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Extraction of Antifungal Substances from *Burkholderia cepacia* with Antibiotic Activity against *Colletotrichum gloeosporioides* on Papaya (*Carica papaya*)

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ABSTRACT

An experiment was conducted to extract and determine the nature of antifungal substances produced by *Burkholderia cepacia* strain B23 that were inhibitory towards *Colletotrichum gloeosporioides.* In addition, the effect of different culture media on the production of antifungal substances by *B. cepacia* B23 was also investigated to have improved efficacy of this biocontrol agent. *B. cepacia* B23 grew faster in nutrient broth medium and the cell concentration in this liquid medium reached the highest level at 72 h after inoculation. Consequently, this strain of *B. cepacia* produced more antifungal substances in a nutrient broth medium than other tested media. Higher dilution (1:8) of the antifungal substances in crude supernatant from *B. cepacia* B23 was found to inhibit the mycelial growth and spore germination of *C. gloeosporioides* by 41% and 100%, respectively. Pyrrolnitrin and other antifungal compounds were detected on TLC plates, which were thermostable. Improvement in biocontrol efficacy may be possible by adjusting cultural conditions to optimize the production of antifungal substances by *B. cepacia* B23.

Key Words: *Burkholderia cepacia*; Antifungal substances; Biocontrol; *Colletotrichum gloeosporioides*; Papaya

INTRODUCTION

Anthracnose of papaya caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. is the most important postharvest disease of papaya in Hawaii and many tropical countries, where it is grown (Paull *et al*., 1997). Control of this disease is usually achieved by hot water treatment, heat or chemical fungicides (Couey & Farias, 1979; Couey *et al.*, 1984). However, in papaya, heat treatment leads to enhanced senescence, while chemical treatments can cause damage that diminish postharvest quality of fruit (Lay-Yee *et al*., 1998).

Elucidating non-chemical control methods to reduce postharvest decay is becoming increasingly important. Consumers are demanding less chemical residue on produce and many fungi are developing resistance to commonly used fungicides (Conway *et al*., 2004). Moreover, the use of chemical fungicides is becoming more restricted due to environmental and health concerns (Mari & Guizzardi, 1998; Janisiewicz & Korsten, 2002). It is therefore necessary to develop alternatives to synthetic fungicide to reduce environmental risks and raise consumer confidence.

Naturally occurring antagonists on host surfaces are a promising component of biological crop protection (De Costa & Erabadupitiya, 2005). Biological control using antagonistic bacteria has been successful in controlling many postharvest pathogens as well as it is environmental friendly. *Burkholderia cepacia* has shown antagonistic activity against a broad range of plant pathogens (Jayaswal *et al*., 1990; Cartwright & Benson, 1995). The bacterium is known to produce a wide range of secondary metabolites such as pyrrolnitrin, phenazine, cepabactin and other unidentified volatile or non-volatile compounds (Roitman *et al*., 1990; Cartwright *et al*., 1995). Several studies suggested that pyrrolnitrin production by *B. cepacia* as well as other *Pseudomonas* spp, is closely associated with biocontrol of plant diseases (Burkhead *et al*., 1994; Cartwright *et al*., 1995; Hwang *et al*., 2002). In this study, *B. cepacia* strain B23, locally isolated from fructosphere of papaya, was used as a biocontrol agent. This strain may be different from other strains of *B. cepacia* in respect of antibiotic production as well as biocontrol activity. It is reported that isolates with same phenotype may differ significantly in their genotype and biocontrol activity (Schena *et al*., 1999). It is therefore, necessary to test the potentiality of this local strain to produce antifungal substances and biocontrol activity against *C. gloeosporioides*.

Natural compounds produced by antagonistic bacteria are generally regarded as the source of biopesticide and special conditions are required for extraction and optimization of antibiotic metabolite substances from antagonistic bacteria (Dikin *et al*., 2005). Optimization of antibiotic production by biocontrol agents may enhance their capacity to control diseases. Cultural conditions such

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as nutritional factors, temperature and pH of the culture media can affect the growth of biocontrol agents and accumulation of antibiotics (Roitman *et al*., 1990; Slininger & Jackson, 1992). The objective of this study was to select a suitable culture medium and optimal conditions for the production of antifungal substances by a local strain *B. cepacia* B23, and to identify the antifungal substances produced by this strain in the selected medium.

