ISSN 1330-9862
(FTB-2346)

preliminary communication

Effect of Lactoperoxidase System on the Control of *Colletotrichum musae* on Bananas

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Received: September 17, 2009
Accepted: September 6, 2010

Summary

Postharvest diseases are a major concern for plant products, leading to considerable postharvest losses. *Colletotrichum musae* is responsible for anthracnose and is also involved in crown rot, the two main postharvest diseases of banana. The use of antimicrobial agents such as the lactoperoxidase system (LPS) represents an interesting alternative to the use of conventional fungicides for the control of postharvest diseases of banana. Conidial germination and fungal growth of three different strains of *Colletotrichum musae* originating from Cameroon (C 52 and C 62) and Guadeloupe (C 46.12) were monitored in the presence of LPS or Eau Activée® (an industrial derivative of LPS). *In vivo* studies were also conducted on bananas preinoculated with strain C 46.12 and then subjected to a soaking treatment in LPS and Eau Activée® solutions. However, this postharvest treatment did not show any significant effect of the LPS or Eau Activée®. The *in vitro* studies showed a difference in the behaviour of *Colletotrichum musae* strains subjected to LPS and Eau Activée® treatments. A highly visible inhibitory effect of LPS was observed on fungal growth of strains C 52 and C 62. Furthermore, LPS gave better results than its industrial derivative, Eau Activée®.

Key words: LPS, Eau Activée®, *Colletotrichum musae*, postharvest diseases, crown rot, anthracnose

Introduction

Disease control in banana is an essential component of fruit quality after harvest (*1*). This quality is affected by postharvest diseases, of which the most important are anthracnose caused by *Colletotrichum musae* (Berk. & M.A. Curtis) Arx, and crown rot, which can be caused by *C. musae* but also by a wide fungal complex (*2*). Anthracnose appears as lesions on the fruit due to the development of the fungus. The initially brown lesions blacken and become covered with salmon-coloured acervuli (*3*). Crown rot affects the tissues joining the fruit pedicels with each other. When infection is severe, the rot may reach the pedicel and ultimately the banana pulp (*4*). Postharvest diseases of banana are mostly controlled in many areas such as Windward Islands, Guadeloupe and

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Cameroon by postharvest fungicides such as bitertanol, thiabendazole, imazalil and benomyl (*1*,*5*,*6*). However, *Colletotrichum musae* and the fungal complex involved in crown rot have aquired some resistance to these fungicides, necessitating doses to be increased (*7*–*9*). One current challenge is thus to find adequate treatments for preserving the fruits in good condition while avoiding environmental, ecological, and human health problems associated with fungicide use. Indeed, the increases in treatment doses can result in fungicide residues on the marketed products (*10*) and thus harm consumer health. The substitution of chemical treatments with natural treatments of vegetables or products of animal origin is less dangerous to human health and less damaging to the environment.

Many studies show that compounds derived from certain plants and/or animals have an antimicrobial activity. The lactoperoxidase system (LPS) found in milk is one such complex that has a potential in the treatment of bacteria (*11*). This system is composed of three elements which are: lactoperoxidase enzyme, thiocyanate ion and hydrogen peroxide. It is the reaction between thiocyanate and hydrogen peroxide, catalysed by lactoperoxidase that produces the most antimicrobial element of the system, namely the hypothiocyanite ion ($OSCN^-$) (*12*). Inhibitory effects of this system have been shown against a number of bacteria, fungi and viruses (*11*,*13*–*15*).

The purpose of this work is to study the effect of LPS on various strains of *Colletotrichum musae* isolated on infected bananas (identified and stocked in CIRAD collection, Montpellier, France) responsible for postharvest diseases of the fruit. The industrial pilot product Catallix® 30 reproduces the reaction of LPS and produces a continuous supply of Eau Activée® (*16*) able to control the microbial flora on foodstuffs such as eggs, sausages and cured meat, cheese, fish and poultry. It can be integrated into the production lines of these foodstuffs and it enables the 'activation' of water treatment, thereby conferring disinfectant properties.

Materials and Methods

Lactoperoxidase system

Lactoperoxidase system (400 IU/mg) was donated by Bio Serae Laboratories (Bram, France). Sodium percarbonate ($Na_2CO_3 \cdot 1.5\ H_2O_2$), used as a peroxide generator, and sodium thiocyanate (NaSCN) were purchased from Sigma-Aldrich (Lyon, France).

Eau Activée® solution

Reagents (Activix, SCN^-, H_2O_2, coagulant) for the production of Eau Activée® were donated by TMI Europe (Lyon, France). Essentially, Catallix® system draws on an enzymatic reaction to create a natural antibacterial agent. Catallix® biogenerator immobilizes and recycles a natural enzyme in order to continuously produce Eau Activée®, which is able to control the contamination of food products by microorganisms.

