Integrated biological control of crown rot of bananas with *Candida oleophila* strain O, calcium chloride and modified atmosphere.

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Abstract

The antagonistic activity of a yeast strain (*Candida oleophila* strain O), was evaluated at three different concentrations (10^6, 10^7, 10^8 cfu/ml) for the biocontrol of crown rot on banana clusters artificially inoculated with *Colletotrichum musae* (10^3 conidia/ml) and a parasitic complex (*C.musae* (10^3 conidia/ml), *Fusarium moniliforme* (10^4 conidia/ml), *Cephalosporium sp* (10^4 conidia/ml). At the concentrations used, *C. musae* was most pathogenic than the fungal complex. Antagonistic effects were observed both on *C. musae* and on the fungal complex. The yeast applied at 10^8 cfu/ml showed the highest protective level (PL of 47-54 %).

In an integrated approach, it has been shown that modified atmosphere packaging (MAP) of fruits (PL of 20%) and the combination of *C. oleophila* strain O at 10^7 cfu/ml with MAP (PL of 21%), significantly reduced crown rot on bananas severely infected with *C. musae*, in contrast with $CaCl_2$ (2%) alone (PL of 6%). The combination of the yeast with $CaCl_2$ (2%) improved the biocontrol activity (PL of 36%), but the highest biocontrol was achieved through the synergistic combination of the yeast, $CaCl_2$ (2%) and MAP (PL of 49%). Interest of this biological control for banana exportation is discussed.

Keywords: banana, crown rot, *Colletotrichum musae*, *Fusarium moniliforme*, *Cephalosporium sp*, biological control, *Candida oleophila*, $CaCl_2$, modified atmosphere.

Résumé

L'activité antagoniste d'une levure (*Candida oleophila* souche O), a été évaluée à trois concentrations (10^6, 10^7, 10^8 cfu/ml) pour le contrôle biologique de la pourriture de couronne sur des bouquets inoculés artificiellement avec *Colletotrichum musae* (10^3 conidies/ml) et un complexe parasitaire (*C.musae* (10^3 conidies/ml), *Fusarium moniliforme* (10^4 conidies/ml), *Cephalosporium sp* (10^4 conidies/ml)). Dans cette gamme de concentrations, *C. musae* a un pouvoir pathogène plus important que le complexe parasitaire. Des effets antagonistes de la levure ont été observés pour le *C. musae* et le complexe fongique. La levure appliquée à 10^8 cfu/ml a permis d'obtenir la meilleure protection biologique (PL de 47-54 %).

Dans une approche intégrée, il a été montré que l'emballage en atmosphère modifiée (MAP) des fruits (PL de 20%) et la combinaison de la levure à 10^7 cfu/ml sous atmosphère contrôlée (PL de 21%), réduisaient significativement les pourritures de couronnes sur des bouquets sévèrement infectés par le *C. musae*, contrairement au $CaCl_2$ (2%) seul (PL de 6%). La combinaison de la levure avec le $CaCl_2$ (2%) améliore encore la protection biologique (PL de 36%), mais la meilleure protection a été obtenue par l'action synergique de la levure, du $CaCl_2$, (2%) et de l'atmosphère contrôlée (PL de 49%). L'intérêt de cette lutte biologique dans le cas de la filière d'exportation est discuté.

Mots clés: banane, pourriture de couronne, *Colletotrichum musae*, *Fusarium moniliforme*, *Cephalosporium sp*, lutte biologique, *Candida oleophila*, $CaCl_2$, atmosphère modifiée.

INTRODUCTION

Crown rot disease of bananas is widespread in producing countries and is considered as the most important post-harvest disease of exported bananas (Muirhead and Jones, 2000). A wide range of organisms are involved in crown rot of bananas, but even if it is not always predominant in this fungal complex, C*olletotrichum musae* is regarded as the most pathogenic

agent (Finlay and Brown, 1993). In most banana-growing areas, crown rot is principally controlled by a postharvest fungicide treatment (Slabaugh and Grove, 1982) combined with sanitation practices. Nevertheless, this chemical control strategy is not always efficient (Krauss and Johanson, 2000) and not sustainable, mainly because of (i) the emergence of resistance to some commonly used fungicides (de Lapeyre de Bellaire and Dubois, 1997); (ii) environmental problems linked to the dumping of fungicide mixtures used in packing stations; and (iii) consumer aversion to chemical residues in food. In such context there is a strong incentive for alternative control methods.

