

Comparison of Effects of Chitin and Chitosan for Control of *Colletotrichum* sp. on Cucumbers

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Experiments were conducted to test the ability of chitin or chitosan to control anthracnose (*Colletotrichum* sp.) on cucumber (*Cucumis sativus* L.). Practical grade chitin and unpurified chitin (ground crab shells) were added to compost at concentrations of 0.05 and 0.1% and chitosan was applied to the leaves of the plant at the concentrations of 0.05 and 0.1%. A fungicide (penncozeb) was used as a positive control while water and untreated plants were used as negative controls. Conidia of *Colletotrichum* sp. were applied to all the plants to initiate even disease development. All three treatments of chitin, at various concentrations, resulted in control of *Colletotrichum* sp. that was comparable to the fungicide. These results showed that *Colletotrichum* sp. on cucumber could be controlled by the use of chitin or chitosan and that both gave comparable levels of control.

Keywords: Anthracnose; Crab chitin; Nonchemical control; Biocontrol; Thailand.

Anthracnose caused by *Colletotrichum* spp. are economically important pathogens.¹ Cucurbit anthracnose is distributed widely, wherever cucurbits are grown in the world and can cause serious losses in varieties of cucumber and melon.² Anthracnose of cucumber is caused by the fungus *Colletotrichum orbiculare* (syn. *C. lagenarium*).³

The symptoms of cucumber anthracnose occur on all above ground parts of the plant. Lesions that appear on the cotyledons are small, water-soaked and pale in colour, chlorotic (yellow) or necrotic (brown). The lesions then progress, becoming larger and coalescing before the cotyledon dries up and dies. The symptoms on the leaves are similar. Other parts of the plant can also become infected, including the fruit. The

conidia of *C. orbiculare* are oval or pill-shaped, clear and have no cross walls.³

Much work has been done on *Colletotrichum* spp. that cause anthracnose of chillies (*Capsicum* spp.) from within Thailand^{4,5} and from the rest of the world.⁶

Four mixtures of plant growth-promoting rhizobacteria (PGPR) strains were tested under field conditions in Thailand against anthracnose of long cayenne pepper and mosaic disease of cucumber among other diseases and hosts.⁷ PGPR strains, not from Thailand, have been tested as possible means of inducing systemic resistance in cucumber to *C. orbiculare*.⁸ Wei et al.⁸ found that when PGRP strains were applied to cucumber seeds, some systemic resistance to *C. orbiculare* could be induced. A promising strain of endophytic *Streptomyces* sp. has also been identified for biological control of *C. orbiculare* on cucumber.⁹ A study on the extracts of 203 weed species found that several of the extracts inhibited *C. orbiculare*

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on cucumber while three of the extracts completely inhibited the anthracnose infection.¹⁰

Chitin is a major component of fungal cell walls and insect exoskeletons.¹¹ Plants produce chitinase when under attack by fungal pathogens.¹¹ When a mutant line has been produced that lacks the gene for production of chitinase the fungal infection is worse than in the wild type.¹² Chitinases have been isolated from *Streptomyces violaceusniger* where they acted as antifungal compounds.¹³

Chitin, extracted from shellfish sources, is a unique biopolymer based on the *N*-acetyl-glucosamine monomer. When the number of *N*-acetyl-glucosamine units is higher than 50%, the biopolymer is called chitin, and when the number of *N*-glucosamine units is higher than that of the *N*-acetyl-glucosamine units, it is called chitosan. For over 40 years this biopolymer has been studied for use in biomedical applications.¹⁴ Chitin is a major constituent of waste from shellfish processing, the management of which is a pressing need.¹⁵

Chitin, added to the soil, has been used to control several different fungal pathogens of plants. The soil borne infection, *Verticillium* wilt, has been controlled by adding powdered crab shells to the soil at a concentration of 2%.¹⁶ Control of *Fusarium oxysporum* on peas, at a concentration of 0.3%, and celery was achieved by the addition of chitin to the soil.^{17,18} While rose blackspot has also been controlled by the addition of powdered chitin to the soil.¹⁹

