14. Analysis of the mechanisms of action of Mycosphaerella fijiensis toxins during the development of Black leaf streak disease - Busogoro, J.P[23], J.J. Etamé[24], G. Lognay[25], J. Messiaen[26], P. van Cutsem[27], P. Lepoivre[28]

Abstract

The goal of our programme is to understand the role of *M. fijiensis* toxins in Black Leaf Streak disease to facilitate early selection of banana genotypes resistant to the disease. Toxic effects induced by crude extracts, as well as by purified juglone released by the pathogen in culture filtrates, were analysed on two reference cultivars of banana. Injection of the pathogen metabolites into banana leaves showed a light-dependent toxicity revealed by the development of necrosis and a rapid decrease of the relative vitality index determined from chlorophyll fluorescence measurements. We demonstrated that juglone, a purified metabolite of *M. fijiensis*, has a direct inhibitory effect on the electron transfer properties of purified banana chloroplasts. Regardless of the bioassay performed, the susceptibility of the two reference cultivars to this metabolite was correlated with their respective sensitivity to the pathogen infection. On the other hand, some preliminary data showed that juglone initiates oxidation of ascorbic acid, the most abundant antioxidant system in plants. On this base, we hypothesized that oxidative damage could be involved in the action of *M. fijiensis* toxins.

1. INTRODUCTION

Among the fungal leaf diseases affecting bananas, Black Leaf Streak (BLS) disease or black Sigatoka, caused by *Mycosphaerella fijiensis* Morelet, together with the yellow Sigatoka caused by *Mycosphaerella musicola*, are considered the most serious economic threats to growing bananas [1]. The BLS disease, distributed worldwide, is more virulent and affects a wider range of banana genotypes than yellow Sigatoka [2]. Symptoms caused by BLS disease, which appear like elongated necrotic lesions surrounded by chlorosis, suggested a possible involvement of pathogen phytotoxic compounds [3,4]. Based on this hypothesis, different authors have developed bioassays that have confirmed the release of toxins in extracts of *M. fijiensis* culture filtrates. The toxicity due to crude extracts was characterised by a water-soaked appearance of banana tissues [3]. Purification of the crude extracts allowed identification of a set of phytotoxic metabolites found in the pathogen culture filtrates [5]. These compounds are 2,4,8-trihydroxytetralone, 5-hydroxy-1,4-naphtalenedione (commonly called juglone), 2-carboxy-3-hydroxycinnamic acid, dimethyl ester of 2-carboxy-3-methoxycinnamic acid, isoochracinic acid and 4-hydroxycytalone. Fijiensin, another metabolite of *M. fijiensis* [4] characterised by a more complex structure than the previous molecules, exhibited no differential reaction to various cultivars despite the specificity of this molecule on banana.

Breeding banana for resistance to *M. fijiensis* is the most appropriate strategy to control BLS disease in subsistence agriculture. The sterility of banana represents an important limitation to the classical improvement of this plant. According to this situation, new techniques of tissue culture might be exploited in breeding schemes to widen the genetic variability within banana

populations. If the involvement of *M. fijiensis* toxins in BLS disease can be demonstrated, they could be used as early screening agents within variant populations generated by tissue culture procedures. Screening the regenerates with *M. fijiensis* toxins requires a deep knowledge of their mechanisms of action. In this area, a set of bioassays (induction of necrosis on banana leaves, electrolyte leakage assay, and measurement of chlorophyll fluorescence) have previously been developed to document the effects of extracted metabolites (ethyl acetate crude extract or EaCE) from culture filtrates of *M. fijiensis* [6]. In these assays, swelling of chloroplasts, a rapid effect on chlorophyll fluorescence, and a strict light-dependency of this toxicity were observed in a susceptible cultivar regardless of the bioassay used. These observations suggested that banana chloroplasts could represent a potential target site for *M. fijiensis* toxins.

In this paper, we present a study of the mode of action of *M. fijiensis* toxins during the development of BLS disease. Identification of the toxic components in the crude extracts was done before using purified juglone to analyse the physiological basis of the toxicity. To understand the physiological basis of the toxicity, the effects of juglone on intact chloroplasts were quantified for two reference banana cultivars. A possible link between juglone and oxidative stress was also considered by assessing the interactions between the purified metabolite and ascorbic acid.

2. MATERIALS AND METHODS

2.1. Plant materials

Banana cultivars Grande Naine (susceptible to BLS) and Fougamou (exhibiting partial resistance to the disease) were used to investigate the role of *M. fijiensis* toxins. These genotypes were micropropagated and transferred to the soil in a greenhouse supplemented with a 16 h light photoperiod at a temperature of about 25°C. The fully expanded second leaf of at least 8-9-week-old acclimatised banana plants were either injected with *M. fijiensis* metabolites or used for chloroplast isolation.

