Enhancing the Efficacy of *Burkholderia cepacia* B23 with Calcium Chloride and Chitosan to Control Anthracnose of Papaya During Storage

M. A. Rahman[1]*, T. M. M. Mahmud[2], J. Kadir[3], R. Abdul Rahman[4] and M. M. Begum[1]

[1]*Fruit Research Station, BARI, Binodpur, Rajshahi-6206, Bangladesh*
[2]*Department of Crop Science,* [3]*Department of Plant Protection,* [4]*Department of Food Technology, University Putra Malaysia.*

The efficacy of the combination of *Burkholderia cepacia* B23 with 0.75% chitosan and 3% calcium chloride (CaCl$_2$) as a biocontrol treatment of anthracnose disease of papaya caused by *Colletotrichum gloeosporioides*, was evaluated during storage. The growth of *B. cepacia* B23 in papaya wounds and on fruit surfaces was not affected in presence of chitosan and CaCl$_2$ or combination throughout the storage period. The combination of *B. cepacia* B23 with chitosan-CaCl$_2$ was more effective in controlling the disease than either *B. cepacia* B23 or chitosan or other combination treatments both in inoculated and naturally infected fruits. Combining *B. cepacia* B23 with chitosan-CaCl$_2$ gave the complete control of anthracnose infection in artificially inoculated fruits stored at 14°C and 95% RH for 18 days, which was similar to that obtained with fungicide benocide®. Moreover, this combination offered a greater control by reducing 99% disease severity in naturally infected fruits at the end of 14 days storage at 14°C and 95% RH and six days post ripening at 28±2°C, which was superior to that found with benocide® or other treatments tested. Thus, postharvest application of *B. cepacia* B23 with chitosan-CaCl$_2$ as enhancers represents a promising alternative to synthetic fungicides for the control of anthracnose in papaya during storage.

Keywords : Antagonists, *Burkholderia cepacia* B23, biocontrol efficacy, chitosan, calcium chloride, postharvest pathology, papaya

Papaya (*Carica papaya* L.) is a highly perishable fruit that suffers severe postharvest losses both in terms of quality and quantity. Among the postharvest diseases, anthracnose caused by *Colletotrichum gloeosporioides* is the major disease in tropical countries (Snowdon, 1990). This fungus is also an important postharvest pathogen attacking a wide variety of other tropical and subtropical fruits. Chemical control is a feasible option to control postharvest diseases, however, environmental and health risks are high. Moreover,

*Corresponding author.
  Phone) +88-0155-875-6589, FAX) +88-02-9261495
  E-mail) atiqur_2004@yahoo.com

consumer demand is increasing for fruit which has been non-chemically treated for postharvest pathogen. Therefore, there is a need for alternative disease management practices able to reduce rot development without risk to consumers and workers.

The search for biocontrol strategies against postharvest diseases of fruits and vegetables have been intensified in recent years, particularly with the banning of several pesticides. Success of different natural antagonists such as fungi, bacteria and yeast especially for the control of wound-invading secondary pathogens of fruit has been reported (Janisiewicz and Korsten, 2002). Being a quiescent infection, anthracnose is a postharvest disease which is difficult to control by postharvest treatment (Yakoby et al., 2001). Thus, only a few reports on successful control of quiescent infections by postharvest applications of biocontrol agents have been reported (Koomen and Jeffries, 1993; Korsten and Jeffries, 2000; De Costa and Erabadupitiya, 2005).

In a previous study, the antifungal activity of *Burkholderia cepacia* strain B23 has been demonstrated in petri plate assays (Rahman et al., 2007). *B. cepacia* and other *Pseudomonas* species has also been shown to protect against or decrease the severity of various postharvest diseases of fruits, including postharvest infections of apples and pears caused by *Penicillium expansum* and *Botrytis cinerea* (Janisiewicz and Roitman, 1988), banana infection caused by *Colletotrichum musae* (De Costa and Subasinghe, 1999) and green mold decay of lemons caused by *Penicillium digitatum* (Smilanick and Denis-Arrue, 1992).

