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Antimicrobial Activities of Chitosan and Calcium Chloride on *in vitro* Growth of *Colletotrichum gloeosporioides* from Papaya

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ABSTRACT

The antimicrobial activities of chitosan and calcium chloride ($CaCl_2$) on the growth, spore germination, and hyphal morphology of *Colletotrichum gloeosporioides*, isolated from naturally infected papaya fruits, was investigated in in vitro studies. Chitosan was found to inhibit the radial growth and spore germination of the fungus significantly at higher concentrations. Radial growth was inhibited by 52 and 82% with 0.75 and 1% chitosan, respectively. Spore germination was completely inhibited in Potato Dextrose Agar (PDA) medium containing 0.5% and above chitosan concentrations. Light microscope observations showed that chitosan induced morphological changes, including abnormal branching, swelling hyphal tips, vacuolation and distortion. Calcium chloride at 1 to 4% had no pronounced effects on mycelial growth inhibition. However, PDA amended with 3 and 4% $CaCl_2$ significantly ($P \leq 0.05$) inhibited about 26% of spore germination relative to the control. Findings from these experiments demonstrated that chitosan has suppressive activity against *C. gloeosporioides* of papaya and could be used as part of a disease management program. Nevertheless, to a lesser extent, $CaCl_2$ can also be included as part of the program.

Keywords: Non-chemical control, natural compounds, postharvest pathogen, papaya

INTRODUCTION

Papaya (*Carica papaya* L.) is considered one of the most important fruit crops throughout the tropical and subtropical countries with high consumer demand worldwide. The fruits are very susceptible to diseases caused by many microorganisms especially fungi, as they are rich in moisture and nutrients (Sankat and Maharaj, 1997). At postharvest stage, many diseases greatly reduce the storage life and quality of papaya, out of which anthracnose caused by *Colletotrichum gloeosporioides* is the major disease of this fruits in tropical countries (Snowdon, 1990).

Anthracnose in papaya can be controlled by prochloraz or propiconazole (Sepiah, 1993) and hot water dip treatment (HWT) at 43-49°C for 20 min (Couey *et al.*, 1984). However, HWT affect the ripening process in papaya (Paull, 1990) and resistant strains of *C. gloeosporioides* have already been developed against some commonly used fungicides (Bautista-Banos *et al.*, 2003). Furthermore, residues of fungicides present on the fruit may be harmful to consumers. All of these issues prompted an investigation of potential safer approaches to disease management. Recently, biologically active natural products have become an effective alternative to synthetic fungicides as a means to control fungal decay (Spadaro and Gullino, 2004; Tripathi and Dubey, 2004).

Chitosan is a polycationic biopolymer that can be produced industrially by chemical deacetylation of chitin, which is found in arthropod exoskeletons and the cell walls of some plant pathogenic fungi (Hernandez-Munoz *et al.*, 2006). This natural compound is associated with its fungistatic or fungicidal properties against pathogens of various fruits and vegetables (Bautista-Banos *et al.*, 2003). Several studies have shown that growth of some important postharvest fungi such as *Alternaria alternate* and *Fusarium oxysporum* is inhibited on nutrient media

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amended with various concentrations of chitosan (Benhamou, 1992; Bhaskara Reddy *et al.*, 1998). In *in situ* studies, the fungicidal effect of chitosan on strawberries against *Botrytis cinerea* and *Rhizopus stolonifer* has also been reported (El Ghaouth *et al.*, 1992a).

Many organic and inorganic salts have been shown to be active antimicrobial agents against a range of phytopathogenic fungi. In particular, postharvest treatment with $CaCl_2$ has been proposed as safe and effective alternative means to control postharvest rot of fruits and vegetables (Smilanick *et al.*, 1999; Ippolito *et al.*, 2005). Prior studies have shown that the addition of $CaCl_2$ to nutrient media reduced spore germination and germ tube growth of *Rhizopus stolonifer*, *Penicillium expensum* and *Botrytis cinerea* (Wisniewski *et al.*, 1995; Narayanasamy, 2006). However, not much had been reported on using chitosan and $CaCl_2$ as postharvest treatment against anthracnose of papaya especially with cv. Sekaki caused by *C. gloeosporioides*. Therefore, the objectives of this study are to evaluate the effects of chitosan and $CaCl_2$ on *in vitro* growth, spore germination and hyphal morphology of *C. gloeosporioides* of papaya.