2.2. Fungal strains and toxin production

The *M. fijiensis* monospore strains 049 HND (from Honduras), 281 COOK (from Cook Islands), and 282 TON and 283 TON (from Tonga) were provided by Dr. X. Mourichon, Laboratory of Plant Pathology, CIRAD-FLHOR (Montpellier, France). These strains were maintained according to the technique previously described [7].

Toxins were obtained from 500 ml M1D medium [8] inoculated with a suspension of *M. fijiensis* spores (5 ml of 10^5 conidia per millilitre) harvested from 12-day-old colonies grown on V8 modified medium. The cultures were incubated under mild agitation (120 r.p.m.) for 4 weeks at 25°C with a 16 h light photoperiod. An equal volume of methanol was added and the culture was incubated at 4°C overnight before discarding the mycelium by filtration through several layers of cheesecloth. The extraction of *M. fijiensis* toxins from the culture filtrates with ethyl acetate was performed according to the technique previously described [8]. The ethyl acetate extract was concentrated to dryness under reduced pressure at 30°C, and the residue was dissolved in 10% methanol. This final solution was the ethyl acetate crude extract (EaCE).

2.3. Fractionation of EaCE and HPLC investigation

Fractionation of the EaCE was performed by column chromatography (80 mm height, 10 mm I.D.) on silica gel G60 (70-230 mesh from Macherey-Nagel, Germany). The elution was performed starting with pure chloroform and then with increasing amounts of methanol in chloroform to reach the ratio of 10:1 (v/v) chloroform/methanol. The collected fractions were concentrated under reduced pressure at a maximum of 40°C and further examined for purity in the abovementioned conditions.

To investigate the constituents of selected purified fractions, HPLC (Hewlett-Packard HP1050 LC chromatograph) analyses were performed. The fractions obtained after column chromatography of EaCE were analysed on a Inertsil ODS-2 (250 × 4.6 mm, 5 µm) column (Chrompack, The Netherlands) using the following conditions: gradient elution with acetonitrile-water 30:70% to 70:30% for 10 min with a final hold of 10 min; flow rate, 0.7 ml min^{-1}; temperature, 24°C. Chromatographic profiles were monitored at 254 nm.

2.4. Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS investigations were performed on a Hewlett-Packard 5972 mass spectrometer coupled to an HP 5890 Series II gas chromatograph equipped with an HP-crosslinked 5% phenyl-methylsiloxane column (30 m × 0.25 mm I.D.; film thickness 1 µm) using the following operating conditions: split-splitless injector at 250°C (splitless mode); gas carrier, helium at 1 $ml.min^{-1}$; temperature programme: from 50°C (1 min) to 140°C at 20°C min^{-1} then from 140°C to 290°C at 10°C.min^{-1}, with a final hold of 10 min at 290°C. The mass spectra were recorded in the electron impact mode at 70 eV (source temperature, 180°C; scanned mass range, 35-500 amu). The detected peaks were identified by their retention data and their fragmentation patterns. The mass spectra were finally compared with those of the NBS 75K.L and Wiley 138.L computer MS libraries.

In the absence of high-purity standards for each reported *M. fijiensis* metabolite, a solution (100 $µg.ml^{-1}$) of 5-hydroxy-1,4-naphthoquinone (Sigma) was used as a reference. The minimum detectable quantity (MDQ) was established at 1 ng for full mass spectrum recordings and 100 pg in the SIM (selected ion monitoring) mode (selected ions at m/z = 174, 146 and 118).

2.5. Injection bioassays

Different compounds (total EaCE, semi-purified fractions, and juglone) were injected into banana leaves of the two reference cultivars at different concentrations. Before the injection tests, banana plants were preincubated for 24 h in a humid chamber at 25 ± 2°C with a 16 h light photoperiod. The injection was then performed on the lower surface of the fully-expanded second leaf with a microsyringe at two to four sites per leaf (20 µl per injection site) and four plants per treatment. Injected plants were incubated at 25 ± 2°C with a relative humidity of 100%. Comparison was performed between incubation in the light (16 h light photoperiod) and incubation in the dark after the injection.

2.6. Chlorophyll fluorescence measurement

To evaluate the effect of this injection, chlorophyll fluorescence was measured on plants incubated at different times following the injection. The measurements were performed on the adaxial side of the injection site, at room temperature and natural O_2 concentration, using a modulated fluorescence system (Kfm 002; Technical University, Atomic Physics Dept.,

Wustaden, Germany). Minimal fluorescence (F_0) was measured after 30 min of dark adaptation. Maximal fluorescence (F_{max}) and the steady-state fluorescence (fs) were recorded at 690 nm after submitting the injected site of the leaf to a saturation pulse of about 500 µmol m^{-2} s^{-1}. The functioning of the photosynthetic apparatus was evaluated by the fluorescence decrease (fd) from the maximum fluorescence (F_{max}) to steady-state fluorescence (fs) which allowed the calculation of the vitality index (Rfd = fd/fs) [9].

