susceptible to BLSD and juglone and cv. Fougamou partially resistant to BLSD and highly tolerant to juglone). The production of active oxygen species (AOS) and the enhancement of the enzymatic and/or non-enzymatic AOS-scavenging systems were investigated after treatment of the two cultivars with juglone. The time-course of AOS-production and AOS-scavenging was shown to be the key difference between these two tested cultivars after treatment with juglone. Thus, an early release of AOS (O_2^- radical and H_2O_2) and a quick stimulation of a preferment anti-oxidant system (superoxide dismutases, catalases, and peroxidases) was observed for cv. Fougamou as compared to cv. Grande Naine for which a late and weak generation of AOS accompanied by a late stimulation of the anti-oxidant systems were detected.

Introduction

Bananas and plantains are crops of worldwide economic importance (Lescot, 1999; Jones, 2000). The black leaf streak disease (BLSD), caused by *Mycosphaerella fijiensis*, is the most destructive disease of these crops within 2/3 of the producing countries (Jones, 2000). In spite of its large use within the industrial plantations, chemical spray could not be adopted for small farmer plantations because of many socio-economical and environmental reasons. Banana breeding programmes, launched in order to reduce an extensive utilisation of fungicides within industrial plantations and to cover this lack of exploitation, aim to generate new BLSD-resistant varieties (Bakry et al., 1997).

Mycosphaerella fijiensis is a hemibiotrophic fungus of a high level of genetic diversity (Carlier et al., 1996; Müller et al., 1997; Hayden

et al., 2003). It produces a number of low molecular weight secondary metabolites (i.e., fijiensine, tetralone, juglone), which are toxic to banana plants (Molina and Krausz, 1988; Stierle et al., 1991; Harelimana et al., 1997; Lepoivre, 2000; Hoss et al., 2000). These metabolites are suspected of contributing to disease progression (Lepoivre, 2000). Toxins such as juglone are non-host specific phytotoxins and can cause symptoms on bananas as well as on many other plant species (Molina and Krausz, 1988). Although its role in plant disease remains unclear, juglone is generally considered not to be required for pathogenicity but probably functions as an aggressiveness factor as its production results in an increase in disease severity (Molina and Krausz, 1988; Upadhyay et al., 1989; Lepoivre, 2000). At the sub-cellular level, juglone induces many biochemical effects such as disturbing the proton electrochemical gradient across the plasmalemma membrane and increasing electrolyte leakage (Lepoivre et al., 2002). Such effects lead to a decrease in the amount of cellular ATP and most probably to the inhibition of some enzymes involved in the respiratory impairment (Lepoivre et al., 2002).

Plants in contact with pathogens or their secondary toxic metabolites are inevitably exposed to different states of stress (Heiser et al., 1998; Daub and Ehrenshaft, 2000). Oxidative bursts generate active oxygen species (AOS) such as superoxide anion (O_2^-, half-life of 2–4 μs) (Knox and Dodges, 1985; Sutherland, 1991), single oxygen ($^1O_2$), hydrogen peroxide (H_2O_2, half-life of 1 ms) (Levine et al., 1994; Chamnongbol et al., 1998) and hydroxyl radical (OH, half-life less than 1 μs) (Halliwell and Gutteridge, 1989); these have been shown to be one of the underlying agents causing plant tissue injury after infection with pathogens or with their phytotoxins (Baker and Orlandi, 1995; Lamb and Dixon, 1997; Noctor and Foyer, 1998; Daub and Ehrenshaft, 2000). These oxygen species are highly reactive and damage membranes, lipids, proteins, pigments, and nucleic acids, thus resulting in dramatic reduction and deterioration of normal functioning, finally causing the death of plants (Foyer et al., 1994). Such events have been postulated to serve the pathogenesis mechanism and plant defence responses but their real role still unknown (Grant and Loake, 2000; Palatnik et al., 2002).

Plants have evolved various protective mechanisms to reduce or to completely eliminate AOS (Mehdy, 1994; Mehdy et al., 1996). One of the protective mechanisms is the enzymatic antioxidant system (Figure 1), which operates with a sequential and simultaneous action of many enzymes (Hegedüs et al., 2001) including superoxide dismutases (SOD, EC 1.15.1.1), peroxidases such