MATERIALS AND METHODS

Culture of Fungus and Study Site

Colletotrichum gloeosporioides, the causal agent of anthracnose 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). All the experiments were conducted at the Plant Protection Laboratory, Faculty of Agriculture, Universiti Putra Malaysia in 2007.

Preparation of Chitosan Solutions

One hundred milliliters of 0.1, 0.25, 0.5, 0.75 and 1.0% chitosan solutions were prepared, where 0.1, 0.25, 0.5, 0.75 and 1.0 g of chitosan (Shrimp shell chitosan, Chito-Chem (M) Sdn Bhd, Malaysia) were dissolved in 75 mL distilled water and 2 mL 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 N 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).

Preparation of Conidial Suspension of C. gloeosporioides

Isolates of *C. gloeosporioides* were grown on PDA agar (Merck) at 28±2°C for seven days. Spores were subsequently harvested by flooding the surface of the media with distilled water containing 0.1% Tween 80 (v/v) and the plate was gently agitated 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 x 10^5 conidia mL^{-1} with sterile distilled water using a haemacytometer (Obagwu and Korsten, 2003).

Effect of Chitosan on C. gloeosporioides

The effect of chitosan on the mycelial growth of *C. gloeosporioides* was studied using agar plates. The viscous stock solution of chitosan (1.25%) was autoclaved and subsequently diluted with sterile, molten PDA to obtain chitosan concentrations of 0.1, 0.25, 0.5, 0.75 and 1.0%. Twenty milliliters aliquots of this solution were immediately dispensed into 9-cm-diameter petri plates. Each of the amended PDA plate was inoculated with a 6-mm-diameter mycelial plug taken from the margin of 4-day-old culture of *C. gloeosporioides*. The plates were then incubated at 28±2°C for a maximum period of seven days. The radial measurements of growth were taken after seven days of incubation until the fungus reached the edge of the control plates. The percent inhibition of radial growth (PIRG) was calculated according to the formula described by Sivakumar *et al.* (2000).

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

where, R1 = Radial growth of *C. gloeosporioides* in control plate

R2 = Radial growth of *C. gloeosporioides* interacting with antagonistic bacteria.

Cultures of *C. gloeosporioides* in PDA without chitosan served as the control. To observe the morphological changes, hyphal strands from the end of the fungal colony were removed aseptically and examined under a microscope for abnormalities.

For spore germination test, 100 µl aliquots of the spore suspensions (1x10^5 conidia mL^{-1}) of *C. gloeosporioides* were pipetted onto each PDA plate amended with chitosan and spread with a

10. 70/2008-Rahman 224 24/3/05, 12:00 PM

sterile bent glass rod. Control plates contained PDA only. Inoculated plates were incubated at 28±2°C for 13 h. Data on spore germination were recorded at every 2 h interval starting from 7 h after inoculation. Germination of 100 spores per plate was determined microscopically. A spore was considered to have germinated when the germ tube length equaled or exceeded the length of the spore (El-Ghaouth *et al.*, 1992a).

Effect of Calcium Chloride on C. gloeosporioides

The effect of $CaCl_2$ on mycelial growth and spore germination of *C. gloeosporioides* was also observed on PDA plates amended with different concentrations of $CaCl_2$ (1, 2, 3 and 4%). Experimental design and procedures were the same as described earlier for chitosan except that the incubation period was six days instead of seven days for the measurement of radial growth of the test fungus. The incubation period for spore germination was 7 h unlike 13 h with chitosan.

Experimental Design and Statistical Analysis

All experiments were arranged in a Completely Randomized Design (CRD) with five replications and were performed twice unless stated otherwise. Since most of the data were quantitative and there was no significant variability among the trials, they were pooled. All percentage data were arcsine transformed before subjected to analysis of variance (ANOVA) and means separation was done by the Tukey's Studentized Range (HSD) Test using SAS version 8.1.