2.7. Chloroplast isolation and Hill reaction

The bioassay on isolated chloroplasts was optimised with the cultivar Grande Naine (susceptible to *M. fijiensis* infection) before testing the other reference cultivar (Fougamou). Young banana leaves were harvested and rinsed with distilled water, cut into small pieces and ground in isolation buffer (330 mM sorbitol, 0.4 mM KCl, 0.5 mM $CaCl_2.2H_2O$ and 2 mM Hepes, pH 7.6) maintained continuously at 4°C. The homogenate was filtered through one layer of Miracloth (22-25 µm) filter (Calbiochem). The filtrate was centrifuged at 49 *g* for 5 min. The supernatant was re-centrifuged at 2000 *g* for 5 min. The pellet obtained after this centrifugation step was resuspended in cold isolation buffer, layered onto an equal volume of 35% Percoll prepared in isolation buffer, and centrifuged at 250 *g* for 5 min. The green layer, containing the intact chloroplasts, was harvested and transferred to cold isolation buffer. This suspension was centrifuged at 2000 *g* for 5 min and the supernatant discarded. The final chloroplast pellet was resuspended in cold isolation buffer at a final concentration of 14×10^6 chloroplasts per millilitre.

The Hill reaction was used to evaluate the physiological activity of isolated chloroplasts. In practice, 990 µl of the chloroplast suspension was placed in a spectrophotometric cuvette and mixed with 110 µl of 1 mM DCPIP (dichlorophenoindolphenol, the electron acceptor) prepared in the isolation buffer. The chloroplast solutions were illuminated with a 60 W incandescent lamp (23 $\mu mol.m^{-2}.s^{-1}$). The decrease of absorbance at 595 nm, due to the reduction of DCPIP by the chloroplasts, was followed for 20 min. Samples of the same suspension of chloroplasts maintained in the dark after addition of DCPIP were used as controls.

2.8. Effect of juglone on isolated chloroplasts

Chloroplasts (14×10^6 chloroplasts per millilitre) isolated from the two reference cultivars were mixed with increasing concentrations (5,10,20,30,40 and 60 p.p.m.) of juglone in 10% methanol solution. The Hill reaction was then performed as previously described. The juglone toxicity was calculated as an inhibitory effect (%) on the physiological activity (electron transfer properties) of chloroplasts using the formula $100-(100d_j/d_c)$ where d_j represents the absorbance decrease in the chloroplast suspension treated with juglone and d_c the absorbance decrease in the untreated control suspension (containing 10% methanol without juglone).

2.9. Interactions between juglone and ascorbate

The chloroplast isolation buffer was mixed with 0.5 mM ascorbic acid and incubated with 60 p.p.m. juglone. The control for this assay was 10% methanol solution without juglone. Ascorbic acid content was followed by measuring the absorbance at 290 nm immediately after the addition of juglone, and again 1 h after this treatment.

3. RESULTS AND DISCUSSION

3.1. Toxicity of the EaCE

The toxicity due to EaCE, previously demonstrated [6], was confirmed. Injection of the EaCE into Grande Naine leaves was followed by development of necrosis and a decrease of the relative vitality index calculated from chlorophyll fluorescence data. Table 1 shows chlorophyll fluorescence data obtained 6 h after injection of the EaCE of the strain 049HND into leaves of the susceptible cultivar. There was a significant decrease of the Rfd value for plants incubated in continuous light, but there was no significant effect of EaCE on plants incubated in darkness after injection. On the basis of these data, the toxicity due to EaCE could be considered as light-dependent. Injection into Fougamou leaves gave rise to a similar situation but the significance of the Rfd decrease was lower than that observed on Grande Naine plants. From these observations, there appears to be a correlation between the sensitivity of the two reference cultivars to EaCE and their sensitivity to field infection. This kind of correlation, which was also revealed in the past [3], might suggest the involvement of *M. fijiensis* extracts in BLS disease, but does not represent experimental evidence.

Table 1 Effect on chlorophyll fluorescence status of Grande Naine leaves injected with EaCE of the strain 049HND

Incubation conditions[a]	Rfd[b] of leaves injected with EaCE	Rfd of control leaves (10%methanol)	Rfd of EaCE-treated leaves (% of the control)
Continuous light	0.385 ± 0.07	0.554 ± 0.05	69
Darkness	0.589 ± 0.08	0.571 ± 0.10	103

[a] Before incubation, plants were injected with EaCE (50 p.p.m.) and the chlorophyll fluorescence measurements were performed 6 h after injection.