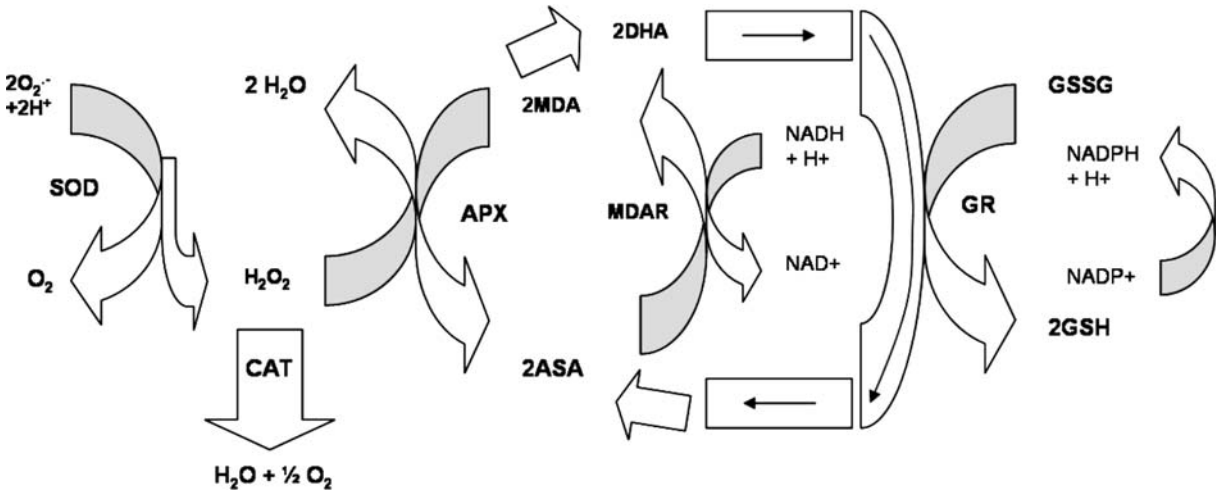

Figure 1. Diagram showing several connections between the enzymatic antoxidant system involved in the detoxification of active oxygen species. SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; ASA, ascorbic acid (oxidized form of ascobate); MDA, monodehydroascorbic acid; MDAR, monodehydroascorbate reductase; DHA, dihydroascorbic acid (reduced form of ascorbate); GR, gluthatione reductase; GSSG, oxidized form of gluthatione; GSH, reduced form of gluthatione.

as guaiacol peroxidases (GPO, EC 1.111.7), catalases (CAT, EC 1.11.1.6), ascorbate peroxidases (APO, EC 1.11.1.11) and dehydroascorbate reductases (DHAR, EC 1.8.5.1). Superoxide dismutases are located in various cell compartments and catalyse the disproportionation of two O_2^- radical to H_2O_2 and O_2 (Salin, 1987). The generated H_2O_2 is eliminated by different antioxidant enzymes such as catalases, which are located in peroxisomes/glyoxisomes and mitochondria, to water and oxygen (Asada, 1992; Palatnik et al., 2002). Peroxidases convert H_2O_2 to water using a wide range of electron donors (Bolwell and Wojtaszek, 1997; Grant and Loake, 2000). Ascorbate peroxidases located in either chloroplasts or cytosol are the key enzymes of the ascorbate cycle, and act by eliminating peroxides by converting ascorbic acid to dehydroascorbic acid (Asada, 1999; Mano et al., 2001). Another protective mechanism, which acts simultaneously with the first one, involves the low-molecular weight anti-oxidants such as ascorbate (ASA) and glutathione (GSH), scavenging ultimately H_2O_2 at the expense of NADPH or NADH (Foyer and Halliwell, 1976; Noctor and Foyer, 1998; Asada, 1999; Gullner and Dodge, 2000). Ascorbic acid is one of the most powerful antioxidants in eucaryotes (Foyer and Halliwell, 1976; Gullner and Dodge, 2000; Horemans et al., 2000). It is involved in removing AOS and generating α-tocopherol, an important lipid-phase antioxidant (Asada, 1994).

The aim of this study was to investigate the differential response of a susceptible and partially resistant banana cultivar to treatment with juglone. The importance of the active oxygen species and the enzymatic and non-enzymatic anti-oxidant systems in the mechanism of the action of juglone is highlighted.

Material and methods

The plants

Two banana cultivars showing either distinct juglone-responses or BLSD-resistance phenotypes were used in this study. The BLSD-susceptible cv. 'Grande Naine, AAA, sub-group Cavendish' has a low tolerance to juglone while the BLSD-partially resistant cv. 'Fougamou, AAB, sub-group Pisang Awak' was highly tolerant. Both cultivars were produced from tissue culture and grown under controlled conditions in the glasshouse (16 h photoperiod, 350 μmol m^{-2} s^{-1} light intensity, and temperature of 25 °C day/night). Fertilisers and pesticides were used when necessary. Plants were used for juglone treatments at the age of six months after potting.

The induction of necrosis with juglone

Before use, plants of the two tested cultivars were kept under a saturated atmosphere for 48 h. To induce necrosis, a commercial juglone product (Sigma-Aldrich) was used. Solutions of 100 and 500 ppm concentrations were prepared in 10% methanol and used as well as the 10% methanol solution as control. Twenty microliter of juglone or 10% methanol solutions were injected into the lower surface of the first fully expended leaf using a syringe with a rubber stopper covering its needle. Four replicates per concentration and per leaf were conducted on three plants of each cultivar and the whole experiment was repeated independently three times. Plants were incubated after the injections at 25 °C day/night under a 16 h photoperiod. Leaf samples (whole leaf) were harvested following the time schedule of 1, 2, 4, 8, 24, and 48 h after the injection. Immediately after harvest, leaf samples were frozen in liquid nitrogen and stored at −80 °C until used for the extractions and the antioxidant analysis.

Determination of AOS production

The direct release of H_2O_2 and the production of the anion superoxide or hydroxyl radical were followed in leaf squares (1 × 1-cm) from both tested cultivars. Leaf squares were incubated either in water, or in 10% methanol or in juglone solutions (100 and 500 ppm) during 1, 2, 4, 8, 24, and 48 h. Five fresh leaf squares of non-treated controls or juglone-treated were used per treatment.

O_2^- production

The production was quantified by the measurement of the nitro blue tetrazolium (NBT) reducing activity as described by Doke (1983). Leaf squares were washed using distilled water and immersed in 3 ml of 10 mM potassium phosphate buffer pH 7.8

containing 0.05% NBT and 10 mM NaN_3 for 1 h. The mixture was then heated at 85 °C for 15 min and cooled rapidly. The activity of the leaf squares to reduce NBT was followed by measurement of the absorbance at 580 nm.

H_2O_2 production

The production was determined using the method described by Tiedemann (1997). Leaf squares were washed using distilled water and incubated in 2 ml of a reagent mixture consisting of 50 mM phosphate buffer pH 7.0, 0.05% guaiacol and peroxidase for 2 h at room temperature in the dark. The release of H_2O_2 was followed by measurement of absorbance at 450 nm.