RESULTS AND DISCUSSIONS

Effects of Chitosan on C. gloeosporioides

All chitosan concentrations tested significantly ($P \leq 0.05$) inhibited the radial growth of *C. gloeosporioides* after seven days of incubation, with a marked effect at higher concentrations (*Fig. 1*). The highest inhibition of mycelial growth (82%) was recorded with 1% chitosan solution followed by 0.75% chitosan with radial growth inhibition of 52.2%. Growth inhibition ranging from 2.6 to 26.8% was obtained with chitosan at concentrations ranging from 0.1 to 0.5%.

By visual observation, it was found that overall sporulation was lower on PDA plates amended with chitosan when compared with the control. However, no spore was formed on PDA amended with ≥ 0.75% chitosan

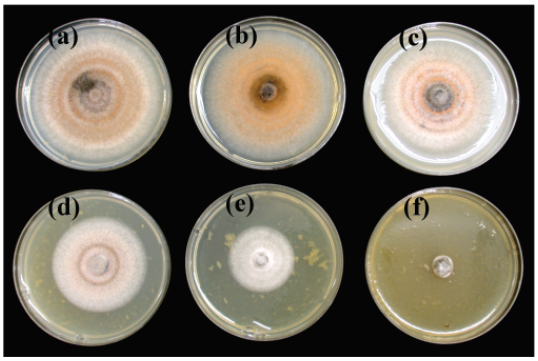

Fig. 1: Effect of different concentrations of chitosan on the radial growth of C. gloeosporioides *after seven days of incubation at 28±2°C.* ***(a)*** *= 0.0% (Control);* ***(b)*** *= 0.1%;* ***(c)*** *= 0.25%;* ***(d)*** *= 0.5%;* ***(e)*** *= 0.75% and* ***(f)*** *= 1.0% chitosan amended with PDA*

concentration after seven days of incubation; whereas, numerous spores were formed on 0.1% chitosan amended medium and in control plates. Chitosan at higher concentrations markedly reduced spore germination of *C. gloeosporioides* (*Figs. 2* and *3*). Spore germination was completely inhibited with ≥ 0.5% chitosan concentrations even after 24 h of inoculation (*Fig. 3e*). Whereas, on PDA plates amended with 0.25% chitosan, germination was inhibited by 75% after 7 h of inoculation (*Fig. 3d*), which decreased considerably with incubation and reached 0.0% inhibition after 11 h of inoculation that was similar to the control. Lowest concentration of chitosan (0.1%) used in this experiment did not inhibit spore germination (*Fig. 3c*).

Chitosan showed a significant influence on growth and sporulation of *C. gloeosporioides* in these *in vitro* experiments. Results of this study showed that *C. gloeosporioides* was very sensitive to chitosan since growth was affected even at lower concentrations including 0.25%. Mycelial growth, spore germination and hyphal morphology were affected by chitosan indicating that it affected various stages of development. Similar results were found by Asgar *et al.* (2004), who worked on Eksotika variety and reported that mycelial growth, spore germination and germ tube elongation of *C. gloeosporioides* were markedly reduced by chitosan with greater effect at higher concentrations. It has been widely reported in the literature that the level of inhibition of fungi is highly correlated with chitosan concentration, indicating that chitosan performance is related

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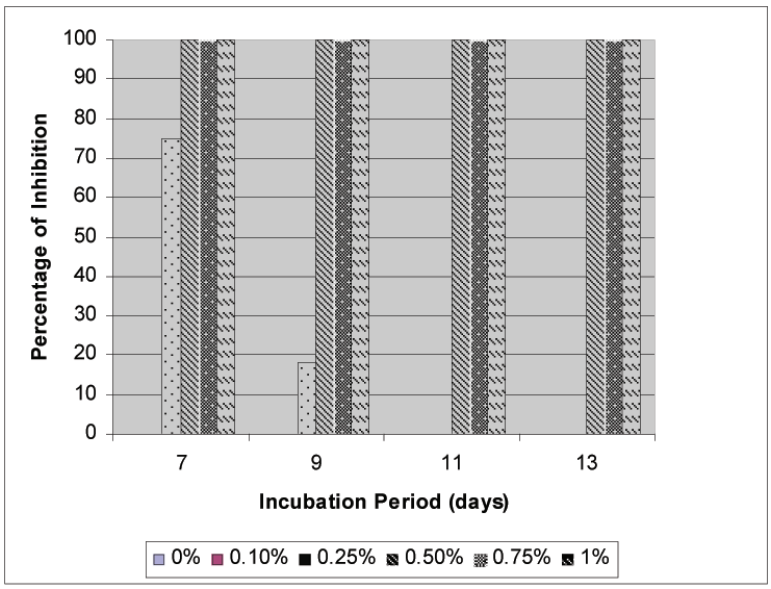