Postharvest biological control is particularly promising because (i) the target area is limited to the fruits; (ii) environmental conditions are defined and stable during storage; and (iii) products for postharvest treatment are a high-value market (Jijakli *et al.,* 1999). The aim of the present study was to evaluate the *in vivo* antagonistic activity of a yeast, *Candida oleophila* strain O, against crown rot of bananas. This approach has been extended to an integrated strategy in combination with (i) an adjuvant, $CaCl_2$, that is known to reinforce its protection level (Jijakli et al., 1993); and (ii) the use of Modified Atmosphere Packaging (MAP) of fruit in a 20µm banavac.

MATERIAL AND METHODS

Fruit sampling

The banana cultivar used was Grande Naine (*Musa acuminata* AAA, Cavendish subgroup). All fruits were harvested at the same physiological age of 900°C.day (Jullien *et al.* 2008).

In the first experiment, 5 homogenous bunches were harvested in Guadeloupe. The 2nd and 3rd hand of these bunches were collected. Each bunch constituted one replicate for the different treatments studied. For the second experiment the 2nd and 3rd hand from 20 bunches were harvested in Cameroon. Each bunch was considered as a block.

Artificial inoculations

Three pathogens frequently observed in the complex were used separately or together: *Colletotrichum musae*, *F. moniliforme*, and *Cephalosporium* sp. Before use, they were grown at 25°C in PDA medium or on modified Mathur culture medium (*C. musae)* for 7 to 10 days. In the first experiment, suspensions were adjusted to 10^4 conidia/ml for *F. moniliforme* and *Cephalosporium sp*. and to 10^3 conidia/ml for *C. musae*. For the parasitic complex, *F. moniliforme*, *Cephalosporium sp*, and *C. musae* were mixed and the respective final concentrations of these species were 10^4, 10^4, and 10^3 conidia/ml. In the second experiment, inoculations were performed with a *C. musae* conidial suspension adjusted to 10^4 conidia/ml.

For all experiments, hands were separated in clusters of 4 fingers randomly attributed to one modality of essay. Smoothly cut crowns were obtained with a sharp knife leaving as much crown tissues as possible. Latex from crown tissues was dried after 30 minutes with absorbent paper and crowns were surface-sterilized by submerging in 50% ethanol. In the first experiment 100 µl of conidial suspension was applied to the centre of the freshly exposed crown tissue and covered with a small paper filter; 50 µl were used in the second experiment.

Preparation of yeast suspensions

In the first experiment, *C. oleophila* strain O was used as a fresh suspension from plate cultures. Before use, the yeast strain was successively subcultured on PDA medium at 20°C and incubated for 24 h. Cells of the third generation were removed by flooding the plates with sterile isotonic solution (NaCl 8.5g/l). In the second experiment, the antagonistic yeast was prepared from cultures grown in a fermentor (biomass) and formulated as dispersible granules. The suspension of the biomass was prepared at 10^7 cfu/ml as following: 1g biomass was rehydrated for 2 hours with a solution of 3 l NaCl at 8.5 g/l.

Experiment 1. Influence of yeast concentration on biological control of crown rot

Antagonistic effects of the yeast strain was evaluated at three different concentrations (10^6, 10^7, 10^8 cfu/ml) against each pathogen separately and against the fungal complex. Thus, for each pathogen and for the complex, 5 treatments were performed on 5 clusters of bananas from 5 different bunches. The treatments were: (UC) untreated control; (FC) thiabendazole 500 mg/l - 1 min; (O6) yeast at 10^6 cfu/ml; (O7) yeast at 10^7 cfu/ml; (O8) yeast at 10^8 cfu/ml. Yeast treatments were applied 3 hours after inoculation of the crowns which were immersed for 10 s in the yeast suspension. One hour after the postharvest treatment, clusters were packed in perforated polyethylene bags, placed in commercial boxes, and stored at 13°C for 10 d. Then artificial ripening was initiated by exposing the bananas to 1000 ppm ethylene for 24 h at 20°C. After strong ventilation they remained at 20°C for another 2 days. This experiment was repeated 6 times for each pathogen and for the complex.