Chitosan has been used to control fungal pathogens including *Fusarium solani* f. sp. *glycines* on soybean at concentrations ranging from 1-5%,²⁰ *Fusarium oxysporum* f. sp. *apii* on celery¹⁷ and *Blumeria graminis* f. sp. *hordei* on barley at 2%.²¹ The post-harvest fungal pathogens *Botrytis cinerea* and *Rhizopus stolonifer* on strawberries at concentrations of 10% and 15%²² as well as *B. cinerea* and *Penicillium expansum* on tomatoes at concentrations ranging from 0.01-1%.²³

On cucumbers chitosan has controlled *B. cinerea* at concentrations of 0.1%²⁴ and *Sphaerotheca fuliginea* at concentrations of 1% and 2.5%.²⁵ *Pythium aphanidermatum* has also been controlled by chitosan alone²⁶ and when combined with an application of *Lysobacter enzymogenes* strain 3.1T8.²⁷

Anthracnose, on post-harvest fruits, has been controlled by chitosan on papaya, at concentrations ranging from 0.1% to 3%,^{28,29} tomatoes and grapes at concentrations of 1% and 2.5%.³⁰

Cucumber is an important crop in Thailand, particularly in the Northeast of Thailand.³¹ The possible use of chitin to control anthracnose would result in a reduction of the amount of fungicides used and would provide a use for a waste product. This study aims to test the effectiveness of applications of practical grade chitin, unpurified chitin and chitosan at a range of concentrations against a locally isolated *Colletotrichum* sp. on cucumber.

MATERIALS AND METHODS

Fungal isolation

Cucumbers with visual symptoms of anthracnose were obtained from a market near Mahasarakham, Northeast Thailand. They had been grown locally.

Sections (1 cm²) of the samples, where the symptoms were visible, were cut using a scalpel and placed on a potato dextrose agar (PDA) (HiMedia Laboratories Pvt. Ltd., India) plate. The samples were cultured at 25°C for up to two weeks. Individual colonies were sub-cultured onto further PDA plates and incubated at 25°C for two weeks again.

Colonies that were determined to be a species of anthracnose were re-plated to develop a stock from which test plants could be infected. They were also transferred to PDA slants and stored at 4°C.

Chemicals

Chitin was obtained from Sigma-Aldrich Co., USA, as 'Chitin from crab shells, practical grade'. Chitosan was obtained from Fluka BioChenika, Japan. The chitosan was prepared for use following the method of Lui et al.²³ It was dissolved in 1% HCL by stirring. This solution was then diluted to the required concentrations and the pH value was adjusted to 5.4 with NaOH.

Crab shells were ground to produce unpurified chitin. Crabs were obtained locally, the meat was removed and the shells were ground to produce a fine powder. This was stored in a sealed container at room temperature until use.

In-addition two products, available commercially in Maha Sarakham were also used. The first product (product 1) contained biological extracts and enzymes but no chitin. The label listed *Colletotrichum* as one of the species that it could control. The other product (product 2) contained 3% chitosan, from shrimps. This product did not list *Colletotrichum* as a species that it could control.

A fungicide (penncozeb, Elf Atochem) was used as a positive control.

Experimental design

Cucumber seeds, variety Ninja 179 (Chia Tai Seed, Thailand), were obtained from a local supplier. Three seeds were grown in each pot (14 cm diameter) containing locally sold compost. A controlled release fertilizer containing 14% nitrogen, 14% phosphorus pentoxide and 14% potassium oxide was added to the compost, as per the instructions on the container, before it was placed in the pots.

The treatments were practical grade chitin at 0.05 and 0.1% (w/w), unpurified chitin at 0.05 and 0.1% (w/w), chitosan at 0.05 and 0.1% (w/v), two commercial products (applied as per the label), fungicide (applied as per the label), water and untreated.