[b] The Rfd value was determined as the ratio fd/fs where fd is the decrease of fluorescence from the maximum (F_{max}) to the steady-state fluorescence (fs).

Table 2 Fractions separated from the EaCE of *M. fijiensis* and their respective biological activity according to their injection into Grande Naine leaves

Fractions[a]	Chlorophyll fluorescence data (Rfd value and percentage of the control)[b]		Induction of necrosis 48 h after injection[d]	
	Light	Darkness	Light	Darkness
0.90< R_F <1	0.433 (74)B[c]	0.619 (89)A	+	-
0.76< R_F <0.88	0.450 (77)B	0.658 (94)A	±	-
0.66< R_F <0.76	0.475 (82)AB	0.672 (96)A	-	-
0.56< R_F <0.64	0.480 (82)AB	0.687 (98)A	-	-
0.50< R_F <0.55	0.515 (88)A	0.664 (95)A	-	-
0.36< R_F	0.442 (76)B	0.658 (94)A	+	-

<0.47				
0.22< R_F <0.36	0.510 (88)A	0.666 (95)A	-	-
0< R_F <0.14	0.440 (76)B	0.622 (89)A	+	-
Control[e]	0.582 (100)A	0.697 (100)A	-	-

[a] Fractions are characterised by their relative migration (R_F) compared with that of the solvent.

[b] Values in parenthesis correspond to percentage of the Rfd of the considered fraction to that of the control (10% methanol solution).

[c] Averages followed by a same letter are not significantly different

[d] + = necrosis at 1000 p.p.m., ± = necrosis at 5000 p.p.m., - = no necrosis.

[e] For the control, a 10% methanol solution was injected.

3.2. Fractionation of EaCE and toxicity of the semi-purified fractions

It was possible to separate eight fractions using column chromatography. Table 2 shows the different separated fractions according to their relative migration compared to that of the solvent used for the fractionation. Results of the injection bioassays of these fractions are also presented in the same table.

Among the eight fractions isolated, only four were biologically active according to the results of injection into banana leaves. As with the EaCE, plants maintained in the light exhibited necrosis and a significant decrease of the relative vitality index (Rfd), while plants incubated in the darkness were not affected by the toxic fractions. All four bioactive fractions exhibited properties similar to those of the EaCE in terms of induction of necrosis, effect on chlorophyll fluorescence, and light-dependency. The total toxicity detected in the *M. fijiensis* crude extracts could be considered as a combination of the effects of each of the four individual fractions.

A comparative study was performed on the two reference cultivars (Grande Naine and Fougamou) by injecting the different toxic fractions previously identified (Table 3). Regardless of the bioassay performed, the cultivar Fougamou showed higher resistance to the different fractions tested than the cultivar Grande Naine. These data are correlated with the response of the two cultivars to pathogen infection in the field or under experimental inoculation. To demonstrate whether the different fractions are involved in the disease development, it would be necessary to verify this correlation with a larger range of banana genotypes. Moreover, the identification of the different metabolites present in each fraction would help to understand the mechanisms of action of *M. fijiensis* toxins.

Table 3 Comparative analysis of the behaviour of the two reference cultivars (Fougamou and Grande Naine) after their injection with the four bioactive fractions of *M. fijiensis* EaCE

Fraction[a]	Relative vitality index (chlorophyll fluorescence) [b]		Necrosis induction[d]	
	Grande Naine	Fougamou	Grande	Fougamou

			Naine	
0.90< R_F <1	0.509 (73)B[c]	0.428 (85)A	+	-
0.76< R_F <0.88	0.548 (79)B	0.483 (96)A	+	-
0.36< R_F <0.47	0.538 (77)B	0.476 (95)A	+	-
0.22< R_F <0.36	0.522 (75)B	0.442 (88)A	+	-
Control[e]	0.696 (100)A	0.501 (100)A	-	-

[a] Fractions are characterised by their relative migration (R_F) compared with that of the solvent.

[b] Values in parenthesis correspond to percentage of the Rfd of the considered fraction to that of the control (10% methanol solution).

[c] Averages followed by the same letter are not significantly different.

[d] + = necrosis at 1000 p.p.m., - = no necrosis.

[e] For the control, a solution of 10% methanol solution was injected.

3.3. GC-MS analysis of the EaCE

The GC-MS analysis allowed the identification of juglone (5-hydroxy-1,4-naphthoquinone) in the most toxic fraction (0.90< R_F <1) of the *M. fijiensis* EaCE (strain 049HND). This metabolite was resolved as a sharp symmetrical peak at 12.95 min. In our culture conditions, other metabolites previously detected [5] were not identified. Juglone belongs to the large group of naphthoquinones found in fungal extracts which are phytotoxic, as described by Medentsev and Akimenko [10]. By our analyses, it was demonstrated that juglone was present in EaCE of three other strains of the pathogen originating from Asia. According to these findings, we investigated the toxicity of this purified metabolite on banana.