Experiment 2. Integrated biocontrol of crown rot

The efficacy of *C. oleophila* strain O, $CaCl_2$ at 2% and MAP has been evaluated against *C. musae* as stand-alone treatment and in combination. The following post-harvest treatments were compared for crown rot control on artificially inoculated clusters: (a) sterile distilled water, (b) calcium chloride, (c) sterile distilled water + MAP, (d) $CaCl_2$ + MAP, (e) biomass + MAP, (f) biomass + $CaCl_2$, (g) biomass + $CaCl_2$ + MAP, (h) bitertanol solution -0.7 ml/l.

One hour after inoculation, crowns were immersed for 10 s into the various suspensions for post-harvest treatments. One hour after the postharvest treatment, bananas were packed in commercial boxes. Fruits conserved under MAP were packed in banavacs (20µm), since fruits not conserved under MAP were packed in perforated polyethylene bags. Boxes were stored at 13°C for 10 d. Then, bananas were dipped for a short time in a 1ml/l solution of Ethrel (480 g/l) for ripening and placed at 20°C for 3 d. Assays were performed 3 times.

Assessment of crown rot development and statistical analysis

Assessment of rot progression was carried out 13 d after inoculation. The internal progression of rot was determined by cutting the crown longitudinally and measuring the surface of rot in the crown. This "internal necrotic surface" (INS) was measured and expressed in mm^2. From this result a Protective Level (PL) was calculated according to the formula: $PL = [(INS_C - INS_O)/INS_C] * 100$; where: INS_C = mean INS of the untreated control, INS_O = mean INS of the treatment. For each experiment, the mean of the INS was subjected to three-way ANOVA performed by MINITAB. Mean separations were calculated using the Newman and Keuls test at 5% probability level.

RESULTS AND DISCUSSION

Influence of yeast concentration on biological control of crown rot

As compared to the untreated control, all strain-concentration combinations showed a significant and similar antagonistic effect against *C. musae* (Fig. 1.A), *F. moniliforme*, (Fig. 1.B), and the fungal complex (Fig. 1.D), the PL ranging from 33,4 to 54,4 %. The Higher concentration provided always the best protection.

The agents of the complex do not have the same pathogenic capacity. Although inoculated at 10-fold lower concentration (10^3 conidia/ml), *C. musae* was by far more aggressive than *F. moniliforme* and *Cephalosporium* sp. (Figure 1A-C). None of the biological treatments controlled any of the three pathogens or their combination as much as the fungicide treatment (PL between 96.9% and 99.5%) (Fig.1A-D). Further experiments were then conducted to reinforce the PL offered by the biological treatment (Experiment 2).

Integrated biocontrol of crown rot with C. oleophila strain O, calcium chloride and MAP

While $CaCl_2$ alone had no significant effect for crown rot control, the combination of *the yeast* with $CaCl_2$ resulted in a significant enhancement of biocontrol activity (Figure 2). This ability of $CaCl_2$ to improve the biocontrol activity of antagonistic yeasts has been widely

demonstrated (Jijakli *et al*., 1993; Droby *et al.*, 1997). MAP packaging in banavacs of 20 µm effectively reduced crown rot during storage (PL of 20%). Under MAP, the addition of $CaCl_2$ to the yeast suspension appeared to be essential for crown rot control, and the best protection was achieved for the combination of the yeast with calcium chloride and MAP (PL of 49%). Nevertheless, fungicide control of crown rot was stronger than any of the biological treatments tested (PL of 99%).

CONCLUSION

In the biocontrol assays, the PL of crown rot control acheived by the combination of the yeast with $CaCl_2$ and MAP was not as effective as synthetic fungicides. Nevertheless, in this study, crowns were inoculated with a high conidial inoculum of *C. musae* which is a very selective test particularly because *C. musae* is considered as the most pathogenic fungi of the crown rot complex (Finlay and Brown, 1993). Furthermore, the inoculation of *C. musae* alone created more severe inoculation conditions than the inoculation of a fungal complex, because of the natural antagonism existing among the different pathogens of the complex causing crown rot of banana. Consequently, the consistency of the results obtained with this yeast combined with $CaCl_2$ and MAP under conditions most conducive for the development of crown rot indicate that this integrated biological control has great potential for crown rot in the conditions of natural contaminations.

