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Effect of chitosan-tripolyphosphate to suppress anthracnose (*Colletotrichum* spp.) in post-harvest chili

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ABSTRACT

Anthracnose disease (Colletotrichum spp.) in chili can lead to low fruit quantity and quality from planting to the postharvest stage. The environmentally friendly management using chitosan (CS) is expected to suppress anthracnose disease and increase the shelf-life of chili. This study aimed to determine the effect of the chitosan-tripolyphosphate (CS-TPP) to suppress anthracnose on chili during postharvest storage through in-vitro and in-vivo assays. In the in-vitro assay, CS-TPP solution with a ratio of [5:2] and [3:1] was applied into a warm Potato Dextrose Agar (PDA) medium. It was grown by Colletotrichum pathogen, while on in vivo assay, CS-TPP was applied to chili fruit before being inoculated by the pathogen. A control treatment was prepared without CS-TPP application. Results revealed that the CS-TPP ratios affected the growth of Colletotrichum spp. at the in-vitro assay. The CS-TPP [5:2] ratio was more effective than CS-TPP [3:1] in reducing the growth of Colletotrichum spp. with the fungal inhibition of 62,65% and 55,56%, respectively, compared to the control treatment. Moreover, it also showed anthracnose disease suppression on chili fruit of 51%, and 29%, respectively, compared to control treatment at in-vivo assay. This study showed the potential use of CS-TPP as a coating application for anthracnose disease management on storage chili, however further study such as viability and longevity of formula need to be done.

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INTRODUCTION

Chili (*Capsicum annum* L.) is one of the essential vegetable commodities in Indonesia, with a planting area of 165,000 ha, and is considered among the main cultivation crops compared to other vegetables (BPS 2018).

Fungal disease attacks that infect plant from nursery to postharvest is one of the constraints causing low yield production of chili (Dev et al. 2012, Hassan and Chang 2017, Feliziani et al. 2015). The anthracnose disease caused by *Colletotrichum* spp. is often found in chili (Diao et al. 2017). Symptoms are blackish-brown on leaves, twigs, branches, and dead shoots (Correa-Pacheco et al. 2017). *Colletotrichum* spp. can reduce fruit yield and quality (Pamekas et al. 2009). Therefore, eliminating the pathogen during seedling growth and storage is very important.

Management of the disease is mainly done by fungicides application. However, the use of synthetic fungicides poses a health risk to consumers. Therefore, alternative disease control is needed. Chitosan (CS), a polysaccharide from Crustacean skin/shell waste, is reported to be used as an eco-friendly substance. The CS application induces plant resistance response to pathogen infection (Al Eryani-Raqeeb et al. 2009, Alam et al. 2015, Ali et al. 2013, Riad et al. 2013). CS was able to inhibit the growth of *Colletotrichum* spp. through inhibition of conidium germination and fungal hyphae lysis (Pamekas et al. 2009). The application of CS mixed with organic and inorganic acids as a coating material was reported to increase the effectiveness of postharvest disease control on strawberries (Romanazzi et al. 2013, Feliziani et al. 2015). The CS seed treatment application to seeds infected with pathogenic fungi still varies (Alam et al. 2015, Azura et al. 2017). In addition, CS is influenced by the large molecular weight (MW) which affects the decrease ineffectiveness in its application. This shows the limited effect of the potential CS polymer in agriculture. Thus, for wider utilization, modifications are needed i.e., the use of enzymes to obtain CS low MW (CS-LMW), followed by the ionic gelation method using cross-linker with sodium tripolyphosphate (Na-TPP) to change the particle into a smaller size.

In a previous study, it was reported that enzymatically CS hydrolyzed by chitinase from *B. firmus* E65 followed by ionic Na-TPP produced an MW of 511.85 kDa, an average particle size of 228.74 nm, and the most effective CS-TPP ratio [3:1] in inhibiting the growth of *C. gloeosporioides* on mango (Mulyaningtyas et al. 2016). The types of chitinase produced by bacterial isolates and the different ratios of CS: TPP are thought to have varied effects on the inhibition of pathogens. Therefore, efforts are still needed to evaluate the production of CS hydrolyzed by chitinases from other bacteria and the optimal ratio between CS-TPP in suppressing pathogenic fungi. The result of the previous study reported that several isolates of phyllosphere bacteria and endophytes-producing chitinase could also be used as antagonist microbes capable of controlling anthracnose disease in mango (Suryadi 2019, *unpublished data*).