OH production

The production was determined using the method described by Tiedemann (1997). Leaf squares were washed using distilled water and immersed in 1 ml of 1 mM of 2-deoxyglucose then incubated at room temperature in the dark for 45 min. Five hundred microliter of this solution were added to 500 µl of 1% (w/v) thiobarbituric acid and 1 ml of 2.8% (w/v) trichloracetic acid. The mixture was boiled for 10 min and immediately cooled for 10 min in ice. The production of the hydroxyl radical was followed by measurement of absorbance at 540 nm.

Determination of ascorbate content

Leaf samples treated or not-treated with juglone (1.5 g FW) were crushed in a mortar pre-frozen with liquid nitrogen and homogenized in 1 ml of 6% trichloracetic acid (TCA). The extract was then kept in ice for 15 min. Before centrifugation (15,600 *g* at 4 °C), 1 ml of 6% TCA was added to the extract. The ascorbate content amount was evaluated immediately on the supernatant fraction as described by Okamura (1980). Thus, an aliquot of 100 µl of the extract was mixed with 0.2 M phosphate buffer at pH 7.4. Ten micromolar dithiothreitol were added to the mixture and tubes were incubated at room temperature for 15 min. After incubation, 0.5% of *N*-ethylmaleimide, 10% of trichloracetic acid, 42% of *o*-phosphoric acid, 4% of 2, 2′bipyridyl, and 3% of $FeCl_3$ were added to each sample. After vigorous stirring, the samples were kept at 42 °C for 1 h and the absorbance was recorded at 525 nm.

Assay of antioxidant enzymes

Preparation of enzyme extracts

The leaf samples were crushed in mortar pre-frozen with liquid nitrogen and homogenized in 1 M NaCl in 50 mM potassium phosphate buffer pH 7.0 containing 1% PVP and 1 mM EDTA. The homogenate was centrifuged (15,000 *g* at 4 °C) and the supernatant was used as an enzyme extract to assay superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPO), ascorbate peroxidase (APO) and dehydroascorbate reductase (DHAR) activities. Total content of proteins were determined using the method of Bradford (1976), by reference to a standard curve pre-established using bovine serum albumin.

Enzyme assays

The SOD activity assay was based on the method of Beauchamp and Fridovich (1971), where 3 ml mixture containing 50 mM phosphate buffer pH 7.8, 13 mM L-methionine, 75 µM NBT, 0.1 mM EDTA, 2 µM riboflavin and the enzyme extract were used. The reaction was started by placing the tubes under two 15 W fluorescent lamps and terminated after 10 min by removing the light source. Non-illuminated and illuminated tubes without enzyme extract served as controls and the absorbance was recorded at 560 nm. CAT activity was measured spectrophotometrically according to Dhindsa et al. (1981). The assay mixture contained 50 mM potassium phosphate buffer pH 7.0, 15 mM H_2O_2 and the enzyme extract. Absorbance of the mixture was measured at 240 nm. APX activity was assayed following the oxidation of ascorbate to dehydroascorbate at 265 nm as described by Nakano and Asada (1981). The assay mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.25 mM sodium ascorbate, 25 µM H_2O_2 and the enzyme extract. The GPO activity was assayed according to the method of Maehly and Chance (1954) and Chance and Maehly (1955). The reaction mixture contained 50 mM sodium acetate buffer pH 5.6, 5 mM guaiacol, 15 mM H_2O_2 and the enzyme extract. The absor-

bance was recorded at 470 nm. All the spectrophotometric assays were performed using a UV–Visible spectrophotometer (Ultrospec II 4050 model, LKB Biochrom) at room temperature.

Data analysis

All the experiments were randomly designed complete blocks. In each block, three plants per cultivar were used. For juglone infiltration, four replicates per concentration and per leaf were conducted on three plants of each cultivar and the whole experiment was repeated independently three times. All the enzymatic assays were conducted in triplicate and the variability was given for each treatment as the standard error. For AOS and ascorbate quantification experiments, five leaf squares were used per assay and each whole experiment was repeated three times. The results were presented as mean ± standard error. Quantitative results obtained were submitted to variance analysis using the STATISTICA software v. 5.0 (Statsoft, France, ed. 1999). Variables were transformed or expressed in a relatively to the non-treated controls when it was necessary. The significant differences between all homogenate groups were detected using the Newman–Keuls test at $P < 0.05$. The correlations between all variables were evaluated using the correlation module of the same software at different levels of significance.

Results

The AOS content

No significant differences were observed between the two cultivars for their H_2O_2 contents either when they were not treated or when they were treated with only 10% methanol (Figure 2). However, when cv. Fougamou was treated with juglone a quick release of H_2O_2 was observed within 2–4 h following the treatment. The increase of H_2O_2 content reached 16–17 and 19–90-fold of those recorded in the non-treated controls for the 100 and 500 ppm of juglone-treated leaves, respectively. As for cv. Grande Naine, this increase occurred later (8–24 h after the treatment) and the concentration of H_2O_2 increased 5–6 and 10–28-times those observed in the non-treated controls for the 100 and 500 ppm of juglone-treated leaves, respectively.

One to 2 h after the juglone treatment, an increase of the production of superoxide anion was observed in cv. Fougamou as compared to cv. Grande Naine. In the latter, such an increase was detectable only 8 h after the treatment (Figure 2). The observed increase of the production of the superoxide anion seemed to be not specific to the juglone-treatment as the 10% methanol solution and wounding induced the same effect especially in cv. Fougamou.