3.4. Toxicity of juglone on banana plants

The results of juglone injection into banana leaves of the two reference cultivars are presented by Table 4 in terms of chlorophyll fluorescence data as well as necrosis induction.

Table 4 Assessment of juglone toxicity on banana leaf tissues after their injection with different increasing concentrations of the metabolite

Juglone concentration (p.p.m.)[a]	Relative vitality index (chlorophyll fluorescence)[b]				Necrosis induction[c]			
	Grande Naine		Fougamou		Grande Naine		Fougamou	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
500	0.148 (26)	0.250 (49)	0.158 (28)	0.436 (72)	+	+	+	+
250	0.228	0.320	0.274	0.442	+	+	+	-

	(41)	(62)	(49.37)	(73)				
100	0.274 (49)	0.370 (72)	0.372 (67.03)	0.479 (79)	+	+	+	-
50	0.301 (54)	0.445 (87)	0.429 (77.30)	0.498 (82)	+	-	-	-
25	0.354 (63)	0.500 (97)	0.464 (83.60)	0.547 (90)	+	-	-	-
12.5	0.441 (79)	0.502 (98)	0.502 (90.45)	0.582 (96)	-	-	-	-
Control[d]	0.559 (100)	0.513 (100)	0.555 (100)	0.604 (100)	-	-	-	-

[a] The concentration of juglone is expressed in mg/l (p.p.m.).

[b] Values in parenthesis correspond to percentage of the Rfd of juglone to that of the control (10% methanol solution).

[c] + = necrosis, - = no necrosis.

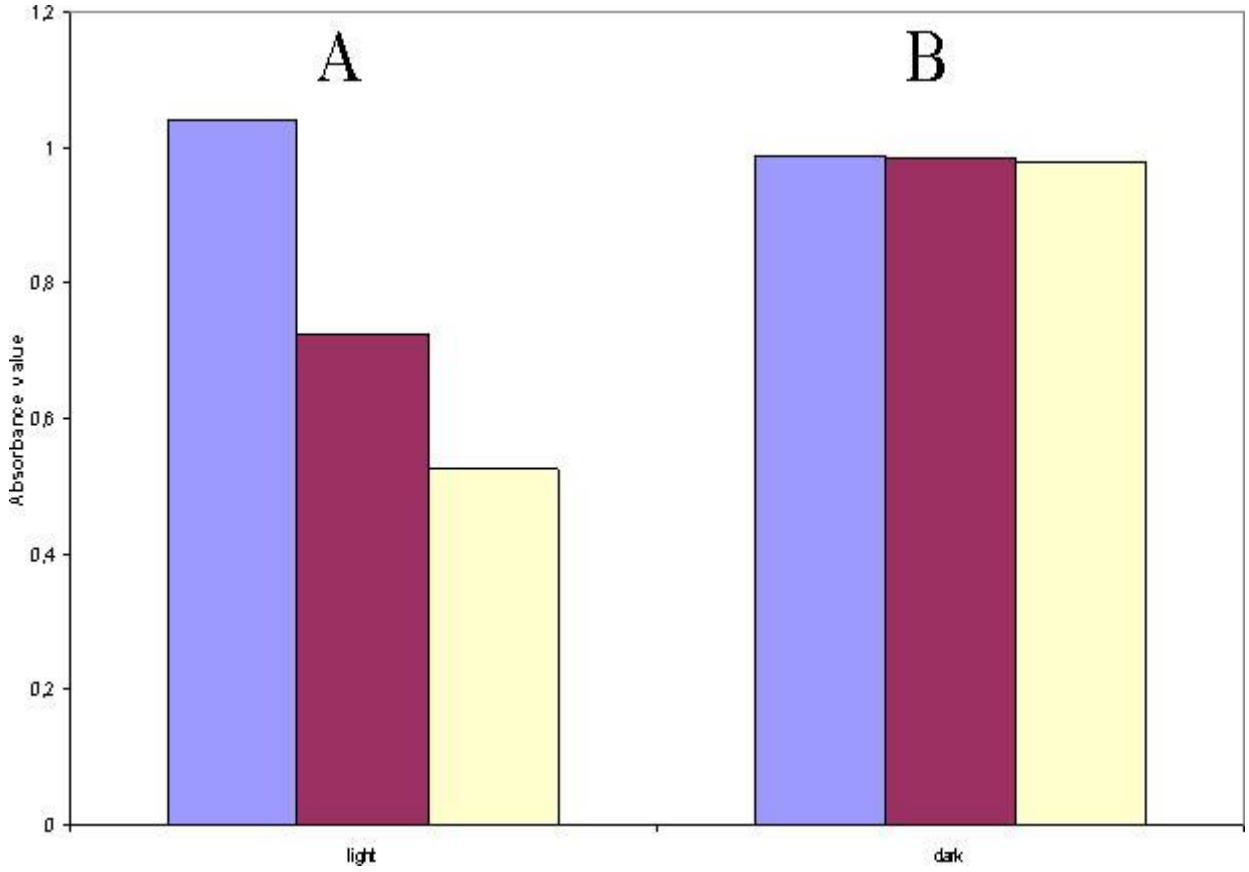
[d] For the control, a 10% methanol solution was injected.