Fig. 2: Effect of different concentrations of chitosan on spore germination of C. gloeosporioides *after 7, 9, 11, and 13 h of inoculation at 28±2°C. Means separation was done on the arcsine transformed values at P≤0.05 according to Tukey's Studentized Range (HSD) Test*

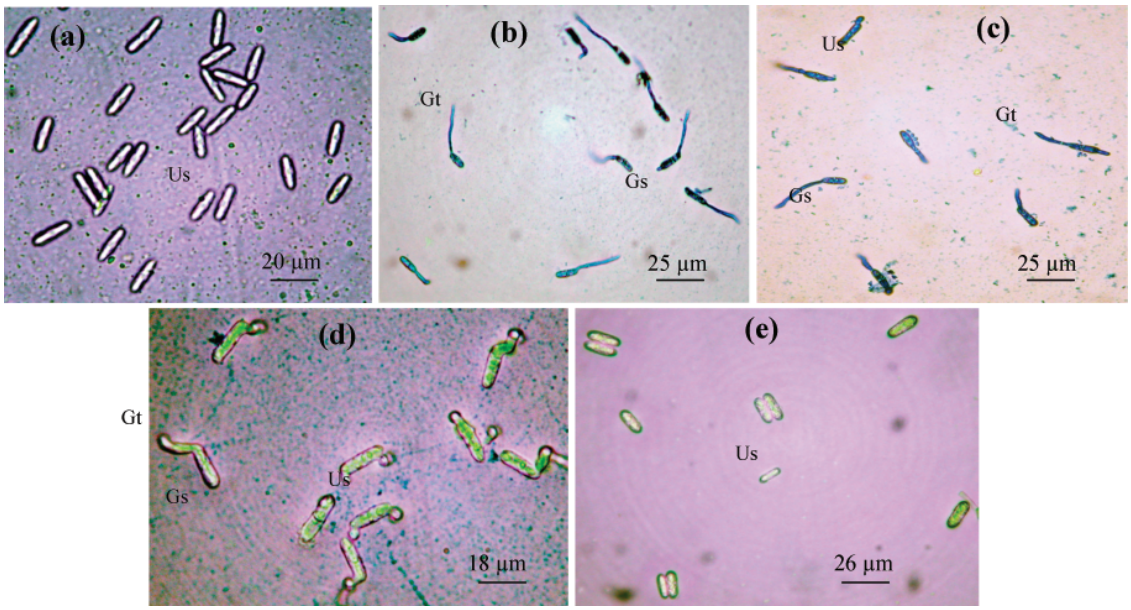

Fig. 3: Effect of different concentrations of chitosan on spore germination of C. gloeosporioides *on PDA after 7 h of incubation at 28±2°C.* ***(a)*** *= Typical ungerminated spores;* ***(b)*** *= Germinated spores in control plate;* ***(c)*** *= Germinated spores with 0.1% chitosan;* ***(d)*** *= Germinated spores with 0.25% chitosan and* ***(e)*** *= Ungerminated spores with 0.5 to 1.0% chitosan. Gs = Germinated spore; Us = Ungerminated spore; Gt = Germ tube*

10. 70/2008-Rahman 226 24/3/05, 12:01 PM

to rate of application. There are strong evidences that mycelial growth can be inhibited or retarded when the growth media of fungi are amended with chitosan. El-Ghaouth *et al.* (1992a) reported that chitosan inhibited spore germination and radial growth of *Botrytis cinerea* and *Rhizopus stolonifer in vitro*. Inhibition of radial growth and spore germination of *B. cinerea* were 95.5 and 98.7%, respectively at chitosan concentration of 6 mg mL^{-1}.