MATERIALS AND METHODS

Fungal pathogen. *Colletotrichum gloeosporioides* was isolated from naturally infected papaya fruits using the methods described by Gamagae *et al*. (2003). Pure cultures were maintained on Potato Dextrose Agar (PDA) slants at 28 ± 2˚C. Conidial suspension of *C. gloeosporioides* was prepared (5 x 10^5 conidia mL^{-1}) following the procedure as described by Obagwu and Korsten (2003).

Antagonistic bacterial isolate. A bacterial strain identified as *Burkholderia cepacia*, isolated from fruit surfaces of papaya, and was found to suppress anthracnose of papaya caused *by C. gloeosporioides*, was used in this study as a biocontrol agent. The mode of action of this strain for disease suppression was the production of antifungal substances (Rahman *et al*., 2007).

Selection of liquid culture media for the growth of *B. cepacia* and accumulation of antifungal substances. Five liquid culture media with different nutritional compositions were used in this experiment for extraction of antifungal substances. The media were M1 (13 g L^{-1} nutrient broth), M2 (1 g L^{-1} cassamino acids; 10 g L^{-1} peptone; 10 g L^{-1} mannitol), M3 (1 g L^{-1} cassamino acids; 10 g L^{-1} peptone; 10 g L^{-1} mannitol; 5 g L^{-1} $CaCl_2$. $2H_2O$), M4 (1 g L^{-1} cassamino acids; 10 g L^{-1} peptone; 10 g L^{-1} glucose) and M5 (40 g L^{-1} lactose; 10 g L^{-1} neopeptone). The initial pH of each medium was adjusted to 6.0. The experiment was carried out in 500 mL Erlenmeyer flasks containing 200 mL of medium. Each of the medium was inoculated with 2 mL of a 24 h preculture of *B. cepacia* B23. Inoculated flasks were incubated at 28 ± 2˚C on a rotary shaker at 110 rpm for five days. The antimicrobial activity of *B. cepacia* continued to increase during stationary phase to reach maximal activity at 120 h (El-Banna & Winkelmann, 1998). Based on this result, the incubation period of *B. cepacia* was fixed for five days for the maximum production of antifungal substances. Bacterial growth in each culture medium was measured periodically by spectrophotometer as an increase of optical density at 600 nm. After completion of the incubation period, each of the liquid culture was centrifuged at 10,000 rpm for 20 min. The cellular pellet was discarded and the supernatant was acidified to pH 2.5 with 6 M HCl. The solution was autoclaved at 0.75 atm for 10 min and then centrifuged again at 8,000 rpm for another 10 min and the precipitate was again discarded. The supernatant was adjusted to a final pH of 7.0 with phosphate buffer (1 M, pH 8). This solution was designated as crude supernatant (Bernal *et al*., 2002).

The best culture medium for the production of antifungal substances by *B. cepacia* was selected according to the percentage inhibition of radial growth of *C. gloeosporioides* in the agar plate.