The practical grade chitin and the unpurified chitin were added to the compost as the seeds were being planted. The chitosan and two commercial products were applied, using a hand held pressurized sprayer, as the first true leaf was emerging on the seedlings. Four days after this the *Colletotrichum* sp. conidia were inoculated onto the plants. The conidia were harvested from the plate cultures and the conidial suspension was adjusted to a concentration of 50,000 +/- 5000 conidia per ml with a haemocytometer.³² Then four days after that the fungicide was applied. Each treatment was applied to three pots. The pots were arranged in a randomized block design and kept externally in a shaded location (average temperature 29.4°C).

A cotyledon and the first true leaf to emerge, on each plant, were tagged and repeatedly scored for the incidence and severity of symptoms twice a week using a seven point scale (0 = no visible symptoms, 1 = small pinprick lesions, 2 = 1-20% of leaf surface covered with lesions, 3 = 21-40%, 4 = 41-60%, 5 = 61-80% and 6 = 81-100%).

Statistics

The three plants in each pot had their measurements combined to form an average for the pot. The combined measurements were used to determine the area under the disease progress curve (AUDPC) for each of the three pots. The AUDPCs were compared by analysis of variance (ANOVA) with Fisher's least significance difference test (LSD) (OpenStat). $P < 0.05$ was considered to be statistically significant.

RESULTS

The cotyledons showed no significant differences between any of the treatments and the fungicide or the untreated (data not shown).

The leaves of the untreated plants and the leaves treated with water had the highest amount of disease symptoms (Fig. 1). While the plants treated with the fungicide had the least amount of disease symptoms (Fig. 1). The symptoms that did develop appeared up to 8 d after the symptoms first appeared on the untreated plants.

Product 1 provided control that was statistically different from the untreated and similar to the level given by the fungicide over the period of this experiment. For the first 12 d the disease level was low but it started to increase rapidly after the twelfth day towards the end of the experiment (Fig. 1). Product 2 did not reduce the disease symptoms at all (Fig. 1).

Practical grade chitin at both concentrations provided control on the leaves that was statistically better than the untreated and no different from the fungicide. The control given closely followed that of the fungicide (Fig. 2).

Unpurified chitin at both concentrations provided control on the leaves that was statistically better than the untreated and no different from the fungicide. Here the control given did not follow that given by the fungicide as closely as with the practical grade chitin (Fig. 3).

Chitosan at the concentration of 0.05% gave control on the leaves that was statistically better than the untreated and no different from the fungicide. The concentration of 0.1% provided control that was statistically better than the untreated but not as good as the fungicide (Fig. 4).

There were no significant differences

between the levels of control, on the leaves, provided by the three different types of chitin that were tested in this work (Table 1).

DISCUSSION

This study found that chitin, as either practical grade or unpurified, and chitosan provided control of this *Colletotrichum* sp. on cucumber. The level of control given by both the concentrations, which the different forms of chitin were used at, was significantly better than the untreated and in all but one case that was the same as that given by the fungicide.

There were significant differences between the treatments on the leaves. The untreated and the water were the same, showing that water alone does not have an inhibitory effect on the development of this *Colletotrichum* sp. on cucumber and so any inhibitory effects seen from the treatments must be due to the treatment. The fungicide significantly inhibited the development of this *Colletotrichum* sp. when compared to the untreated control. Since biocontrol methods, such as the use of chitin, are often less effective, as a

single treatment, than a traditional chemical control method any results that give control comparable to that given by the fungicide would show great promise for future commercial use.

Product 1 gave control of this *Colletotrichum* sp. that was statistically comparable to the fungicide but the level of diseased leaf area was starting to increase towards the end of the experiment, possibly suggesting that the control given was not long lasting and the product would need reapplications. Product 2 did not give significant control of this *Colletotrichum* sp. on cucumber. Product 1 listed *Colletotrichum* spp. on the label as one of the suitable targets for the product but Product 2 did not, but it did contain chitosan at 3% and so was included in the study. Any other benefits that the products might have given to the plants were not investigated in this study.