Overall, sporulation of fungi treated with chitosan was generally reported to be lower than in untreated fungi. In this study, no spore was formed at higher chitosan concentrations (0.75 to 1.0%) amended plates, whereas, numerous spores were produced by *C. gloeosporioides* at lower chitosan concentration (0.1%) as well as in control plates. The inhibition of spore formation was also found by other researchers in *F. oxysporum, R. stolonifer, C. gloeosporioides, A. alternate* f. sp. *lycopersici* and *A. niger* (Bhaskara Reddy *et al.*, 1998; Bautista-Banos *et al.*, 2003; Plascencia-Jatomea *et al.*, 2003). Nevertheless, chitosan sometimes stimulates sporulation. It is reported that spore formation of *P. digitatum* when grown on 0.5 and 1.5% chitosan was significantly greater than the control treatment (Bautista-Banos *et al.*, 2006) and suggesting that this high sporulation could be due to a stress response induced by chitosan.

Spore germination of *B. cinerea* was reported to be inhibited by 50% even at lower concentrations of chitosan (20-30 µg mL^{-1}) with complete inhibition at 50 µg mL^{-1} (Ben-Shalom *et al.*, 2003). El-Ghaouth *et al.* (1992a) found that chitosan reduced the spore germination and germ tube elongation of *R. stolonifer* by more than 90 and 75% with 10 mg mL^{-1}chitosan concentration. Similarly, this study showed that spore germination of *C. gloeosporioides* was completely inhibited by chitosan at higher concentrations (0.5 to 1.0%). The long-term fungicidal effect of chitosan can also be related to concentration and incubation time. It was observed that the inhibition of spore germination decreased with incubation time, when grown on PDA amended with 0.25% chitosan. This finding is in agreement with Benhamou (1992) who reported that inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* grown at two of the lowest concentrations (1.0 and 2.0 mg mL^{-1}) decreased with increased incubation time. However, the differences regarding inhibition of mycelial growth and spore germination of fungal pathogens may be due to the molecular weight and concentrations of chitosan used and as well as fungal species response (El-Ghaouth *et al.*, 1992b).

Morphological Changes

Observations on inhibition of fungal growth *in vitro* was carried out further by light microscope investigations (*Fig. 4*). When fungal plugs were deposited on chitosan-free agar, mycelial growth was found to be regularly septate in which hyphae were branched at diverging angles (*Fig. 4a*). In contrast, noticeable morphological changes occurred in hyphae when the fungus was grown on chitosan amended agar medium. At all concentrations, except 0.1%, hyphae growth was abnormal in shape and many hyphal tips were swollen (*Fig. 4c*). A greater degree of contortion was observed at chitosan concentrations of 0.5 to 1.0%, where significant increase in hyphal vacuolation and shriveling (*Fig. 4d*), coiling of hyphae and hyphal tips (*Fig. 4e* & *4f*) and abnormal branching (*Fig. 4b*) were observed.

Previous studies have shown that chitosan is not only effective at halting the growth of pathogens, but also induces marked morphological changes, structural alterations and molecular disorganization of fungal cells (El Ghaouth *et al.*, 1992b; Ait Barka *et al.*, 2004). In this study, microscopic observation of *C. gloeosporioides* treated with chitosan showed that it can affect the morphology of the hyphae. Hyphal tips of the fungus became malformed, and hyphae were thickened and vacuolar compared with hyphae in the control plate. Many swellings occurred in the hyphae, whereas normal hyphal walls were smooth with no swellings or vacuolation. These findings are in agreement with El Ghaouth *et al.* (1992b) who reported that chitosan not only inhibited the radial growth of major postharvest pathogens, but also induced severe morphological alterations in *Rhizopus stolonifer* and *Botrytis cinerea*, as well as increased cellular leakage in both fungi, presumably by interfering with fungal plasma membranes. Other observations carried out on fungi such as *F. oxysporum* f. sp. *radicis-lycopersici, R. stolonifer* and *S. sclerotiorum* treated with chitosan, showed excessive mycelial branching, abnormal shapes, swelling, and hyphal size