Determination of pyrrolnitrin in the crude supernatant from *B. cepacia* on thin layer chromatography (TLC). For this experiment the crude supernatant, extracted from *B. cepacia* in nutrient broth medium was concentrated and dried by rotary evaporation at 56°C (Buchii Rotavapor R-200). The remaining residue was stirred with methanol and filtered through a Whatman No. 50. The filtrate was dried again, redissolved in methanol and further filtered through a 0.45 µm filter (Janisiewicz & Roitman, 1988). This purified preparation was analyzed by TLC [0.25-mm Silica Gel 60 F_{254} (10 x 20 cm), aluminum-backed, Merck] sheets and hexane/acetone system (3:2, v/v). Firstly, retention factor (R_f) values for the compounds in crude supernatant were determined for various solvent system. After that hexane/acetone system (3:2, v/v) was chosen to optimize the locations of the major antibiotic spots. A 100 µL of the extraction was dropped on the edge of TLC plates and air dried in laminar chamber. The edge of the plates was dipped in the mobile phase of hexane/acetone (3:2, v/v). The spots developed on the TLC plates marked with a soft pencil. Visualization of the spots was performed by exposing the developed sheets to air in laminar chamber for 10 min and monitoring under UV-irradiation at 315 nm. Standard Pyrrolnitrin (Sigma) was also used in this experiment for detecting the pyrrolnitrin produced by the *B. cepacia* strain. The R_f value of the standard pyrrolnitrin was recorded as 0.70. Different spots developed on TLC plates were scraped separately to determine their antifungal activities against *C. gloeosporioides*. This experiment was repeated twice.

Determination of the inhibitory activity units (IAU) of the antifungal substances in the crude supernatant. The crude supernatant obtained by liquid culture (in nutrient broth media) of *B. cepacia* was sequentially mixed with molten PDA cooled to 45˚C to get final ratio of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8. PDA plates without amended supernatant served as a control. Each of these dilutions was tested against *C. gloeosporioides* in order to establish the minimum inhibitory concentration of the antifungal substances in crude supernatant. Radial growth of the test fungus on these amended medium was recorded after seven days of incubation at 28 ± 2˚C. The experiment was conducted in a randomized complete design with five replicates. One IAU was defined as the higher dilution of the crude supernatant that inhibits *in vitro* the growth of the target pathogen.

Thermostability of the antifungal substances in the crude supernatant from *B. cepacia*. A 100 mL of crude supernatants were poured in sterilized conical flasks. The flasks were treated as maintained at room temperature (28 ± 2ºC) (T1), heated water bath at 50ºC for 20 min (T2), heated water bath at 100ºC for 20 min (T3) and autoclaved at

121ºC for 20 min (T4). After treatment, each set of supernatant was amended with molten PDA to get final ratio 2:3 (v/v). The amended medium was inoculated with test fungus, incubated for seven days and observed for radial growth.

Statistical analysis. Data were statistically analyzed using SAS software for analysis of variance and when the treatments were significant, means were separated using LSD ($P < 0.05$). The percentage data were transformed into arcsine transformation before subjected to ANOVA (Gomez & Gomez, 1984).

RESULTS

Effect of liquid culture media on the growth of *B. cepacia* and accumulation of antifungal substances. Growth of *B. cepacia* was highly affected by the composition of culture media. In nutrient broth medium, growth rate of the strain B23 was greater for first three days of incubation compared to the growth in other tested media (Fig 1 & 3). Cell concentration in NB medium reached the highest level (5.9×10^{15} cfu mL^{-1}) at 72 h after incubation, whereas maximum cell concentration in other media was observed at 168 h after incubation. No significant difference in cell concentration of *B. cepacia* B23 was found in any culture medium after five and seven days of incubation.

Results of this study revealed that the nutritional compositions of culture media also significantly affected the production of antifungal substances by *B. cepacia*. The production of antifungal substances increased when this strain was inoculated in nutrient broth (NB) medium compare to that of other tested media. Antifungal substances produced in NB medium inhibited the mycelial growth by 82.67% in relation to the control and was significantly higher than other media (Fig. 2).

Determination of pyrrolnitrin in the crude supernatant from *B. cepacia*. Pyrrolnitrin and some other antifungal substances in the crude supernatant produced by *B. cepacia* were assessed by TLC. Four spots with different retention factors (R_f) were detected on TLC plates under UV light, which inhibited the growth of *C. gloeosporioides*. The R_f values of these zones were 0.55, 0.63, 0.70 and 0.77 on plates developed with hexane/acetone (3:2, v/v). Based on the comparison between the standard pyrrolnitrin and active substances produced by *B. cepacia* in NB medium it was confirmed that this strain of *B. cepacia* produced pyrrolnitrin and other antifungal compounds, which may play a major role in controlling anthracnose disease of papaya.