The production of hydroxyl radical was also investigated and showed an increase in cv. Grande Naine treated with juglone of about 1.1 to 14-fold of the level observed in non-treated controls at 2, 4 to 8 h after the treatment (Figure 2). In cv. Fougamou an increase of 2 to 27-fold of the level of the non-treated control was observed when it was treated with 100 ppm of juglone and only 2 to 4.5-fold when it was treated with 500 ppm of juglone. The greater part of the hydrogen peroxide observed was generated by either anion superoxide or hydroxyl radical as a significant correlation was recorded between H_2O_2 and O_2^- and $OH^\cdot$ contents ($r = -0.282$; $P = 0.0006$ and $r = 0.226$; $P = 0.0063$, respectively) (Table 1).

The activity of the antioxidant systems

In this study the non-enzymatic (i.e., ascorbate, ASA) and the enzymatic antioxidant systems (i.e., SOD, CAT, GPO, APO and DHAR) were investigated during the same time period used for AOS detection. Over time, the level of free and total ASA in cv. Fougamou was relatively higher or at least equal to that in cv. Grande Naine (Figure 3). An increase of free ASA content was observed in cv. Fougamou treated with juglone during the first 2 h following the treatment. On the contrary, a deficit of free ASA content was observed generally in cv. Grande Naine treated with juglone during the whole period of the experiment as compared to the controls. This variation of the ASA levels was related conjointly to the opposite activities of the two ascorbate cycle key enzymes APO and DHAR, as significant correlations were detected between ASA content and APO and DHAR activities ($r = -0.218$; $P = 0.009$ and $r = 0.301$; $P = 0.00025$, respectively) (Table 1). In cv. Fougamou, a baseline activity of APO associated with

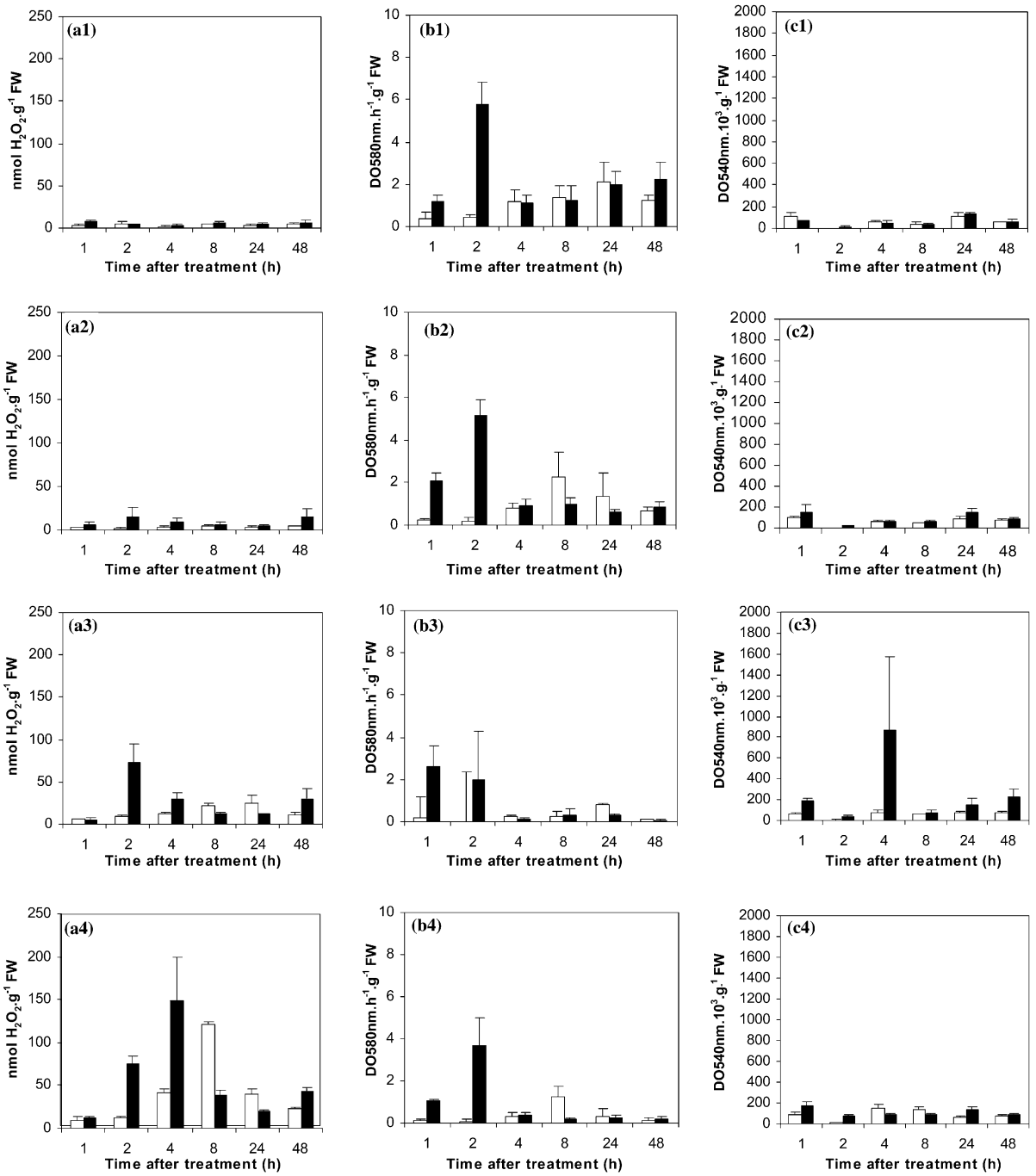

Figure 2. AOS content (a: H_2O_2, b: O_2^-, c: OH^-) of cultivars Grande Naine (□) and Fougamou (■) non-treated (1) and treated with 10% methanol (2) or with 100 ppm (3) or 500 ppm of juglone (4) at different times after the injection. Bars represent the standard deviation.