Similarly to the EaCE and the four bioactive fractions, the injection of juglone induced a necrotic spot at the site of injection and decreased the relative vitality index, calculated from the chlorophyll fluorescence data. The activity of juglone was also light-dependent except for very high concentrations (at least 100 p.p.m.) where necrosis appeared in the dark as well as in the light. Moreover, we observed that cultivar Fougamou was more resistant to juglone than cultivar Grande Naine. In fact, 25 p.p.m. of juglone induced necrosis on Grande Naine leaves while no necrosis appeared on Fougamou leaves injected with concentrations less than 100 p.p.m.. As this purified metabolite exhibits similar properties to those of the EaCE on banana plants, we decided to use juglone to study the mechanisms of action of the *M. fijiensis* toxins.

3.5. Effect of juglone on isolated banana chloroplasts

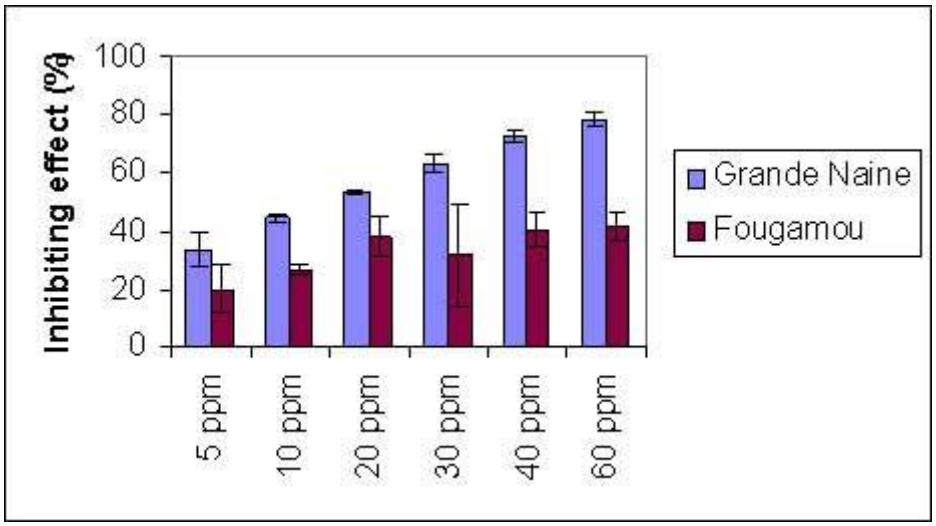
The integrity of the purified chloroplasts isolated from banana leaves was assessed by the Hill reaction [11] which measures their ability to perform the oxidation of water in the presence of light according to the reaction: $H_2O + A \rightarrow AH_2 + ½O_2$ (A being an electron acceptor or 'Hill oxidant') (Figure 1). The absorbance of DCPIP at 595 nm decreased rapidly in chloroplasts maintained in the light, while no decrease of absorbance was noticed in chloroplasts incubated in the dark. The banana chloroplast purification protocol we developed in our lab thus allows the isolation of intact, functional chloroplasts.

Figure 1 Hill reaction on Grande Naine chloroplast suspension in the presence of DCPIP. Reduction of DCPIP was followed by measuring (at 0, 10 and 20 min) the absorbance at 595 nm in the light (A) and in the darkness (B).



To analyse the possible effect of juglone on isolated chloroplasts, a comparative study was performed with the two reference banana cultivars (Fougamou and Grande Naine). After addition of increasing concentrations of juglone (5, 10, 20, 30, 40 and 60 p.p.m. in 10% methanol), the suspensions were mixed with DCPIP and illuminated for 20 min. The inhibitory effect of juglone was determined by comparing the decrease in absorbance at 595 nm in the presence of juglone and the decrease in absorbance in the control chloroplasts for each genotype (Figure 2).

Figure 2 Effect of juglone on the electron transfer properties of chloroplasts of the banana cultivars Grande Naine and Fougamou measured by the Hill reaction. The decrease in absorbance at 595 nm in the presence of juglone was compared with that obtained in the untreated control chloroplasts to determine the inhibitory effect (%).