The practical grade chitin and the unpurified chitin gave control of this *Colletotrichum* sp., at both concentrations, that was the same as that given by the fungicide. These results are comparable to the results of Bell et al.,¹⁷ Guy and Baker,¹⁸ Hall et al.,¹⁹ and Huang et al.,¹⁶

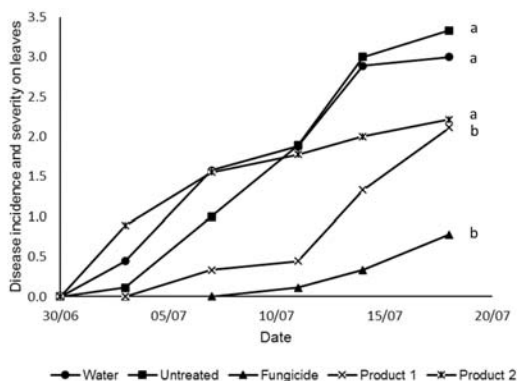


Fig. 1. Incidence and severity of *Colletotrichum* sp. lesions (measured on a scale from 0-6) on the leaves of cucumber plants inoculated with *Colletotrichum* sp. conidia and treated with one of two commercially available products (Product 1 and Product 2) compared to plants that were treated with a fungicide, had water applied or were untreated. Lower case letters indicate significant statistical differences as measured by AUDPC ($P < 0.05$) according to LSD test following ANOVA (OpenStat).

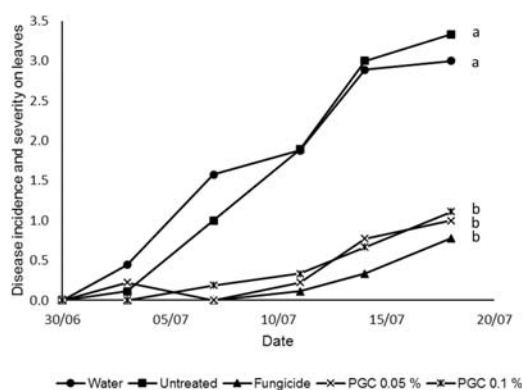


Fig. 2. Incidence and severity of *Colletotrichum* sp. lesions (measured on a scale from 0-6) on the leaves of cucumber plants inoculated with *Colletotrichum* sp. conidia and grown in compost with practical grade chitin (PGC) added at two different concentrations 0.05 and 0.1% compared to plants that were treated with a fungicide, had water applied or were untreated. Lower case letters indicate significant statistical differences as measured by AUDPC ($P < 0.05$) according to LSD test following ANOVA (OpenStat).

who all found that applications of chitin to the soil could result in reduced fungal disease symptoms.

There were no significant differences between the control given by the different concentrations of the practical grade chitin and the unpurified chitin. This suggests that if there was large scale adoption of chitin as a biocontrol product it would be possible to use the unrefined waste from shellfish processing that poses an environmental problem.¹⁵ This would hopefully result in a lower price and so a greater adoption by farmers.

The chitosan at a concentration of 0.05% gave control of this *Colletotrichum* sp. that was similar to that given by the fungicide. Unlike the chitin, the chitosan also resulted in a treatment that was significantly different from the untreated and the fungicide (0.1%). As has been shown by many authors previously, but not on anthracnose of cucumber, chitosan can result in significant control of fungal pathogens.^{17,20,21,22,23,24,26,27,28,29,30} The addition of chitin to the soil is something that could only be archived pre-planting, so the use of a foliar application of chitosan would be able to increase the amount of time that it would be

possible for plant protection to be derived from chitin or its derivative.

The chitosan was applied to the leaves before the inoculation of the leaves as Bautista-Banos et al.²⁸ and Ben-Shalom et al.²⁴ had both shown that this was the most effective way to use chitosan.

There were no differences between the applications of chitin, either as practical grade or unpurified, and chitosan showing that both forms give comparable levels of control.