Despite the efforts that have been focused on the development of microbial biocontrol agents, their effectiveness as the sole method of controlling postharvest pathogens is reported to be low when compared to that of synthetic fungicides (Droby et al., 1998). However, the efficacy of biological control can be improved by manipulation of the environment and integration with other methods of control (Janisiewicz and Korsten, 2002). Some exogenous substances, such as chitosan, calcium chloride and sodium bicarbonate have been studied to enhance biocontrol capability of antagonists against fungal pathogens (El Ghaouth et al., 2000a; Tian et al., 2002; Gamagae et al., 2003). Among

them, chitosan and its derivatives are known to form a semi permeable film and are inhibitory to a number of pathogenic fungi as well as safe for the consumers and the environment (Wilson and El Ghaouth, 2002).

Calcium chloride has also been proposed as safe and effective alternative means to control postharvest rots of fruit and vegetables. It has been shown that the addition of this salt can improve the efficacy of microbial antagonists against postharvest decay of a variety of fruits in previous works. The biocontrol efficacy of *C. oleophila* against *B. cinerea* and *P. expansum* (Wisnewski et al., 1995) and *Pichia guilliermondii* against postharvest rots of grapefruits and apples (Droby et al., 1997; Scherm et al., 2003) was significantly improved by addition of calcium chloride.

One of the unique characteristics of chitosan-based coating is that it can be used as a carrier for incorporating functional ingredients, such as antimicrobial agents and nutraceutical (Park and Zhao, 2004). Therefore, it have been thought that, combining chitosan and calcium chloride with *B. cepacia* B23 will make it possible to exploit the antifungal and eliciting property of these chemicals and biological activity of *B. cepacia* B23. The objective of this study is to evaluate the biocontrol efficacy of postharvest treatments with a local strain of *B. cepacia* B23 with chitosan and calcium chloride as enhancers to control anthracnose of papaya during storage.

Materials and Methods

Fungal culture and preparation of conidial suspension of *C. gloeosporioides*. *C. gloeosporioides* was isolated from naturally infected papaya fruits following the procedures described by Bautista-Banos et al. (2003). Purified cultures were maintained on Potato Dextrose Agar (PDA) slant at room temperature (28±2 °C). Isolate of *C. gloeosporioides* was grown on PDA medium at 28±2 °C for seven days. Spores were subsequently harvested by flooding the surface of the media with sterilized distilled water containing 0.1% Tween 80 (v/v) and gently agitated the plate with a bent sterilized glass rod to dislodge the spores. The resulting suspension was filtered through two layers of sterile muslin cloth. The concentration of conidia in the filtered suspension was adjusted to 1×10^5 conidia mL^{-1} with sterile distilled water using a haemacytometer.

Preparation of aqueous suspension of *B. cepacia* B23. A local strain of *Burkholderia cepacia* B23, isolated from papaya fruit surface, was used as a biocontrol agent in this study. To prepare aqueous antagonist suspension, isolate was grown on nutrient agar (NA) plate at 28±2 °C for 24 h. A colony was then transferred to a 250 mL Erlenmeyer flask containing 50 ml of sterilized nutrient broth (NB), and

incubated on a rotary shaker at 150 rpm for 48 h at 28±2 °C. The isolate was re-cultured in fresh NB and incubated for another 72 h before used. At the time of use, the cell concentration of *B. cepacia* B23 in the suspension was adjusted to approximately 1×10^9 CFU mL^{-1} with sterilized distilled water using spectrophotometer at 600 nm.

Preparation of chitosan solutions. In a preliminary study, it was found that *B. cepacia* B23 was compatible with 0.75% or less concentration of chitosan solutions. Based on this result, 0.75% chitosan was used in this study. One hundred milliliters of 0.75% chitosan solution was prepared, where 0.75 g of chitosan (Shrimp shell chitosan, Chito-Chem (M) Sdn Bhd, Malaysia) was dissolved in 75 mL of distilled water added with 2 mL of glacial acetic acid. The mixture was heated with continuous stirring for proper dissolution of chitosan. The final pH of the solution was adjusted to 5.6 with 2 M NaOH and volume made up to 100 mL with sterilized distilled water. To improve the wettability, 0.1 mL of Tween 80 was added to the solution (Jiang and Li, 2001).