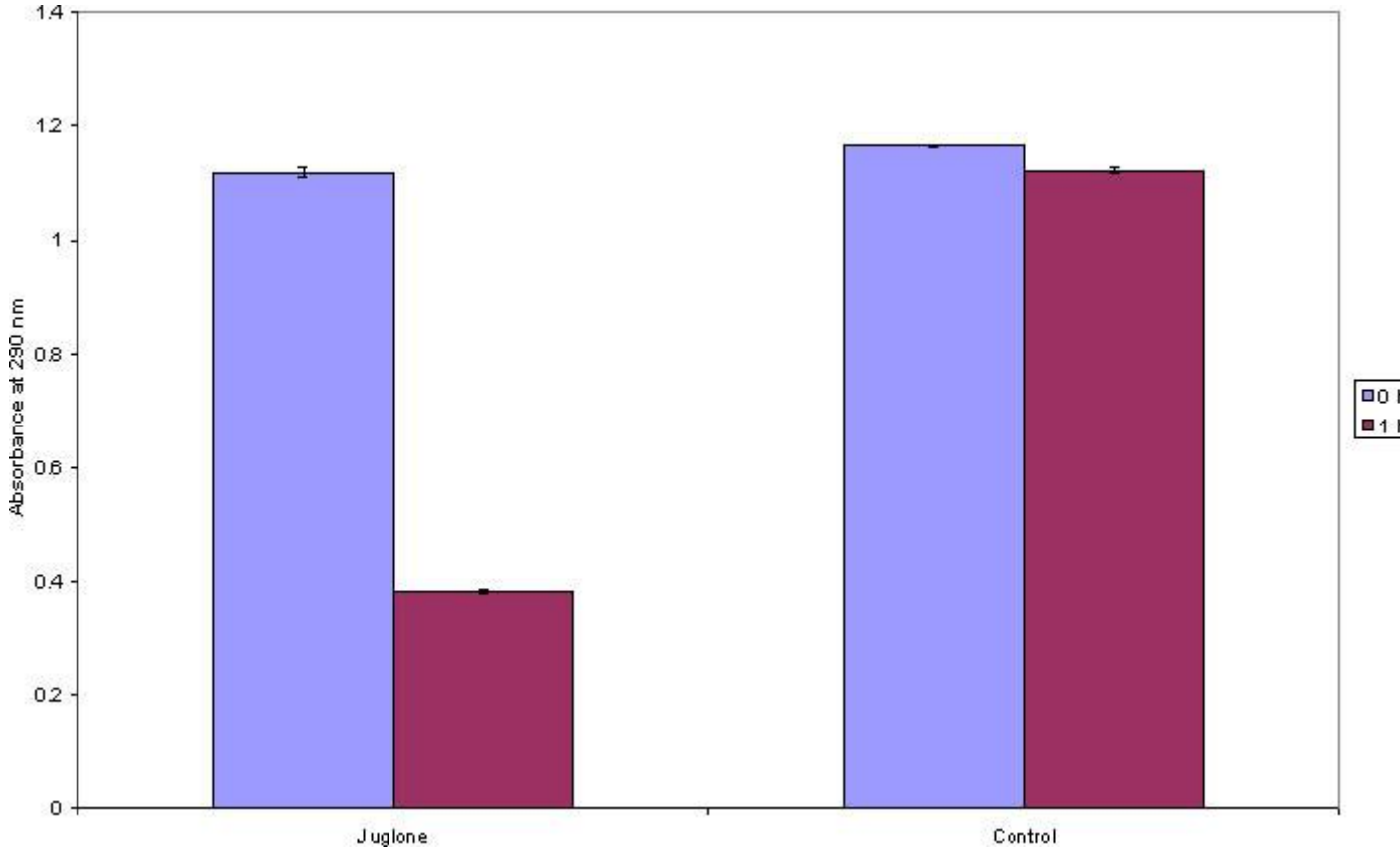


Juglone inhibited the electron transfer properties of isolated banana chloroplasts of the two reference cultivars. The inhibitory effect was higher on Grande Naine chloroplasts than on Fougamou chloroplasts. Inhibition was dose-dependent on Grande Naine chloroplasts, but reached a plateau at 20 p.p.m. juglone for Fougamou chloroplasts. These results can be correlated with the sensitivity of the two reference cultivars based on leaf injection tests performed with EaCE or juglone, and with the susceptibility of the two cultivars to pathogen infection in field conditions. Based on these correlations, juglone appeared to be involved in the infection process, as the genotype with the highest degree of partial resistance to BLS disease seems also to be the less affected by juglone than the susceptible genotype. To confirm this hypothesis, it would be necessary to develop this bioassay on other banana cultivars showing different levels of resistance to BLS disease. These findings suggest that chloroplasts could constitute a potential primary target site for *M. fijiensis* naphthoquinone compounds in banana. It was observed that fungal naphthoquinones lead to chloroplast alteration such as disruption of the organelle membrane, resulting in the release of nucleotides, amino acids, chlorophyll, proteins and mineral salts [10]. Moreover, some naphthoquinone molecules released by fungal pathogens were reported to induce the formation of superoxide anion radicals followed by the oxidation of several cell components in plant tissues [10].