Table 1. Correlations between AOS content and the antioxidant system content or activities

	H_2O_2	O_2^-	OH	ASA	Total ASA	Ratio ASA	SOD	CAT	GPO	APO	DHAR
H_2O_2	*1*										
O_2^-	−0.282	*1*									
	0.0006										
$OH^{\cdot}$	0.226	-0.076	*1*								
	0.0063	*ns*									
ASA	−0.010	0.051	0.297	*1*							
	ns	*ns*	*0.0003*								
Total ASA	0.035	0.096	0.287	0.611	*1*						
	ns	*ns*	*0.0005*	*0.000000*							
Ratio ASA	−0.068	−0.011	0.186	0.790	0.095	*1*					
	ns	*ns*	*0.026*	*0.000000*	*ns*						
SOD	−0.057	0.051	−0.083	0.057	0.347	−0.120	*1*				
	ns	*ns*	*ns*	*ns*	*0.00002*	*ns*					
CAT	0.177	−0.095	0.066	−0.055	−0.054	−0.049	−0.008	*1*			
	0.033	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*				
GPO	0.086	−0.205	−0.240	−0.218	−0.050	−0.215	0.038	0.074	*1*		
	ns	*0.0136*	*0.0038*	*ns*	*ns*	*0.0097*	*ns*	*ns*			
APO	−0.114	−0.355	−0.060	−0.218	−0.213	−0.048	0.126	0.160	0.157	*1*	
	ns	*0.00001*	*ns*	*0.009*	*ns*	*ns*	*ns*	*ns*	*ns*		
DHAR	0.160	0.159	0.400	0.301	0.348	0.154	0.023	0.048	0.004	−0.167	*1*
	ns	*ns*	*ns*	*0.00025*	*0.00002*	*ns*	*ns*	*ns*	*ns*	*0.046*	

Spearman *r* values are followed by the level of significance. ASA, ascorbic acid; total ASA, ascorbic and dehydroascorbic acids; ratio ASA, ASA/total ASA (oxidized + reduced forms); SOD, superoxide dismutase; CAT, catalase; GPO, guaiacol peroxidase; APO, ascorbate peroxidase; DHAR, dehydroascorbic acid reductase; ns, not significant ($p \geq 0.05$).