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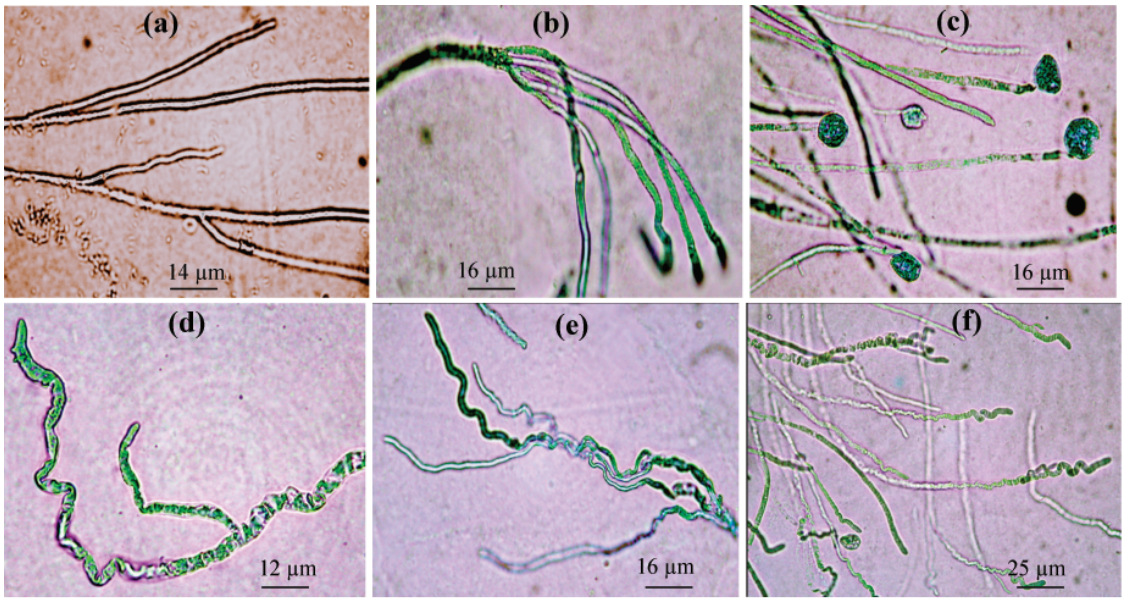

Fig. 4: Effect of chitosan on hyphal morphology of C. gloeosporioides, ***(a)*** *= hyphae with normal brancing (control);* ***(b)*** *= Hyphae with abnormal branching;* ***(c)*** *= swallowed hyphal tips;* ***(d)*** *= Shriveled hyphae;* ***(e)*** *and* ***(f)*** *= Coiling of hyphae and hyphal tips*

reduction (Cheah *et al.*, 1997; Benhamou, 1992; El Ghaouth *et al.*, 1992a, 1992b).

Effects of Calcium Chloride on C. gloeosporioides

Different concentrations of $CaCl_2$ showed significant effect on the radial growth of *C. gloeosporioides* (*Figs. 5* and *7a*). Radial growth (8.5 cm) was significantly ($P \leq 0.05$) higher for PDA amended with 1 or 2% $CaCl_2$ and by 3 and 4% after six days of incubation. The radial growth of the fungus in control plates was 7.6 cm, which was significantly lower than $CaCl_2$ amended plates at any concentration. The results of this study showed that not only was fungal growth uninhibited by $CaCl_2$ in the growth media, but concentrations of $CaCl_2$ up to 3% in the media actually proved to be somewhat stimulatory to the growth of the test fungus.

Although the mycelial growth of *C. gloeosporioides* was not inhibited by $CaCl_2$, spore germination was markedly reduced at higher concentrations (*Figs. 6, 7b* and *7c*). Spore germination (26%) was significantly higher ($P \leq 0.05$) when PDA was amended with 3 and 4% of $CaCl_2$ (*Fig. 7c*) followed by 2% with 6.3% inhibition. The lowest concentration of $CaCl_2$ (1%) did not inhibit the spore germination (*Fig. 7b*). However, spore germination in control and 1% $CaCl_2$ plates was 94 and 95%, respectively.