Determination of the inhibitory activity units (IAU) of the antifungal substances in the crude supernatant. From the sequential dilutions of the crude supernatant, volumetric activity against *C. gloeosporioides* revealed that all of the dilutions tested in this experiment significantly inhibited the radial growth of the tested fungus in relation to the control. The inhibition of radial growth recorded ranged from

Fig. 1. Growth curve of *B. cepacia* in different liquid culture media. Values plotted are means of five replicates. Vertical bars represent standard error

M1=13 gL^{-1} nutrient broth; M2= 1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} mannitol; M3= 1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} mannitol; 5 gL^{-1} $CaCl_2$. $2H_2O$; M4 =1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} glucose; and M5 = 40 gL^{-1} lactose; 10 gL^{-1} neopeptone. The initial pH of each medium was adjusted to 6.0

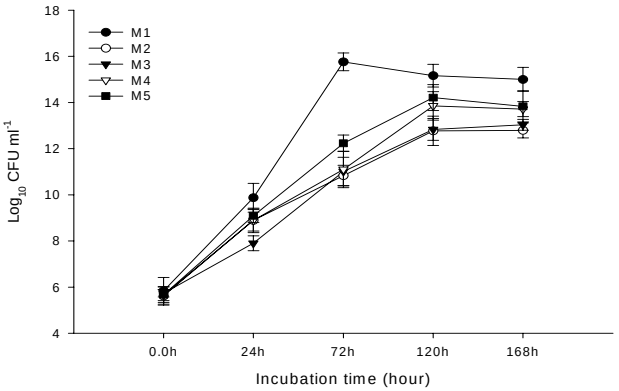
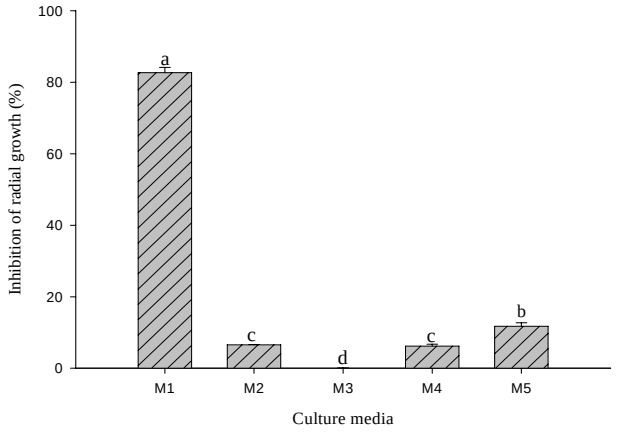


Fig. 2. Inhibition of radial growth of *C. gloeosporioides* by antifungal substances produced by *B. cepacia* in different liquid culture media after 7 days of incubation

Means followed by the different letters are different significantly at 0.05 levels on the arcsine transformed values. Vertical bars represent standard error. M1=13 gL^{-1} nutrient broth; M2= 1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} mannitol; M3= 1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} mannitol; 5 gL^{-1} $CaCl_2$. $2H_2O$; M4 =1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} glucose; and M5 = 40 gL^{-1} lactose; 10 gL^{-1} neopeptone. The initial pH of each medium was adjusted to 6.0



81.75% to 41.33% for the lowest (1:1) and highest (1:8) dilutions of crude supernatant, respectively (Table I). However, spore germination of *C. gloeosporioides* was completely inhibited by minimum inhibitory concentration. Thus, it seemed that spore germination is more sensitive to antifungal substances compare to radial growth.

Thermostability of the antifungal substances in the crude supernatant from *B. cepacia.* Heat treatments did not affect the antibiotic activity of the crude supernatant against *C. gloeosporioides* (Table II & Fig. 4). The

Fig. 3. Growth inhibition of C. gloeosporioides by antifungal substances from B. cepacia produced in different liquid culture media. Photograph was taken after 7 days of incubation at 28 ± 2º C.