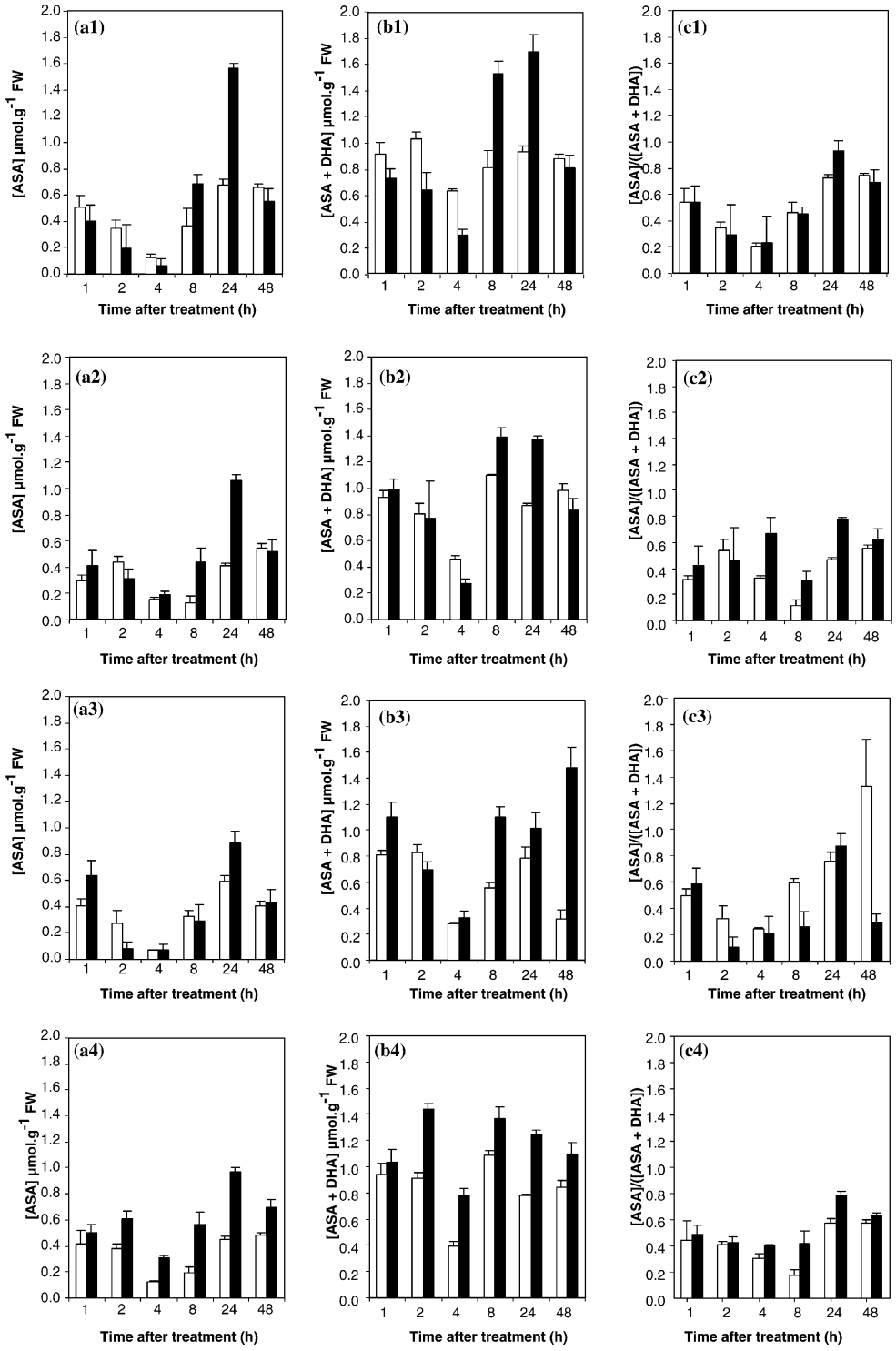

Figure 3. Ascorbate content (a) oxidized form of ascorbate; (b) total ascorbate (oxidized and reduced forms); (c) ratio of free ascorbate of cultivars Grande Naine (□) and Fougamou (■) non-treated (1) and treated with 10% methanol (2) or with 100 ppm (3) or 500 ppm of juglone (4) at different times after the injection. ASA, ascorbic acid; DHA, dehydroascorbic acid. Bars represent the standard deviation.

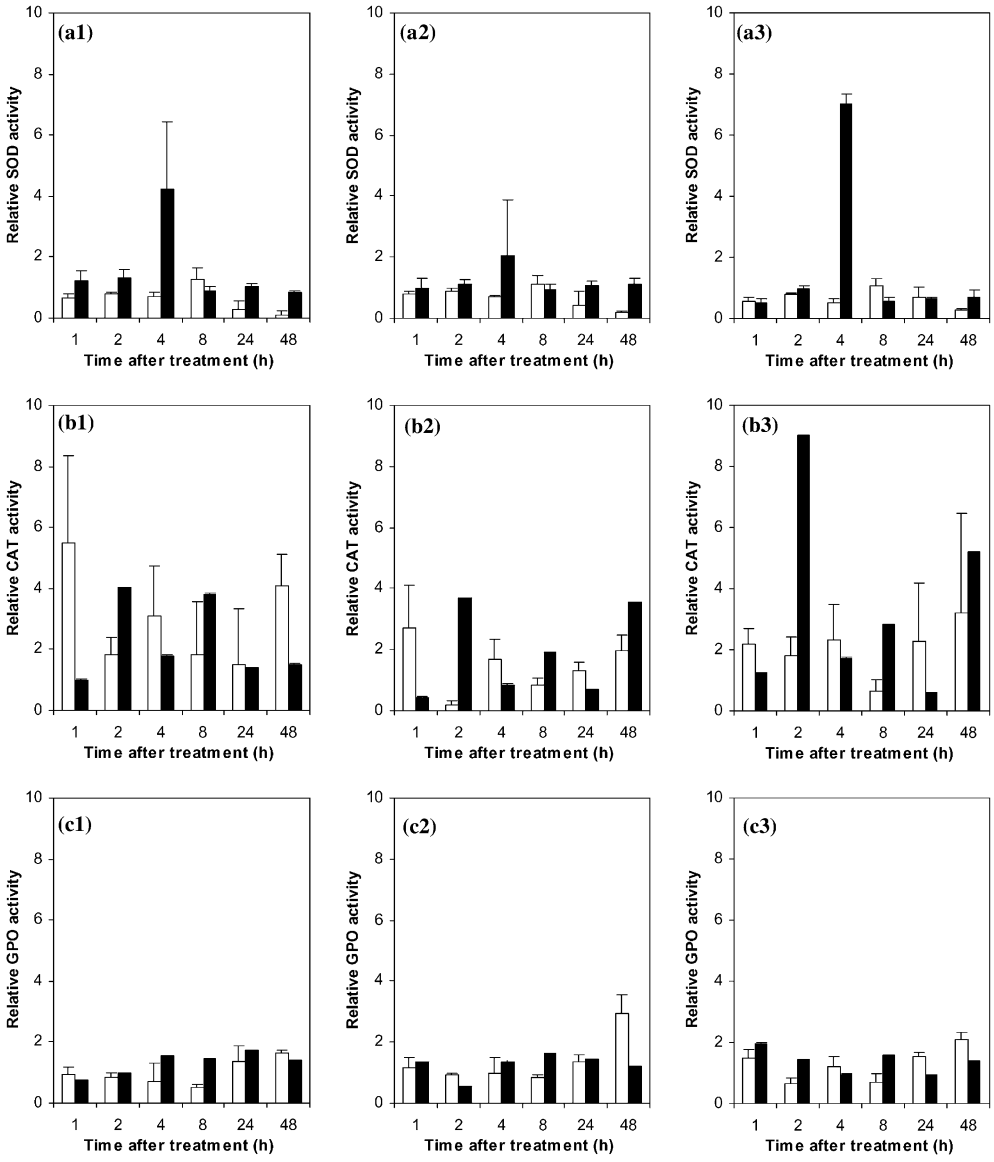

Figure 4. Relative activities (compared to the non-treated control) of some anti-oxidant enzymes (superoxide dismutase (a), catalase (b), and guaicol peroxidase (c)) within cultivars Grande Naine (□) and Fougamou (■) treated by 10% methanol (1) or by 100 ppm (2) or 500 ppm of juglone (3) at different times after the injection. Bars represent the standard deviation.

a higher activity of DHAR allowed free ASA to be available during the whole period of the experiment. On the contrary, in cv. Grande Naine, the activity of APO was high and that of DHAR was low, resulting in a disproportionate level of free ASA (Figure 3).

The redox state, calculated as the ratio between free ASA and total ASA (pool of oxidized and reduced forms) during the juglone treatment kinetic, was variable between 0.11 and 0.87 and between 0.17 and 1.33 for cvs Fougamou and Grande Naine, respectively (Figure 3). The higher index observed for cv. Grande Naine reflects the status of stress of the cultivar after juglone-treatment, though the ascorbate must habitually remain in reduced form.