Microorganisms and inoculum preparation

Three different *Colletotrichum musae* strains obtained from monospore cultures were used: C 52, C 62 and C 46.12. The C 52 and C 62 strains originate from Cameroon from a noncommercial banana plot and the C 46.12 strain from Guadeloupe from an intensive commercial banana farm. These two sources were chosen due to their different cropping systems and scale of cultivation, which could potentially lead to different contamination levels in the field, and therefore to various treatment effectiveness. The strains were identified by CIRAD. All the culture media were purchased from DIFCO (Illkirch, France).

Spore suspensions were chosen as inoculum. Fungal cultures of *Colletotrichum musae* strains (C 52, C 62 and C 46.12) aged 7 to 10 days on potato dextrose agar were flooded with 10 mL of saline water (9 g/L NaCl), containing 0.05 % Tween 80 to prevent conidia clumping (*17*). The conidial suspension was filtered through Watmann filter paper no. 1, and adjusted to a final concentration of 10^4 conidia per mL using a Malassez cell.

Comparison of the antimicrobial activity of the LPS, Eau Activée®, thiabendazole and chlorinated water on conidial germination

A volume of 1 mL of 10^6 conidia per mL of suspension of *Colletotrichum musae* (C 52, C 62 and C 46.12) was diluted in 10 mL of treatment solution: LPS (Bio Serae Laboratories), Eau Activée® (TMI Europe), thiabendazole (TBZ; MSD AGVET, Paris, France) or chlorinated water. The treatment lasted 15 min for LPS (lactoperoxidase enzyme 25 mg/L, sodium thiocyanate 100 mg/L, sodium percarbonate 50 mg/L) (*18*), 20 min for Eau Activée® (700 μM of $OSCN^-$ ion) (*16*), 1 min for TBZ (2 mL/L) (*1*) and 5 min for chlorinated water (80 ppm; as per French legislation for the use of chlorinated water on foodstuffs). A control was prepared using distilled water. To enhance the effects of LPS and Eau Activée®, the pH of these solutions was adjusted to 5.5 using a 0.1 M solution of lactic acid (*18*). The same treatment of acidification was applied to distilled water in order to control any pH-related action.

After treatment, 100 μL of a solution from each tube were incubated on PDA in Petri dishes (three dishes per treatment; DIFCO) using a sterile spreader. The dishes were evaluated using an optical microscope (Laborlux, Paris, France) after 4 h of incubation in an oven at 30 °C and the count was expressed as a percentage of germinated conidia. Each treatment was repeated three times and the experiment was repeated five times.

Evaluation of the antimicrobial activity of LPS, Eau Activée®, TBZ and chlorinated water on fungal growth of C. musae strains in liquid cultures

The culture medium used for these studies was potato dextrose agar (PDA). The pH was adjusted to 5.5 with phosphate buffer (0.2 M $NaH_2O_4 \cdot H_2O/Na_2HPO_4 \cdot 2H_2O$). A volume of 0.75 mL (3 %) of suspension of *Colletotrichum musae* (C 52, C 62, C 46.12) was used to inoculate the main culture (25 mL).

The control cultures (25 mL) contained the same substances without the LPS (lactoperoxidase 25 mg/L, so-

dium thiocyanate 100 mg/L and sodium percarbonate 50 mg/L). The LPS system was replaced by TBZ (2 mL/L) and chlorinated water (80 ppm) for the other two treatments.

Immediately after inoculation and the addition of LPS reagents to the test culture, the flasks were placed on a rotary shaker at 180 rpm and 30 °C. Samples were collected after 0, 6 and 24 h. Fungal growth was determined by the gain in biomass of the main culture. Volumes of 5 mL of the main cultures were filtered on membranes (pore ∅=0.45 μm). These membranes were then dried at 105 °C for 24 h and weighed on a balance to four decimal places until a constant mass was reached (*18*). Each treatment consisted of three replications and the treatment was replicated five times.

Evaluation of crown rot control using LPS, Eau Activée® and Baycor, under artificial inoculation of bananas with Colletotrichum musae strain C 46.12

Inoculation of banana crowns

A total of 24 clusters, each consisting of four Cavendish dessert bananas from Guadeloupe with a ripeness index of 1 (all green), were used. The crowns of the banana clusters were trimmed by thinly slicing off the outermost tissues, and then surface sterilised with 50 % ethanol. After the ethanol had evaporated, the crowns were inoculated with 50 μL of the conidial suspension (10^4/mL). The droplet was covered with sterile Watmann filter paper no. 1 to prevent it from running off. The fruits were then left at ambient temperature for 3 h.