Effect of calcium chloride, chitosan and their combination on fruit colonization by *B. cepacia* B23. The effect of chitosan, $CaCl_2$ and their combination on the population of *B. cepacia* B23 in papaya wounds and on fruit surface was determined immediately after treatment and at the end of 18 days storage. Papaya fruits were wounded (3 mm deep and 5 mm diameter) with a sterilized cork borer and five wounds made on each fruit. Six of the wounded fruits were dipped for 15 min in each solution/suspension as follows: i) aqueous suspension of *B. cepacia* B23 (10^9 CFU mL^{-1}); ii) 3% $CaCl_2$ solution followed by aqueous suspension of *B. cepacia* B23; iii) aqueous suspension of *B. cepacia* B23 followed by 0.75% chitosan solution; and iv) aqueous suspension of *B. cepacia* B23 followed by 0.75% chitosan solution amended with 3% $CaCl_2$. Each of the treated fruit was sleeved with Styrofoam netting, packed in a commercial packaging and held at 14 °C and 95% RH for 18 days. At each sampling time, wounded tissues and peel samples (1 cm^2 piece^{-1}) were removed with a sterilized cork borer (6 mm in diameter) and a sharp knife, respectively from the three fruits selected randomly from each treatment. Each of the five wounds and five pieces of peel samples from the same fruit were ground with mortar and pestle in 5 mL of 0.05 M phosphate buffer (pH 7) (El Ghaouth et al. 2000a). Serial tenfold dilution were made and 100 µL of each dilution was spread on NA plates. The inoculated plates (three plates for each replicate) were incubated at 28±2 °C. Bacterial colonies were counted after 48 h and the results were expressed as the mean number of log_{10} CFU wound^{-1} or cm^{-2}.

Biocontrol activity of *B. cepacia* B23 enhanced with chitosan and calcium chloride on papaya fruits pre-inoculated with *C. gloeosporioides*. Papaya fruits at color stage two (mature green with trace yellow) were used in this study. The fruits were obtained from an exporter (Seng Chew Hup Kee (M) Sdn Bhd, Kajang, Selongor, Malaysia) at the same day of harvesting. A total of 96 fruits were surface sterilized with 75% ethanol followed by rinsed with distilled water and wounded (3 mm deep and 5 mm diameter) with a sterilized cork borer. Two wounds were made at the mid region of each fruit with seven centimeters apart from each other. Wounded fruits were then inoculated with conidial suspension of *C. gloeosporioides* (50 μL wound^{-1}) and held at 28±2°C for 2 h. Each of the 12 inoculated fruits were then dipped for 15 min in i) aqueous suspension of *B. cepacia* B23 (10^9 CFU mL^{-1}); ii) 3% CaCl$_2$ solution and iii) 0.75% chitosan solution. In the combination treatment of CaCl$_2$ and bacterial suspension, 12 inoculated fruits were initially dipped in 3% CaCl$_2$ solution for 15 min and allowed to air dry for 5 min. The fruits were then immersed in aqueous solution of *B. cepacia* B23 for another 15 min. In another combined treatment, 24 inoculated fruits were initially immersed in bacterial suspension for 15 min and allowed to air dry for 5 min. Each 12 of them were then immersed either in 0.75% chitosan solution or in 0.75% chitosan amended with 3% CaCl$_2$ solution for another 15 min. The control treatment consisted of a set of 12 inoculated fruits that were immersed either in sterilized distilled water or commercial fungicide, benocide® (benomyl 50% WP)≅0.33 g L^{-1} acted as negative and positive controls, respectively. Fruits were allowed to air dry for 5 min after treatment. Each fruit was sleeved with Styrofoam netting, packed in a commercial packaging and held at 14°C and 95% RH for 18 days. Data on lesion diameter were recorded each alternate day started on day tenth after inoculation.

Biocontrol activity on naturally infected papaya fruits. Fully matured papayas with similar color stage were obtained from the distributor previously described. Fruits were surface sterilized with 75% ethanol followed by rinsed with distilled water and air dried. A set of the 12 non-inoculated fruits were dipped for 15 min in (i) aqueous suspension of *B. cepacia* B23 (10^9 CFU mL^{-1}) and (ii) 0.75% chitosan solution amended with 3% CaCl$_2$. In the combination treatment, 12 fruits were initially immersed in bacterial suspension for 15 min and allowed to air dry for 5 min. The fruits were again immersed in 0.75% chitosan solution amended with 3% CaCl$_2$ for another 15 min. The control treatments were as described previously. These treatments were selected based on the results of the previous experiment. Treated fruits were then stored for 14 days,

where package and storage conditions were the same as describe before. At completion of storage time, fruits were removed from storage and ripened with calcium carbide at room temperature (28±2°C). Ten gram calcium carbide was placed in each box for 24 h. Calcium carbide was then removed and the fruits were allowed to ripen at room temperature for another six days.