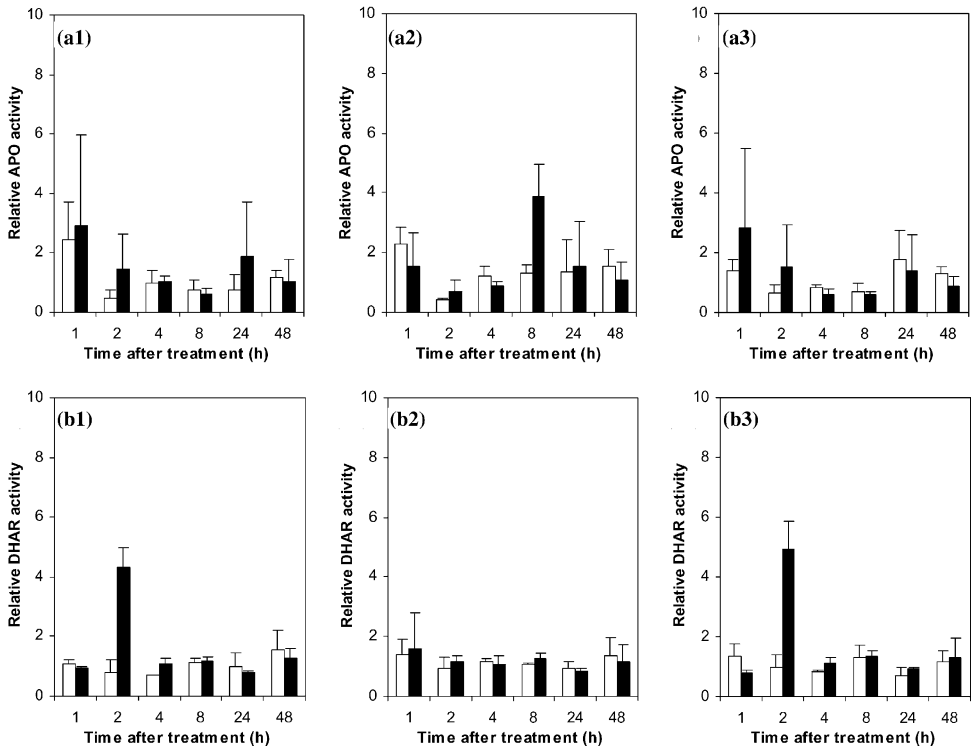

Figure 5. Relative activities (compared to the non-treated control) of the key enzymes of the ascorbate cycle (ascorbate oxidase (a) and dehydroxyascorbate reductase (b)) within cultivars Grande Naine (□) and Fougamou (■) treated by 10% methanol (1) or by 100 ppm (2) or 500 ppm of juglone (3) at different times after the injection. Bars represent the standard deviation.

Many of the antioxidant enzymes investigated have shown an increase in their activities after juglone treatment in both tested cultivars. Such stimulation seems to be not specific to the juglone treatment as baselines of activity were observed with the 10% methanol control. Following the SOD in both tested cultivars, an increase in activity was detected earlier in cv. Fougamou (4 h after the juglone treatment) and later in cv. Grande Naine (8 h; Figure 3). Such increases were of 2 to 7 and 1.06 to 1.25-times that observed in non-treated controls in cvs Fougamou and Grande Naine when they were treated with 100 and 500 ppm of juglone, respectively (Figure 4). The CAT activity was also stimulated earlier in cv. Fougamou (2 h after juglone treatment, specifically with 500 ppm of juglone) and remained unchanged in cv. Grande Naine (Figure 4). The increase in activity was about 3.7 to 9-fold of non-treated controls in cv. Fougamou but only 2.3 to 4.5 in cv. Grande Naine, when they were treated with 100 and 500 ppm of juglone, respectively. This enzyme activity was stimulated throughout all the tested time periods. A significant correlation was detected between CAT activity and H_2O_2 content ($r = 0.177$; $P = 0.033$), suggesting an involvement of the enzyme in the rapid detoxification of H_2O_2 overproduced under juglone treatment. Similarly, the GPO activity was enhanced twice under juglone treatment for both studied cultivars during the first 2–8 h following treatment with juglone compared to the non-treated controls (Figure 4). As was observed for CAT, the initial GPO activity was not restored quickly during the whole time period tested (Figure 4). The ascorbate cycle enzymes (APO and DHAR), activities were much more stimulated in cv. Fougamou than in Grande Naine at different time points, keeping the free ASA amount under control (Figure 5).

Discussion

Active oxygen species such as H_2O_2, O_2^- and OH are detected under diverse stress situations (Mehdy, 1994; Baker and Orlandi, 1995; Mehdy et al., 1996; Wojtaszek, 1997; Grant and Loake, 2000; Palatnik et al., 2002). The involvement of these AOS was reported also during the action of non-host specific toxins such as juglone on many plant species as being responsible for tissue damage (Daub, 1986; Remotti et al., 1997; Heisser et al., 1998; Daub and Eherenshaft, 2000). Against these AOS, plants have evolved a battery of antioxidant systems including some molecular scavengers and main enzymes (Mehdy et al., 1996; Noctor and Foyer, 1998; Hegedüs et al., 2001; Palatnik et al., 2002). In this study, a quantification of AOS released after juglone treatment was investigated in two cultivars, distinct either for their resistance to the disease or their tolerance to the toxin. Cultivar Grande Naine was susceptible to BLSD and of a low tolerance to juglone while Fougamou was partially resistant to the disease and highly tolerant to juglone. The evaluation of some enzymatic and non-enzymatic anti-oxidant systems evolved in the detoxifying mechanism was also conducted.