3.6. Interactions between juglone and ascorbate in solution

The strict light dependency of the symptoms induced by *M. fijiensis* EaCE and juglone, the inhibition by juglone of electron transfer in chloroplasts, and the fact that chloroplasts constitute one of the sites of active oxygen species production in plants [12,13] suggest that the toxicity of juglone might be induced by deleterious oxidative events linked to alterations in the ability to detoxify active oxygen species. In a preliminary experiment (Figure 3), we tested this hypothesis by measuring the impact of juglone in solution on ascorbic acid, one of the most abundant non-enzymatic antioxidants in plants [14].

Figure 3 Oxidation of ascorbic acid as measured by the decrease in absorbance at 290 nm in the presence or absence of juglone.



The presence of juglone in solution induced a decrease of the absorbance at 290 nm corresponding to a decrease in the ascorbate content. This decrease of absorbance, only observed in the presence of juglone, might be due to the oxidation of ascorbate by the naphthoquinone molecule. If this observation is confirmed, reducing the amount of the quantitatively most predominant antioxidant in plant cells [15] might therefore constitute one of the possible ways used by the toxin to initiate damage to banana chloroplasts during BLS development. In the future, this hypothesis could be verified *in vivo* by assessing both ascorbate content and the generation of active oxygen species during juglone treatment within the two banana reference cultivars. Analysing the effect of this metabolite on other banana antioxidant systems would constitute another way to check the eventuality of oxidative damage induced by *M. fijiensis* toxins in bananas.

4. CONCLUSIONS

Necrosis induction and chlorophyll fluorescence measurements were used as easy bioassays to analyse the toxicity of *M. fijiensis* culture filtrate extracts on banana. Globally, injection bioassays into banana leaves allowed the identification of four EaCE semi-purified fractions having similar properties to the crude EaCE. For a more detailed study of the toxic compounds released by *M. fijiensis*, GC-MS analyses were performed on the four EaCE semi-purified fractions; this allowed the identification of juglone (5-hydroxy-1,4-naphthoquinone) within the most toxic fraction. This metabolite was detected within extracts of all the pathogen strains analysed in the area of our study.

Juglone exhibited a similar toxicity on banana leaves to the EaCE or its semi-purified fractions regardless of the bioassay used. To evaluate the possible involvement of this compound in pathogenesis, the different bioassays were performed on two reference cultivars

(Grande Naine as the susceptible cultivar and Fougamou as the partially resistant one). As Fougamou was always more tolerant than Grande Naine to all the toxic compounds or fractions tested, there was a constant correlation between the sensitivity to juglone and the susceptibility to pathogen infection.

The light dependency of the juglone toxicity in banana was a strong indication of the involvement of the photosynthetic apparatus in the action of *M. fijiensis*. To investigate this hypothesis further, a new bioassay was developed using physiologically intact chloroplasts isolated from banana leaves. We showed that juglone clearly inhibited chloroplast function as measured by an alteration of the electron transfer properties of the chloroplasts. Comparison of the two reference cultivars showed that chloroplasts isolated from the partially resistant cultivar Fougamou were less affected by juglone than those obtained from the susceptible cultivar Grande Naine.

Numerous naphthoquinone metabolites, including juglone, produced by different phytopathogenic fungi show different mechanisms of toxicity. Among these is their auto-oxidative property, which is responsible of the oxidation of NADH and NADPH, leading to the removal of these molecules from the oxidative phosphorylation system as potential sources of reduction equivalents for the respiratory chain [10]. This mode of action could be an explanation for the rapid effect of juglone on chloroplast function.

Another mode of action of juglone may affect the ability of cells to protect themselves against oxidative stresses. A first indication of a link between juglone and oxidative stress came from the measurements of the impact of juglone on ascorbate, the predominant antioxidant in plant cells. We showed that juglone rapidly oxidised ascorbate in solution, suggesting that a direct interactions between ascorbate and *M. fijiensis* toxins within banana cells is *per se* not excluded. Oxidation of ascorbate by juglone should therefore deprive bananas partly of their antioxidant ability, and establish a link between oxidative stress and the development of necrotic spots at the injection site. Based on these preliminary results, we hypothesize that differences in chloroplast resistance to juglone between the two reference cultivars might reflect differences in the composition of their respective antioxidant systems.

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