Data on anthracnose incidence and severity were recorded everyday started on third day of post-storage, when disease symptoms began to appear in ripened fruits. Data on disease severity (DS) was recorded using a scale of 0-4, where, 0=no disease symptom, 1=1-10%, 2=11-20%, 3= 21-30% and 4=31% or more diseased area on the fruit surface (Illeperuma and Jayasuriya, 2002). Disease severity was expressed according to the following formula (Singh, 1984):

$$DS(\%) = \frac{\Sigma(\text{Severity rating} \times \text{number of fruits in that rating})}{\text{Total number of fruits assessed} \times \text{highest scale}} \times 100$$

Reduction in disease severity as a measure of the effectiveness of treatments in suppressing disease progress was expressed as area under the disease progress curve (AUDPC). The AUDPC was calculated using the formula (Campbell and Madden, 1990):

$$AUDPC = \sum_{i}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right)(t_{i+1} - t_i)$$

Where,
n = number of assessment
y = Disease severity
t = time

The disease progress rate was obtained from the slopes of the regression lines. The slopes of the curves were obtained by transforming the disease severity data using the logistic model $Y = B1/\{1 + B2 \exp(-B3X)\}$ (Campbell and Madden, 1990), where Y represented disease severity, $B1$ represented the asymptotic stabilization of the curve, $B2$ was the rate of disease progress and X was days. This model was fitted to the severity values by non-linear regression analyses using sigma plot software version 8.02.

Experimental design and statistical analysis. For *in vitro* and *in vivo* studies, experiments were arranged in a completely randomized design, three replications for *in vitro* and 12 for *in vivo* studies per treatment. All data were analyzed using the statistical software SAS version 8.1. An arcsine transformation was applied to percentage data prior to Analyses of Variance (ANOVA). Significant differences among treatment means were determined using Tukey's Studentized Range (HSD) Test.

Results and Discussion

Effect of chitosan and calcium chloride on fruit colonization by *B. cepacia* B23.

Burkholderia cepacia B23 multiplied in papaya wounds and on fruit surface in the presence of chitosan or chitosan amended with $CaCl_2$. The initial concentration of *B. cepacia* B23 in the bacterial suspension was approximately 1×10^9 CFU mL^{-1}. This resulted in bacterial numbers in wounds and on fruit surface of 4.81 log CFU $wound^{-1}$ and 4.69 log CFU cm^{-2}, respectively, immediately after dipping the fruits in the cell suspension of *B. cepacia* B23, which were not significantly different ($P<0.05$) from other treatments (Table 1). After 18 days of storage at 14°C and 95% RH, the population in wounds markedly increased in all treatments. However, the population reached the maximum level of 6.29, 6.06 and 5.64 log CFU $wound^{-1}$ in treated wounds with *B. cepacia* B23-chitosan, the combination of *B. cepacia* B23-chiosan-$CaCl_2$ and *B. cepacia* B23-$CaCl_2$, respectively, which were not significantly different ($P<0.05$) from each other.

Likewise, at the end of the storage period, significantly higher population (5.49 log CFU cm^{-2}) was recorded on fruit surface dipped in the bacterial suspension followed by chitosan coating (Table 1). This was similar to the combined treatment *B. cepacia* B23-chitosan-$CaCl_2$ (5.36 log CFU cm^{-2}). The number of bacterial population on fruit surface slightly decreased when fruits were treated with *B. cepacia* B23 alone or in combination with $CaCl_2$ with the population of 4.25 and 4.57 log CFU cm^{-2}, respectively, which was not significantly different ($P<0.05$) from each other.

Rapid colonization of wounds by the microbe is necessary for it to be a potential antagonist. El Ghaouth et al. (2000a) reported that *Candida saitoana* grew equally well on surfaces and wounds of apple in the presence of glyco-chitosan. Similarly, in this study, both chitosan and $CaCl_2$ had no adverse effect on the growth of *B. cepacia* B23, when tested *in vitro*. Several studies showed that *Pseudomonas* sp. is compatible strain to combine with chitosan (Rhoades and Roller, 2000; Simpson et al., 1997), which

strongly supported the present finding. On papaya surfaces and wounds, *B. cepacia* B23 grew well in the presence of chitosan or chitosan-$CaCl_2$. This result might be due to filmogenic property of chitosan that caused a modified atmosphere environment around the fruits, thus created more favorable conditions for the survival and multiplication of *B. cepacia* B23. Furthermore, during the whole storage period, relative humidity was maintained at 90-95% to avoid desiccation. In these conditions, water was not a limiting factor and the presence of $CaCl_2$ should not have any negative impact on the antagonist survival (Ippolito et al., 2005), thus making it possible to exploit the additive and synergistic effects of biocontrol agents. This finding was corroborated with results found by Gamagae et al. (2004), who observed that wax coating create and a modified atmosphere around the papaya fruits that resulted in an increased recovery of *Candida oleophila* and significantly reduced anthracnose incidence and severity during storage.