M1=13 gL^{-1} nutrient broth; M2= 1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} mannitol; M3= 1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} mannitol; 5 gL^{-1} $CaCl_2$. $2H_2O$; M4 =1 gL^{-1} cassamino acids; 10 gL^{-1} peptone; 10 gL^{-1} glucose; and M5 = 40 gL^{-1} lactose; 10 gL^{-1} neopeptone. The initial pH of each medium was adjusted to 6.0

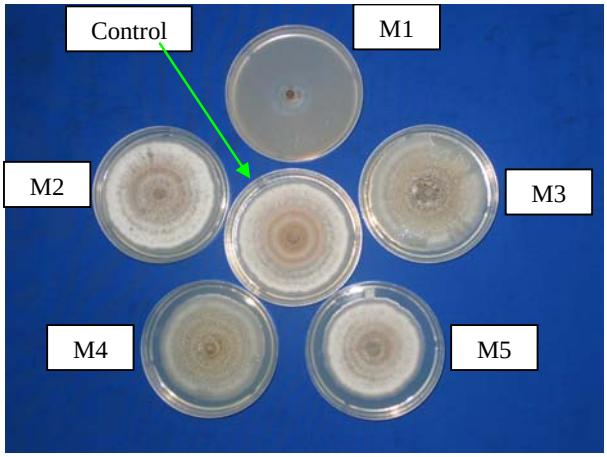
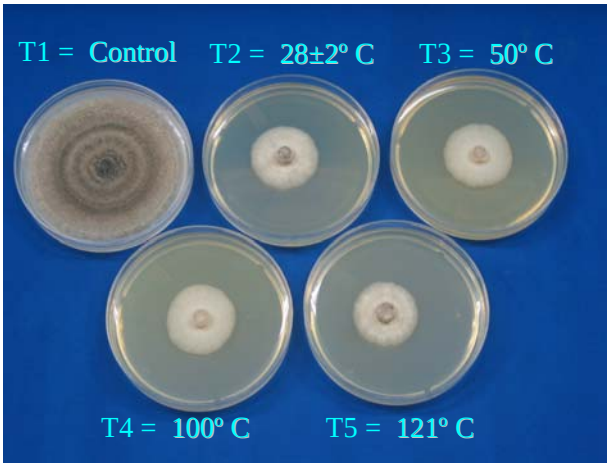


Fig. 4. Effect of different thermal treatments on the antifungal activity of crude supernatant from B. cepacia against radial growth of C. gloeosporioides after 7 days of incubation at 28 ± 2º C.

T1 = Control, T2 = Maintained at room temperature (28±2˚C) for 20 min, T3 = Water bath heated at 50˚C for 20 min, T4 = Water bath heated at 100˚C for 20 min and T5 = Autoclaving at 121˚C for 20 min.



antifungal substances in crude supernatant from *B. cepacia* exposed to different heat treatment significantly inhibited the mycelial growth of *C. gloeosporioides* by about 60% in relation to control growth. However, no statistical difference was found among the treatments. The antifungal compounds produced by the strain were resistant to boiling and to autoclaving at 121°C for at least 20 min. Therefore, the antibiotic produced by *B. cepacia* was thermostable.

Table I. Inhibition of radial growth and spore germination of *C. gloeosporioides* by different concentration of crude supernatant after 7 days of incubation at 28 ± 2ºC

Dilution of crude supernatant	Inhibition of *C. gloeosporioides*	
	Mycelial growth (%)	Spore germination (%)
Control	-	-
1:1	81.7 a	100
1:2	60.0 b	100
1:3	52.6 c	100
1:4	51.0 d	100
1:5	50.0 e	100
1:6	48.1 f	100
1:7	45.3 g	100
1:8	41.3 h	100

In each column, means followed by the different letters are different significantly at 0.05 levels on the arcsine transformed values.