The present findings show that the hydrogen peroxide release peak occurred earlier in cv. Fougamou than in Grande Naine. H_2O_2 is known to serve the plant defence playing a role in plant signal transduction (van Breusem et al., 2001) or the pathogenesis mechanisms responsible for local tissue injuries produced after infection (Noctor and Foyer, 1998; Daub and Ehrenshaft, 2000). Since the concentrations of H_2O_2 observed in the present study were moderate in both tested cultivars, it may be suggested that H_2O_2 plays a role as a secondary messenger to induce antioxidant systems or other defence mechanisms earlier in cv. Fougamou. H_2O_2 release therefore constitutes a part of the early events of the resistance enhancement but a role in the mechanism of pathogenesis cannot also be ruled out.

As in many other plant-pathogen interactions (Baker et al., 1995; Low and Merida, 1996), the juglone-induced time-course of H_2O_2 accumulation was fully coincident with that of the superoxide anion generation with a little time lapse difference. Such results are in agreement with the fact that anion superoxide is an intermediate redox state between oxygen and hydrogen peroxide (Mithöfer et al., 1997). The detection of O_2^- generation and subsequently H_2O_2 during many stress conditions seems to be generated by NAD(P)H oxidase systems (Doke, 1983; Apostol et al., 1989; Lamb and Dixon, 1997). The present findings suggest a difference in the activity time-course of these enzymes between the two tested cultivars. Elsewhere, taking into account the high content of preformed polyphenols observed in cv. Fougamou as compared to cv. Grande Naine (Beveraggi et al., 1995; El Hadrami, 1997) and the high reactivity of the superoxide anion to these compounds, it could be suggested also that O_2^- plays a role in the resistance mechanism of cv. Fougamou by cross-linking cell walls when oxidising phenols, as reported by Yamasaki et al. (1997).

Significant differences were also observed between the two tested cultivars for the hydroxyl radical levels during the whole experimental period. This radical was produced earlier in cv. Fougamou as compared to Grande Naine. However, when the juglone concentration was increased, the liberation of this radical was reduced in cv. Fougamou but not in Grande Naine. This might explain the late appearance of necrosis on cv. Fougamou as compared to Grande Naine due probably to the preservation of cell-wall lipids from peroxidation.

Plants have evolved many antioxidant systems to scavenge AOS and to keep stress conditions under control (Baker and Orlandi, 1995). Our investigations were focused on the evaluation of ascorbate content and activities of some antioxidant enzymes such as SOD, CAT, GPO, APO and DHAR. The present study shows a difference in the time-course involvement of ascorbate in the AOS-scavenging between Fougamou and Grande Naine cultivars. Higher free ascorbate content, generated by the disproportionate relative activities of two ascorbate cycle enzymes APO and DHAR, was observed in cv. Fougamou compared to cv. Grande Naine. This resulted in the maintenance of a lower stress status in cv. Fougamou than in Grande Naine. Similar results were reported in other plants under other stress conditions (Noctor and Foyer, 1998; Asada, 1999).

A stimulation of many antioxidant enzymes such as CAT, SOD, GPO and APO was detected in the present study. This stimulation occurred earlier in cv. Fougamou than in Grande Naine.

CAT activity was stimulated weakly and later in cv. Grande Naine than in Fougamou in spite of its primary role in H_2O_2 detoxification. This result could be explained by the time-course difference in H_2O_2 production between the two tested cultivars and by the low affinity of CAT to H_2O_2 as compared to other enzymes such as APO and GPO as reported by Scandalios (1994) and Creissen et al. (1994). In this case, the CAT served only to remove the bulk of H_2O_2, whereas other enzymes can scavenge H_2O_2 that is inaccessible for catalases because of their higher affinity to H_2O_2 and their presence in different sub-cellular compartments (Creissen et al., 1994). In agreement with this suggestion, GPO activity increased twice for the two tested cultivars and remained stimulated during the whole experimental period.

For the SOD, a time-course difference in the activity and level of stimulation was observed between Fougamou and Grande Naine cultivars after the juglone treatment, even with the non-specificity of the enzyme response. SOD activity increased quickly in cv. Fougamou leading to an early detoxification of O_2^- compared to cv. Grande Naine. As no correlation was detected between O_2^- production and enzyme activity, it might be suggested that juglone susceptibility of the two tested cultivars is not related to the activity of SOD. Also, other mechanisms involved in O_2^- detoxification such as phenols oxidation (Yamasaki et al., 1997) may explain such a weak correlation.

In the light of the present findings it could be hypothesised that the time-course of AOS-production and AOS-scavenging is one of the key differences between the reaction of cvs Fougamou and Grande Naine to juglone and in turn to BLSD. The level of tolerance to juglone observed in cv. Fougamou might then be related to an early release of AOS, which quickly prevented the resistance mechanisms, and to a performing anti-oxidant system, which neutralised in time the overproduction of AOS leading to less tissue injuries. Based on the data reported here, the early superoxide anion generated by the juglone treatment is disproportionate to hydrogen peroxide by SOD, which is then scavenged by other anti-AOS systems such as GPO, APO and CAT, resulting in a low level of H_2O_2. AOS seems to play in this case, a role in resistance enhancement. However, in cv. Grande Naine, the AOS-generation starts late after the toxin treatment, leading to a late stimulation of antioxidant enzymes. This situation might upset the balance between the AOS-generating and the AOS-scavenging systems. In the latter case, AOS seems to affect the pathogenesis process more than the resistance mechanisms, even though no resistance gene is carried by cv. Grande Naine.

Acknowledgements

This research was supported by grants from the International Agency of Atomic Energy (IAA). The first author would like to thank the principal investigator of the project 'Selection for banana resistance to Black Leaf Streak Disease' Professor Philippe Lepoivre.

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