Biocontrol activity on papaya fruits pre-inoculated with *C. gloeosporioides*.

The lesion diameter of anthracnose in inoculated fruits for all treatments was reduced significantly, when compared to the water-treated control (Fig. 1). The combination treatment of *B. cepacia* B23 with chitosan-$CaCl_2$ had greater effect in controlling decay than treatments with bacterial suspension or chitosan or other combinations tested. After 18 days of storage, no lesion developed in inoculated papaya treated with combination of *B. cepacia* B23-chitosan-$CaCl_2$. The level of disease control with this combination was comparable to that obtained with the fungicide benocide®. On the other hand, the highest lesion diameter (4.86 cm) was recorded in water-treated control fruits. Pre-inoculated fruits treated with *B. cepacia* B23, $CaCl_2$+*B. cepacia* B23 and chitosan solution had the lesion diameters of 2.20, 2.23 and 2.25 cm respectively, after 18 days of storage, which were not significantly ($P<0.05$) different from each other. Calcium chloride alone had a little effect in reducing the lesion diameter on inoculated fruits.

Table 1. Populations of *B. cepacia* B23 in papaya wounds and on surface in presence of chitosan, calcium chloride and combined during storage at 14°C and 95% RH for 18 days

Treatments	Wound population (Log CFU $wound^{-1}$)		Surface population (Log CFU cm^{-2})	
	Immediately after treatment	At the end of 18 days storage	Immediately after treatment	At the end of 18 days storage
B. cepacia alone	4.81 a*	5.48 b*	4.69 a*	4.25 b*
B.cepacia + $CaCl_2$	4.94 a	5.64 ab	4.73 a	4.57 b
B.cepacia + Chitosan	4.52 a	6.29 a	4.46 a	5.49 a
B. cepacia + chitosan + $CaCl_2$	4.63 a	6.06 a	4.49 a	5.36 a

*Means in each column followed by the same letter (s) are not significantly different at $P \leq 0.05$ according to Tukey's Studentized Range (HSD) Test.
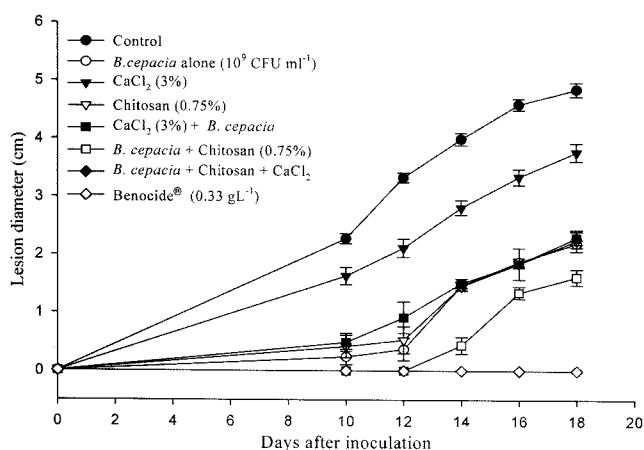
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—●— Control
—○— *B.cepacia* alone (10⁹ CFU ml⁻¹)
5 —▼— CaCl₂ (3%)
—▽— Chitosan (0.75%)
—■— CaCl₂ (3%) + *B. cepacia*
4 —□— *B. cepacia* + Chitosan (0.75%)
—◆— *B. cepacia* + Chitosan + CaCl₂
3 —◇— Benocide® (0.33 gL⁻¹)

Lesion diameter (cm)

0   2   4   6   8   10   12   14   16   18   20
Days after inoculation

Fig. 1. Effect of *B. cepacia* B23, chitosan, calcium chloride and combinations on anthracnose lesion development caused by *C. gloeosporioides* in pre-inoculated papaya fruits stored at 14 °C and 95% RH for 18 days. Means were separated by Tukey's test (HSD) at $P<0.05$. Vertical bars represent standard errors of the means.
Note: In this figure, the results of treatment *B. cepacia* + chitosan + CaCl₂ are overlapping with the results with benocide®. This is because of equal in efficacy of these treatments to control the disease throughout the storage.