Calcium is an essential plant mineral, which plays major roles in plant functions. Postharvest treatment of fruits with low concentration of calcium salt has been found to reduce physiological disorders, mold growth and delay senescence (Conway, 1982; Poovaiah, 1986). In the present study, although $CaCl_2$ did not reduce mycelial growth of *C. gloeosporioides,* reduced germination of spores exposed to $CaCl_2$ has been observed. These findings are in agreement with Conway and Sams (1984) who reported that calcium ions *in vitro* did not reduce the fungal growth of *Penicillium expansum*, and there was also no growth reduction when the fungus was grown on juice extracted from calcium treated fruits. In the same work, the authors also found that calcium had a stimulatory effect on the growth of *P. expansum.* In another study, Biggs *et al.* (1997) reported that $CaCl_2$ did not inhibit the growth of *Monilinia fructicola* on PDA as strongly as some other salts. Nevertheless calcium salts have been shown to reduce mycelial growth of *C. gloeosporioides and C. acutatum,* which are causal agents of bitter rot of apple and *Leucostoma persoonii in vitro* (Biggs *et al.*, 1994;

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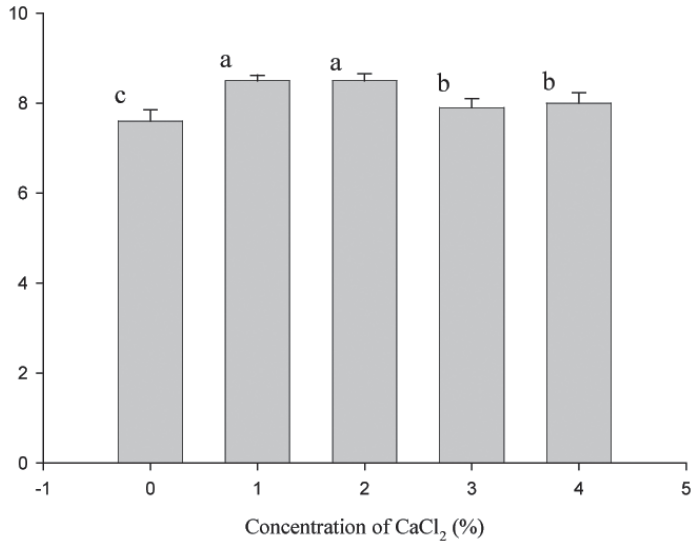

Fig. 5: Effect of different concentrations of calcium chloride on the radial growth of C. gloeosporioides *on PDA after six days of incubation at 28±2 °C. Means (Bars) followed by the same letter (s) are not significantly different according to Tukey's Studentized Range (HSD) Test at P≤0.05. Vertical bars represent the standard error of the means*

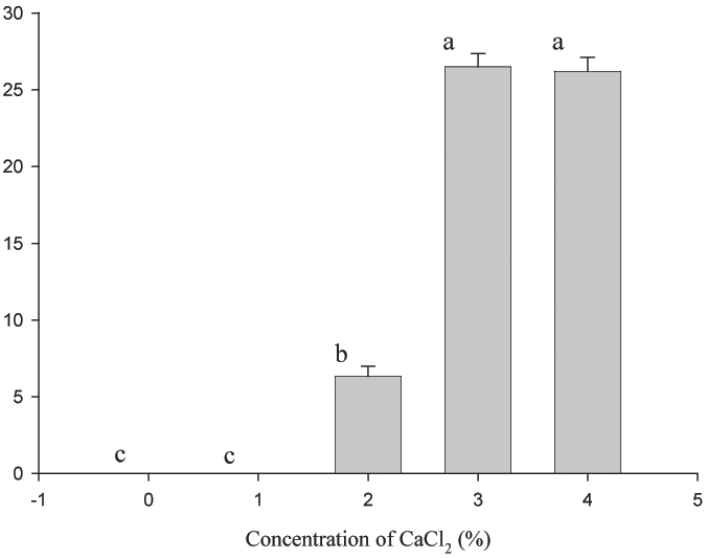

Fig. 6: Effect of different concentrations of calcium chloride on the spore germination of C. gloeosporioides *on PDA after 7 h of incubation at 28±2°C. Means (Bars) followed by the same letter are not significantly different according to Tukey's Studentized Range (HSD) Test at P≤0.05 on the arcsine transformed values. Vertical bars represent the standard error of the means*