Table II. Effect of the different thermal treatments on the antifungal activity of crude supernatant from *B. cepacia* against the radial growth of *C. gloeosporioides* after 7 days of incubation at 28 ± 2ºC

Treatments	Inhibition of radial growth (%)
T1 = Control	-
T2 = Maintained at room temperature (28±2˚C), 20 min	60.05 a
T3 = Water bath heated at 50˚C, 20 min	60.70 a
T4 = Water bath heated at 100˚C, 20 min	60.70 a
T5 = Autoclaving at 121˚C, 20 min	60.93 a

Means in column followed by the same letter are not different significantly at 0.05 levels.

DISCUSSION

Development of antagonistic microorganisms for biocontrol of postharvest diseases of fruits is advancing (Janisiewicz & Korsten, 2002). *Burkholderia cepacia* and other *Pseudomonas* species are some of the most promising biocontrol microorganisms (Cartwright *et al*., 1995). These bacteria produce many antibiotic substances that can be inhibitory to plant pathogens (Flaishman *et al*., 1990; Harrison *et al*., 1993). In our previous study, a bioassay based on clearing zone on agar plate was used to determine the antibiotic activity of antifungal substances produced by *B. cepacia* strain B23 against *C. gloeosporioidses.* A wider inhibition zone produced by this strain revealed the production of a strong antibiotic (Rahman *et al*., 2007).

Production of antibiotics by biocontrol agents in liquid culture can be affected by several factors such as pH, temperature and composition of the culture medium (Slininger & Shea-Wilbur, 1995). Variations in the fermentation environment often resulted in alteration in antibiotic production. The alteration involves change in both yield and composition of the compound (Upadhyay *et al*., 1991). Modified cultural conditions for *B. cepacia* showed that pH, composition of the culture medium and length of incubation period affected the production of pyrrolnitrin in

liquid culture (Roitman *et al*., 1990). El-Banna and Winkelmann (1998) also reported that antibiotic production by *B. cepacia* NB-1 was greatly influenced by nutritional and environmental factors. By adjusting growth conditions, production of antifungal compounds can be optimized in culture. In this present study we found that the growth rate of *B. cepacia* was affected by types of culture medium. This strain of *B. cepacia* B23 incubated in NB medium reached stationary growth phase at 72 h after seeding flasks. Whereas, cell concentration in all other tested media remained in log growth phase until 168 h of incubation. This result is in agreement with Hwang *et al*. (2002), who observed that the maximum cell concentration of *B. cepacia* in NB medium was reached after three days of incubation.

Findings of the present study revealed that *B. cepacia* produced more antifungal compounds when grown in NB medium compare to other tested media. Roitman *et al*. (1990) reported that pyrrolnitrin accumulated by *Pseudomonas cepacia* at the later stage of cell growth in liquid culture. Frequently, antibiotics are produced only after completion of the growth phase of biocontrol agent (El-Banna & Winkelmann, 1998). Considering these, more antifungal substances produced in NB medium due to faster growth rate of *B. cepacia* during the first 72 h of incubation. It is also reported that many microorganisms can synthesize antibiotics, while in growth phase (Bernal *et al*., 2002).

The initial pH of culture medium affected the production of antibiotics by biocontrol agent. The high productivity of phenazine by *P. florescens* 2-79 was achieved with lower pH (Slininger & Shea-Wilbur, 1995), which is consistent with previous findings by Roitman *et al*. (1990), who observed the increase production of pyrrolnitrin at a lower initial pH of the culture medium. In this study, the initial pH of each of the culture medium was adjusted to 6.0, which may enhanced the production of antifungal substances by *B. cepacia* in NB medium. These findings are supported by Hwang *et al*. (2002), who reported that with pH of 5.8, strain of *B. cepacia* produced more pyrrolnitrin in a NB medium than in a minimum salts medium.