Findings of the present study are in agreement with De Costa and Erabadupitiya (2005), who reported that *B. cepacia* was very effective in controlling anthracnose, crown rot and blossom end rot of banana and the greater control was achieved by repeatedly dipping in bacterial solution. Similarly, in postharvest trials under commercial conditions, *Pseudomonas flourescens* strain 558 significantly reduced anthracnose development of mango caused by *C. gloeosporioides* (Koomen and Jeffries, 1993). Like some other previous works (Smilanick et al., 1993; Gamagae et al., 2004), it was observed that the level of disease control provided by *B. cepacia* B23, when used alone, was inferior to control provided by Benocide®. However, the addition of

0.75% chitosan amended with 3% CaCl₂ to *B. cepacia* B23 overcame some of the limitation of this antagonist. Efficacy of the biocontrol agents could be enhanced in the presence of chitosan and/or calcium chloride as reported by some workers (Tian et al., 2002; Wilson and El Ghaouth, 2002; Zhang et al., 2005).

The AUDPC was the lowest in the treatment of the combination of *B. cepacia* B23-chitosan-CaCl₂ and benocide® and significantly higher ($P<0.05$) for the water-treated control fruit (32.96) followed by CaCl₂ treatment (20.03) (Table 2).

In this study, although the combination of *B. cepacia* B23-chitosan delayed the onset of lesion development, it could not control the disease. In contrast, the combination of *B. cepacia* B23-chitosan-CaCl₂ provided complete control (100%) of the disease during the whole storage period (Table 2). Therefore, addition of 3% CaCl₂ into the combination of *B. cepacia* B23-chitosan enhanced the antifungal property of chitosan and the biocontrol activity of *B. cepacia* B23. These findings are in agreement with Zhang et al. (2005), who reported that the efficacy of *Cryptococcus laurentii* in controlling grey mold rots of pear was significantly enhanced by the addition of 2% CaCl₂. Fan and Tian (2001) also reported that CaCl₂ not only reduced grey mold and blue mold of apples, but also enhanced the biocontrol activity of *Cryptococcus albidus*. The mechanism, by which CaCl₂ can improve the efficacy of antagonist, is not fully understood. However, McLaughlin et al. (1990) suggested that the effect of calcium was due to some interaction with the biocontrol agent or its metabolic products at the wound site rather than a direct effect of calcium on the pathogen or fruit tissue.

Biocontrol activity on naturally infected papaya fruits. Anthracnose incidence on naturally infected papaya fruits subjected to different treatments is presented in Fig. 2. Di-

Table 2. Effect of *B. cepacia*, chitosan, calcium chloride and combinations on the reduction of anthracnose disease in pre-inoculated papaya fruits stored at 14 °C for 18 days

Treatments	AUDPC ˣ (unit square)	Lesion expansion rate (mm day⁻¹)	Disease control (%)ʸ
Control	32.96a*	3.21a*	–*
B. cepacia (1×10⁹ CFU ml⁻¹)	7.0c	2.68ab	78.76c
CaCl₂(3%)	20.03b	2.78ab	39.22d
Chitosan (0.75%)	8.35c	2.24b	74.67c
CaCl₂(3%) + *B. cepacia*	9.65c	2.42b	70.72c
B. cepacia + Chitosan (0.75%)	2.23d	2.17b	93.23b
B. cepacia + Chitosan (0.75%) + CaCl₂(3%)	0.0e	0.0c	100.00a
Benocide®(0.33 gL⁻¹)	0.0e	0.0c	100.00a