Soaking treatments

A total of 24 clusters were soaked as follows: 6 clusters in water for 1 min; 6 clusters in Eau Activée® (700 μM of $OSCN^-$ ion) for 20 min; 6 clusters in LPS (lactoperoxidase enzyme 25 mg/L, sodium thiocyanate 100 mg/L, sodium percarbonate 50 mg/L) for 20 min; and 6 clusters in Baycor 300 EC (300 g/L of bitertanol) for 1 min. LPS and Eau Activée® were used at the same concentrations as in the other experiments; besides we also used Baycor, one of the most used industrial fungicides, for dipping bananas. The clusters subjected to the same treatment were packed in a perforated polyethylene bag and stored at 13 °C for 10 days, to simulate shipping. After 10 days, ripening of bananas was artificially induced by an ethylene treatment (1000 ppm) for 24 h at 20 °C in an airtight chamber. The fruits were then aerated and returned to 20 °C for 3 days, corresponding to an artificial ripening stage identical to that carried out in commercial ripening rooms.

The progression of internal lesions was then observed. The internal progression of rot was determined by cutting the cluster crown longitudinally in two and measuring the surface of rot spread into the crown, from the original inoculation point. This internal necrotic surface was calculated by assuming a rectangular shape:

$$A=(L\cdot b)/\mathrm{mm}^2 \qquad /1/$$

where L is length and b is width (*19*). The experiment was repeated three times.

Statistical analysis

Analysis of variance (ANOVA) tests were conducted using the software package SPSS v. 12.0. If the analysis of variance was found to be significant, *post hoc* tests were used to identify significantly different treatments. The method used to establish whether there was equality of variance was the least significant difference (LSD), with a set error risk of 5 %.

Results and Discussion

Antimicrobial effect of LPS on various Colletotrichum musae strains

The data obtained are presented as the percentage of conidia germinated after each treatment for each strain of *Colletotrichum musae* (Fig. 1). For strain C 52, a significant inhibitory effect was observed in all the treatments (LPS, Eau Activée®, TBZ or chlorinated water) in comparison with the control; TBZ and chlorinated water had the greatest effect. There was 32, 28, 0 and 0 % of germination when Eau Activée®, LPS, TBZ and chlorinated water were used, respectively, compared to 44.3 % with distilled water (Fig. 1).

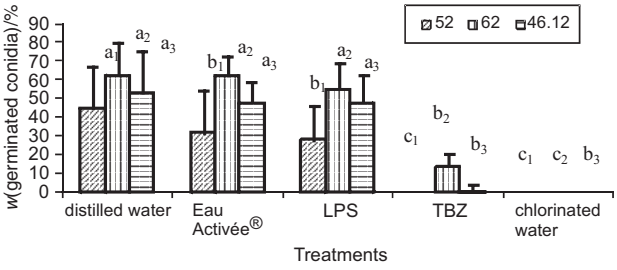

Fig. 1. Percentage of germination of conidia of three strains of *Colletotrichum musae* (C 52, C 62 and C 46.12) after 4 h of incubation at 30 °C following the treatment with distilled water, Eau Activée®, LPS, TBZ or chlorinated water. For each treatment, mean values followed by the same letter and number do not differ significantly at $p<0.05$

For strains C 62 and C 46.12, a significant inhibitory effect was found with TBZ and chlorinated water in comparison with the control. For strain C 62, there was 14 and 0 % germination with TBZ and chlorinated water treatments, respectively, and for strain C 46.12, 1 and 0 % germination with the same treatments (Fig. 1). For these two strains, the LPS and Eau Activée® treatments had no significant effect on conidial germination (62, 61 and 55 % germination respectively for the control, Eau Activée® and LPS for strain C 62; and 53, 48 and 48 % respectively for the control, Eau Activée® and LPS for strain C 46.12). The effectiveness of TBZ, Eau Activée® and LPS differed among the *Colletotrichum musae* strains. However, conidial germination does not systematically lead to the expression of disease symptoms. Therefore, the subsequent growth of *Colletotrichum musae* conidia biomass was measured.

Determination of biomass

The biomass at 0, 6 and 24 h was measured for *Colletotrichum musae* strains C 52, C 62 and C 46.12 grown

on PDA medium, with different treatments (LPS, Eau Activée®, TBZ and chlorinated water). The results obtained for biomass measurements after 24 h of growth are shown in Fig. 2.