TLC was performed using extracts from an analytical-scale fermentation of *B. cepacia* B23. Pyrrolnitrin exhibited a characteristic fluorescent orange spot on TLC sheets under UV-irradiation (Chernin *et al*., 1996). In this study, four spots were detected on TLC sheets with different R_f values, out of which one spot yielded orange color with R_f value of 0.70, when visualization was performed by exposing the developed sheets to air for 10 min and monitoring under UV-irradiation at 315 nm. Standard pyrrolnitrin also yielded an intense fluorescent orange spot on TLC sheet at the same R_f value. These findings are in agreement with Dikin *et al*. (2005), who reported that antimicrobial substances produced by *B. cepacia* developed five bands on TLC sheets with different R_f. The R_f values they found for the pyrrolnitrin ranged from 0.68-0.72, which was very close to our findings. Although the R_f value was more or less similar to the R_f reported by Arima *et al*. (1964) and Burkhead *et al*. (1994) for pyrronitrin, comparisons with data from other studies (Homma *et al*., 1989; McLoughlin *et al*., 1992; Chernin *et al*., 1996) on pyrrolnitrin revealed differences. These variations in TLC R_f values may be explained by the different silica gel absorbents and the different solvent system used (Burkhead *et al*., 1994; Dikin *et al*., 2005). It is reported that *B. cepacia* produces several antibiotics, most of which have antifungal activity, such as pyrrolnitrin (Cartwright *et al*., 1995; Hwang *et al*., 2002; Dikin *et al*., 2005), phenazine (Cartwright *et al*., 1995) and cepacidine A (Lee *et al*., 1994).

The crude extract, obtained by liquid culture of *B. cepacia* B23 sequentially diluted, amended with PDA and was tested for antifungal activity against *C. gloeosporioides in vitro*. Inhibition of mycelial growth and spore germination was achieved by 41% and 100%, respectively even when the crude extract was diluted up to 1:8. It is reported that pyrrolnitrin produced by *E. agglomerans* was very effective against *B. cinerea*, *R. solani* and *S. rolfsii*, in very low concentration (Chernin *et al*., 1996). The *in vitro* minimum inhibitory concentration for pyrrolnitrin against *Sclerotinia sclerotiorum* is 0.01 µg mL^{-1} (McLoughlin *et al*., 1992). Dikin *et al*. (2006) also reported that the mixed antimicrobial substances produced by *B. cepacia* and *Microbacterium testaceum* were effective against *Schizophyllum commune*, even when the crude supernatant was diluted up to 1:50. Findings of this study revealed that the strain of *B. cepacia* might be able to control the anthracnose disease papaya caused by *C. gloeosporioides* even by producing small amounts of pyrrolnitrin.

Different thermal treatments conducted with supernatants from NB cultures of this strain of *B. cepacia*, demonstrated the thermo resistance of the inhibitory antifungal compounds. Growth inhibition zones produced in *C. gloeosporioides* plates were the same for all supernatants, independent of the thermal treatment to which they were subjected. These results are in agreement with Chernin *et al*. (1996) who reported that pyrrolnitrin, produced by *Enterobacter agglomerans* was resistant to heat treatment. Quan *et al*. (2006) reported that *B. cepacia* strain CF-66 produced an antifungal compound CF66I, which is stable to high temperature, proteolytic enzymes and organic solvents. The mixed antimicrobial substances produced by *B. cepacia* and *Microbacterium testaceum* in liquid cultures were found stable up to 75ºC (Dikin *et al*., 2006). The antimicrobial activity of iturin, an antibiotic substance produced by *Bacillus subtilis* was also reported to resistance to boiling and to autoclaving at 121ºC (Bernal *et al*., 2002).

The above results suggest that the usefulness of *B. cepacia* as a biological control agent for papaya may extend beyond postharvest protection against anthracnose caused by *C. gloeosporioides*. At a time when the use of synthetic pesticide is being curtailed biological control agents such as *B. cepacia* and other organisms, which can effectively inhibit target organisms by competition or by natural low-level antibiotic production are commercially attractive.

CONCLUSION

B. cepacia grew faster in nutrient broth medium, where the cell concentration was highest level at 72 h after inoculation. Simultaneously, this strain produced more thermostable antifungal substances including pyrrolnitrin in nutrient broth. The antifungal substances were strong to suppress *C. gloeosporioides* even at lower concentration.

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