ˣ AUDPC=Area Under The Disease Progress Curve.
ʸ Disease control (%) derived from values of AUDPC.
*Values in each column followed by the same letter (s) are not significantly different at $P<0.05$ according to Tukey's test (HSD) ($n=12$).
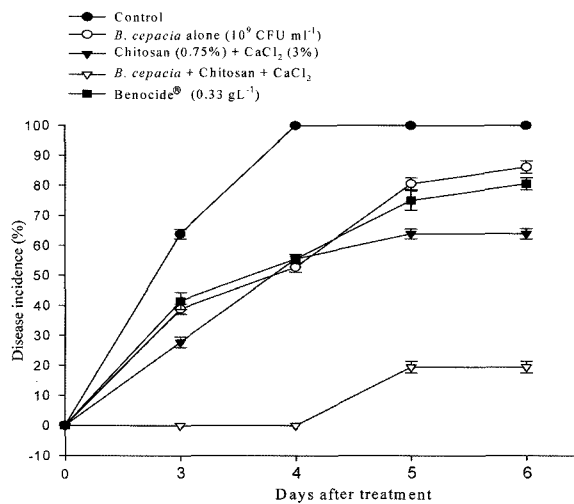
Fig. 2. Effect of *B. cepacia* B23, chitosan, calcium chloride and combinations on the incidence of anthracnose in naturally infected papaya fruits stored at 14 °C for 14 days and six days post ripening under ambient temperature (28±2 °C). Means were separated by Tukey's test (HSD) at $P<0.05$ on arcsine transformed values. Vertical bars represent standard errors of the means.

sease incidence was significantly ($P<0.05$) lower in fruits subjected to all treatments, when compared to water-treated control fruits during 14 days of storage and six days post ripening at ambient temperature (28±2 °C). The combination of *B. cepacia* B23-chitosan-$CaCl_2$ was the most effective treatment, which showed significantly ($P<0.05$) lower incidence on naturally infected fruits than in fruits dipped either in *B. cepacia* B23 suspension, benocide[®] or chitosan-$CaCl_2$. These findings are in agreement with Wilson and El Ghaouth (2002), who found that the combination of *Candida saitoana* with chitosan acetate-$CaCl_2$ was very effective in controlling decay of apple, orange and lemon fruits caused by *Botrytis cinerea*, *Penicillium expansum* and *P. digitatum*. Water-treated control fruits showed anthracnose spots even after the second week of storage followed by increased incidence of 64 and 100% by the third and fourth day of shelf period, respectively. The lowest disease incidence (19%) was recorded in fruits subjected to the combination of *B. cepacia* B23-chitosan-$CaCl_2$ by the end of the ripening period followed by chitosan-$CaCl_2$ (64%). The data showed that the combination of *B. cepacia* B23-chitosan-$CaCl_2$ was not only effective in reducing the disease incidence but also delayed the onset of anthracnose infection. This treatment delayed the anthracnose appearance on fruits by four days compared to water-treated control fruits.

Enhancement of microbial biocontrol agents with chemical and physical additives such as $CaCl_2$, $NaHCO_3$, 2-deoxy-D-glucose, chitosan and heat treatment has been reported by various researchers (El Ghaouth et al., 2000a, 2000b; Gamagae et al., 2004; Huang et al., 1995; Janisiewicz,

1994). Such enhanced biological activity has been attributed to additive and synergistic activities between the additives and the biocontrol agent (El Ghaouth et al., 2000a). Similarly, in the present study the effectiveness of the combination of *B. cepacia* B23-chitosan-$CaCl_2$ might be due to the interplay of the biological activity *B. cepacia* B23 and the antifungal properties of chitosan-$CaCl_2$. The formulation of chitosan-$CaCl_2$ that acted as a carrier for *B. cepacia* B23, a coating which modified the atmospheric compositions surrounding the fruits with reduced O_2 and elevated CO_2, thus delayed ripening process and ultimately suppresses the disease incidence (Ben-Yehoshua et al., 1993).