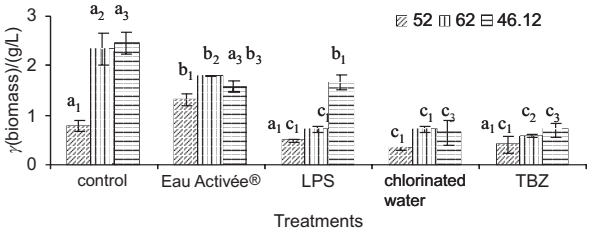

Fig. 2. Biomass of three *Colletotrichum musae* strains treated with distilled water, Eau Activée®, LPS, TBZ and chlorinated water after 24 h of growth on PDA (pH=5.5) at 30 °C. For each treatment, mean values followed by the same letter and number do not differ significantly at $p<0.05$

A difference in the behaviour of *Colletotrichum musae* strains under different treatments was observed. There was a clear inhibitory effect of LPS, TBZ and chlorinated water on strain C 52 after 24 h of growth, but no significant effect of the Eau Activée® treatment. It is also noteworthy that a slow growth of strain C 52 was observed in the control treatment as compared to the other two strains. With Eau Activée® treatment, the growth remained nearly constant (around 1.50 g/L) regardless of the strain. The ineffectiveness of this treatment may be due to the rapid exhaustion of the active ingredient, namely the hypothiocyanate ion in the culture medium.

An equivalent inhibitory effect was observed with LPS, TBZ and chlorinated water treatments on strains C 52 and C 62. The LPS also had an inhibitory effect on the growth of strain C 46.12, but this effect was less important than that of TBZ and chlorinated water treatments.

Le Nguyen *et al*. (*18*) showed that at pH=5.5, LPS had a significant inhibitory effect on the growth of two mango pathogens, *Colletotrichum gloeosporioides* and *Botryodiplodia theobromae*. They showed that the inhibitory effect was due to $OSCN^-$ ions produced by the lactoperoxidase system and not due to the hydrogen peroxide present in the system. Popper and Knorr (*20*) also showed the effect of LPS on other fungi: *Rhodutorula rubra*, *Mucor rouxii*, *Aspergillus niger*, *Byssochlamys fulva* and *Saccharomyces cerevisiae*. Even if the antifungal factor in the two experiments (LPS and Eau Activée®) is hypothiocyanate ion and the enzyme is attached to a support, antifungal efficiency is lower.

Artificial inoculation of banana crowns

After 13 days of treatment, observations were made on the banana clusters. A significant difference was observed between the Baycor treatment and all the other treatments (Fig. 3). For the LPS treatment, the necrotic areas found were smaller than in the control and bananas treated with Eau Activée®, but this difference was not significant. In addition, a difference in cluster colouration was observed with each different treatment applied. Bananas treated with Eau Activée® and the control bananas had the same colouration, corresponding to

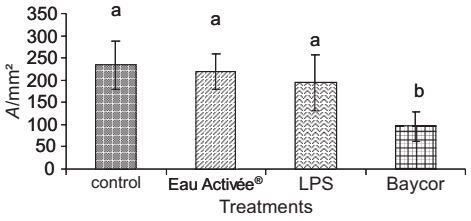

Fig. 3. Average necrotic area of banana crowns inoculated with *Colletotrichum musae* strain C 46.12 and treated with water (1 min), Eau Activée® (20 min), LPS (20 min) and Baycor (1 min). For each treatment, mean values followed by the same letter and number do not differ significantly at $p<0.05$

level 5 on the dessert banana ripeness colour scale. Those treated with LPS and Baycor, on the other hand, had level 4 colouration, which demonstrates an inhibitory effect of LPS on the peel colour change.

Conclusion

Our experiment was carried out to examine the *in vitro* effect of Eau Activée® and LPS on conidial germination and fungal growth of three different *Colletotrichum musae* strains (C 52, C 62 and C 46.12). The artificial inoculation on banana crowns was performed with the strain C 46.12. Regarding the results, different behaviour of the strains was observed in different treatments, and Eau Activée® and LPS did not give the same inhibition of the strains even though they were similar treatments that produce the same active ion. The only difference is in the fact that with Eau Activée® the enzyme is not in contact with the fruit. LPS exhibited similar effectiveness to TBZ and chlorinated water for strains C 62 and C 52.

It would be interesting to know better the mechanism of action of Eau Activée® in order to understand why the same results were not obtained as with the LPS. Moreover, in order to improve and increase the efficiency of LPS, coupling with other substances with antifungal potential can be considered.

Acknowledgements

The authors wish to thank the company TMI Europe (Vaulx-en-Velin, France) for the loan of material for production of Eau Activée® and M. Lebrun for dedicated technical help.

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