There were no significant differences between the AUDPCs of the treatments or controls for the cotyledons. Inagaki et al.¹⁰ found that cotyledons were suitable for the identification of weed extracts that could inhibit anthracnose lesions on cucumbers caused by *C. lagenarium*. In addition chitosan significantly inhibited the powdery mildew incidence on cucumber cotyledons.²⁵ Concentrations of 10^{-6} spores mm^{-1} and concentrations of 4×10^4 and 4×10^5 conidia ml^{-1} were used by Inagaki et al.¹⁰ and Moret et al.,²⁵ respectively. Both authors added Tween-20 to the conidial suspensions at a low level, while in this work the method of Monroe et al.³² was followed

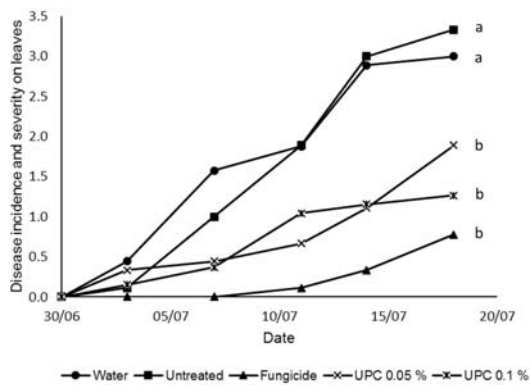


Fig. 3. Incidence and severity of *Colletotrichum* sp. lesions (measured on a scale from 0-6) on the leaves of cucumber plants inoculated with *Colletotrichum* sp. conidia and grown in compost with unpurified chitin (UPC) added at two different concentrations 0.05 and 0.1% compared to plants that were treated with a fungicide, had water applied or were untreated. Lower case letters indicate significant statistical differences as measured by AUDPC ($P < 0.05$) according to LSD test following ANOVA (OpenStat)

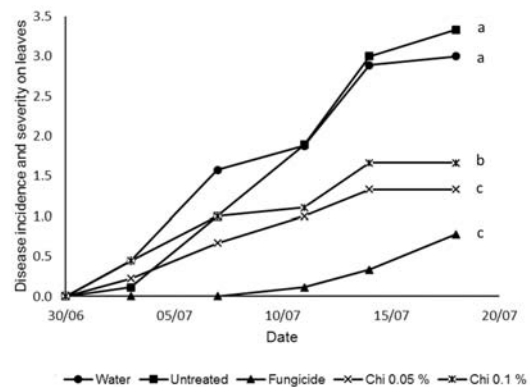


Fig. 4. Incidence and severity of *Colletotrichum* sp. lesions (measured on a scale from 0-6) on the leaves of cucumber plants inoculated with *Colletotrichum* sp. conidia and treated with chitosan (Chi) at two different concentrations 0.05 and 0.1% compared to plants that were treated with a fungicide, had water applied or were untreated. Lower case letters indicate significant statistical differences as measured by AUDPC ($P < 0.05$) according to LSD test following ANOVA (OpenStat)

Table 1. Significant statistical differences as measured by AUDPC ($P < 0.05$) according to LSD test following ANOVA (OpenStat) between cucumber plants that were either grown in compost amended with practical grade chitin (PGC) or unpurified chitin (UPC) at the concentrations of 0.05 and 0.1% and plants which were treated with chitosan (Chi) at the concentrations of 0.05 and 0.1%. * = statistically different, NSD = not statistically different

	UPC 0.05%	UPC 0.1%
PGC 0.05%	NSD	NSD
PGC 0.1%	NSD	NSD
	UPC 0.05%	UPC 0.1%
Chi 0.05%	NSD	NSD
Chi 0.1%	NSD	NSD
	PGC 0.05%	PGC 0.1%
Chi 0.05%	NSD	NSD
Chi 0.1%	NSD	NSD

where the conidial concentration was between the amounts used by the previous authors but without Tween-20. Monroe et al.³² worked on the leaves rather than the cotyledons. In addition, the treatments were applied at the optimal time for application to the leaves rather than to the cotyledons as in the previous papers.

This work has shown that chitin as either practical grade or unpurified and chitosan can all control *Colletotrichum* sp. on cucumber plants. This is the first report of these three types of chitin being used to control a *Colletotrichum* sp. isolated from samples obtained in Thailand. This work has shown that it would be possible to use chitin to control anthracnose on cucumber plants. It has also shown that unpurified chitin is comparable to practical grade chitin or chitosan so the waste products from shellfish processing could be used directly with minimal processing to help reduce the amount of fungicides used in agriculture.

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