In this study, all treatments significantly ($P<0.05$) reduced the disease severity when compared to control (Fig. 3). The naturally infected fruits subjected to the combination of *B. cepacia* B23-chitosan-$CaCl_2$ showed lowest anthracnose severity than in fruits dipped in *B. cepacia* B23 suspension, chitosan-$CaCl_2$ and benocide[®]. The combined treatment *B. cepacia* B23-chitosan-$CaCl_2$ reduced the anthracnose severity with complete absence of symptoms until 14 days of storage at 14 °C and four days of post ripening. At the end of ripening period, disease severity was recorded by 1.38% for the fruits subjected to the combination of *B. cepacia* B23-chitosan-$CaCl_2$. Similar trend of anthracnose control in papaya was also achieved with the combined application of 2% sodium bicarbonate in wax formulation and *Candida oleophila* (Gamagae et al., 2004). In contrast, anthracnose symptoms appeared in water-treated control fruits at the end of the 14 days storage and disease severity increased gradually reaching 61% by the end of ripening. By this time
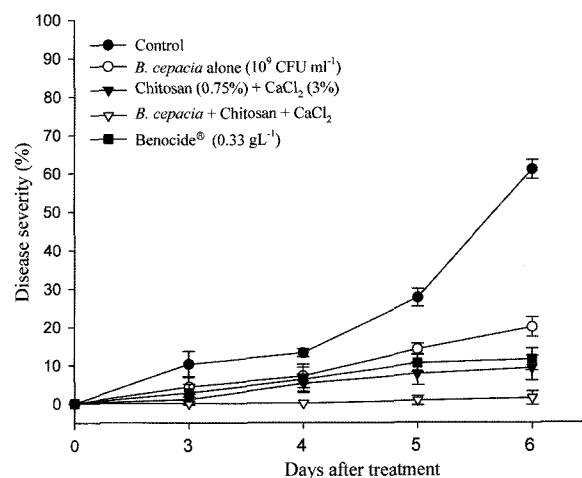

Fig. 3. Effect of *B. cepacia* B23, chitosan, calcium chloride and combinations on the anthracnose severity in naturally infected papaya fruits stored at 14 °C for 14 days and six days post ripening under ambient temperature (28±2 °C). Means were separated by Tukey's test (HSD) at $P<0.05$ on arcsine transformed values. Vertical bars represent standard errors of the means.

Table 3. Effect of *B. cepacia*, chitosan, calcium chloride and combinations on the reduction of anthracnose disease in naturally infected papaya fruits stored at 14 °C for 14 days and six days post ripening under ambient temperature (28±2 °C)

Treatments	AUDPC[x] (unit square)	Lesion expansion rate (mm day^{-1})	Disease control (%)[y]
Control	32.08a*	7.17a*	–*
B. cepacia (1×10^9 CFU ml^{-1})	16.31b	4.73b	49.15b
Chitosan (0.75%) + CaCl$_2$(3%)	9.54b	2.64bc	70.26b
B. cepacia + Chitosan (0.75%) + CaCl$_2$(3%)	0.34c	0.86c	98.92a
Benocide® (0.33 gL^{-1})	12.84b	4.40b	60.0b

[x] AUDPC=Area Under The Disease Progress Curve.
* Values in each column followed by the same letter (s) are not significantly different at $P<0.05$ according to Tukey's test (HSD) ($n = 12$).
[y] Disease control (%) derived from values of AUDPC.

all fruits in control treatment were heavily infected and completely rotten. At the end of ripening, values of disease severities were 20, 11.4 and 9.2% in fruits treated with *B. cepacia* B23 suspension, benocide® and chitosan-CaCl$_2$, respectively.

The efficacy of treatments to delay the onset of disease symptoms as well as to reduce the lesion expansion rate was expressed as percentage disease reduction, which derived from the AUDPC values. At the end of post ripening, the highest AUDPC (32.08) was recorded in water-treated control fruits and the lowest value (0.34) was found in fruits that were subjected to combination of *B. cepacia* B23-chitosan-CaCl$_2$ (Table 3). Other AUDPC values were not significantly different for the treatments of *B. cepacia* B23 suspension, benocide® and chitosan-CaCl$_2$ with the values of 16.31, 12.48 and 9.54, respectively. Similar trend of treatment efficacy was also found in reducing the disease progress rate. The lower performance of benocide® in non-inoculated fruits might be due to the quiescent infection of papaya caused by *C. gloeosporioides*. This is because the infecting hyphae are protected from fungicide once the pathogen has penetrated the plant cuticle (Yakoby et al., 2001) and thus, difficult to control by postharvest treatment (Janisiewicz and Korsten, 2002), even along with synthetic fungicides (Ippolito et al., 2004).

Conclusion

The findings of this study demonstrated that the bio-control activity of *B. cepacia* B23 against anthracnose of papaya was significantly enhanced by the addition of 0.75% chitosan amended with 3% CaCl$_2$. The level of control conferred by the combined treatment *B. cepacia* B23-chitosan-CaCl$_2$ was superior to *B. cepacia* B23 alone or in combination with chitosan or benocide®. Therefore, combining *B. cepacia* B23 with chitosan-CaCl$_2$ can be expected to provide more effective control of anthracnose in papaya.

Acknowledgement

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