Biggs, 1999). In this study, *in vitro* growth stimulation of C. *gloeosporioides* was significantly ($P \leq 0.05$) lower at $CaCl_2$ concentrations of 3 and 4% compared to 1 and 2%. Moreover, significant reduction of spore germination (26%) was found at 3 and 4% $CaCl_2$. This might be due to the toxicity of higher concentrations of calcium to *C. gloeosporioides* by affecting the osmotic balance in the fungal cells and inhibition of pectinolytic enzymes (Arras *et al.*, 1998). These results are

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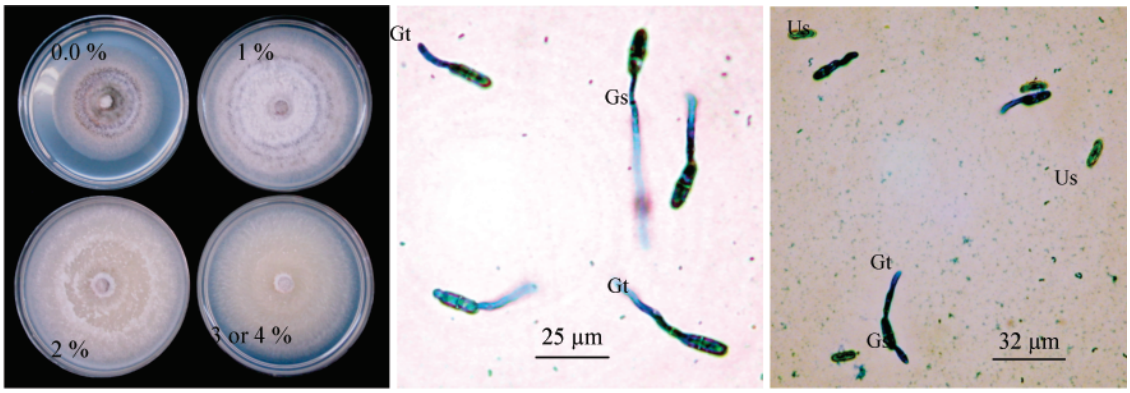

Fig. 7: Effect of different concentrations of calcium chloride on the radial growth and spore germination of C. gloeosporioides *after six days and 7 h of incubation, respectively at 28±2°C. (**a**) = radial growth of fungus on PDA amended with different concentrations of $CaCl_2$; (**b**) = germinated spores with 0.0, 1, or 2% $CaCl_2$; (**c**) = inhibition of spore germination with 3 or 4% $CaCl_2$. Gs = Germinated spore; Us = Ungerminated spore; Gt = Germ tube*

corroborated with the result reported by Narayanasamy (2006), who found that the addition of 2% calcium chloride reduced the spore germination and growth of germ tubes of *Rhizopus stolonifer in vitro*. Reduction of spore germination of *Penicillium digitatum* exposed to calcium chloride has also been observed (Droby *et al.*, 1997), as well as reduced germination and germ tube growth of *P. expansum* and *Botrytis cinerea* (Wisniewski *et al.*, 1995).

CONCLUSIONS

The findings of this *in vitro* study demonstrate that chitosan has significant effects on the growth, spore germination and hyphal morphology of *C. gloeosporioides* from papaya. On the contrary, $CaCl_2$ was not inhibitory but rather stimulates (to a small extend) radial growth of the fungus. However, $CaCl_2$ was ineffective in controlling anthracnose of papaya cv. Sekaki in particular and other benefits of $CaCl_2$ can still be exploited. It is hoped that various combinations of both could be used in postharvest treatments as an alternative to synthetic fungicide in preventing the occurrence of anthracnose in papaya caused by *C. gloeosporioides*.

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10. 70/2008-Rahman 230 24/3/05, 12:01 PM

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