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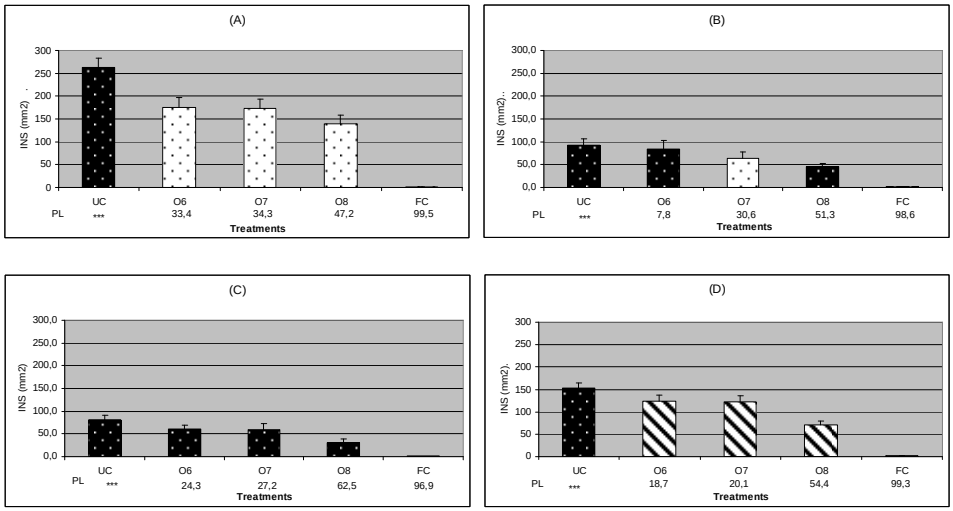

Figure 1. Internal Necrotic Surface (INS) assessed on banana clusters inoculated with (A) *C. musae*, (B*) F.. moniliforme*, (C) *Cephalosporium sp*., and (D) an artificial complex composed of these three pathogens, after treatment with *C. oleophila* strain O at various concentrations. Results with no statistical difference ($P > 0.05$) are represented in the same colour. Protective Levels (PL) acheived by each treatment are indicated.

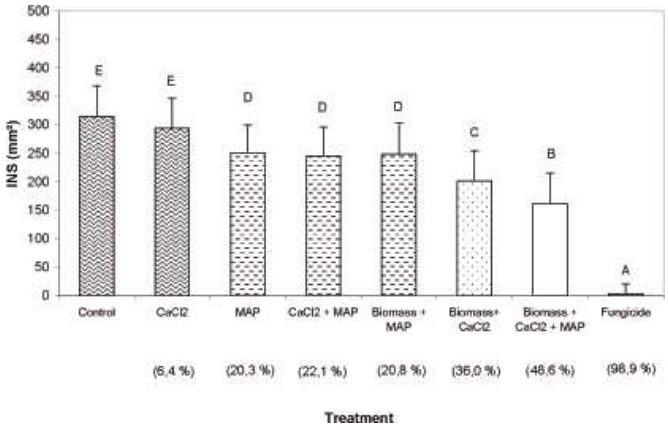

Figure 2. Internal Necrotic Surface (INS, mm²) on banana clusters (20 clusters/treatment) inoculated with *C. musae* (10^4 conidia/ml) and subjected to treatments of *C. oleophila* strain O (1g biomass/3l), 2% calcium chloride and/or modified atmosphere packaging of fruit (MAP) in banavac of 20 µm after 10 days of storage at 13°C and artificial ripening of fruits. Fruits treated with sterile distilled water (Control) or bitertanol at 0.7 ml/l (Fungicide) were used as control. Histograms with different letters are statistically different at the significance level of 0.05 according to the Newman and Keuls test. Standard deviations are represented on each histogram. Assays were performed three times.