The general purpose of the study was to select chili bacterial isolates-producing chitinase activity for enzymatic depolymerization of chitosan as a biocontrol agent for plant disease control. The present study aimed to determine the effect of enzymatically CS using chitinase produced by RB isolate bacteria towards inhibiting chili anthracnose, which was evaluated through in-vitro and in-vivo assays. The research is expected to contribute to the potential use of the enzymatic CS formula as a growth inhibitor of *Colletotrichum* spp., remarkably to reduce postharvest disease infection in chili.

METHODS

Preparation of chitinase by RB isolates.

To determine qualitative chitinase activity, bacterial RB isolate (Biogen-Culture Collection) was cultured in Nutrient broth (NB) for 24 hours using a rotary shaker (IKA, K-260) at 75 rpm. Two ml of RB isolate was grown on solid chitin media and incubated for 7 days. The incubated chitin media was rinsed with congo red 0.3%, rinsed again with sterile distilled water, and left for 24 hours, then the clear zone formation was observed (Kim and Ji 2001).

Quantitative chitinase assay of RB isolate was tested by spectrophotometry using a standard N-acetyl-D-glucosamine (Glc-NAc) solution. The absorbance measurement of the sample was carried out in Duplo by mixing 450 µl of the enzyme, 450 µL of PBS, and 900 µl of colloidal chitin, then shaken with a vortex and incubated at 37^{o}C for 60 minutes. The mixture was centrifuged at 5,000 rpm for 5 min. The 500 µl supernatant

was then added with 500 µl of distilled water and 1 mL of Schales reagent. The mixture was boiled at 100°C for 10 minutes. After cooling, the samples were measured at a wavelength of 420 nm (Hitachi U-2800) (Soeka and Sulistiani, 2012).

Partial purification of chitinase

One loop of RB isolate was cultured in 10 ml of liquid Luria Bertani (LB) media for 32 hours while shaking at 75 rpm using a rotary shaker (IKA K-260), then 1 ml of culture was inoculated into 10 ml of RB isolate on liquid chitin media. The cultures were incubated for five days at room temperature and shaken at 75 rpm. Furthermore, the liquid chitin media containing bacteria was centrifuged (Hettich d-78532) at 10,000 rpm (4°C) for 20 minutes. For the partial enzyme purification, 40 ml of the supernatant (crude enzyme extract) was mixed with saturated 70% (v/v) ammonium sulfate. The mixture was centrifuged at 10,000 rpm (4°C) for 30 minutes. The pellet was then dissolved in saline phosphate buffer (PBS) at pH 6.8 to remove the protein bound to the substrate (Pratiwi et al. 2015). Furthermore, the pure enzyme solution was stored at 4°C before use (Vishu Kumar et al. 2007, Soeka and Sulistiani 2012).

Preparation of CS-LMW and formulation of CS-tripolyphosphate (CS-TPP) by ionic gelation method.

A total of two grams of CS (Sigma, MW 200 kDa) was dissolved in 100 ml of 2% acetic acid pH 3.5. The mixture was stirred using a medium-speed magnetic stirrer until the CS was completely dissolved. The pH of the CS solution was adjusted to 5.3 by adding NaOH. A total of 20 ml of 2% CS solution was hydrolyzed using 0.2 ml of chitinase at 37°C for 24 hours. The hydrolysis process was stopped by heating at 100°C for 5 minutes. The precipitated CS (white residue) was washed with sterile distilled water until the pH was neutral. The precipitate was stored at 4°C (Vishu Kumar et al. 2007, Handayani et al. 2018).

Determination of CS MW was done by a viscometric method using Oswald's viscometer. Standard solutions of CS with various concentrations (0.01%; 0.02%; 0.03%; 0.04%, and 0.05%) (w/v) were prepared in 0.10 M acetic acid and 0.02 M sodium chloride. The flow time is calculated when the liquid flows from the first line (index line) to the second index line. Based on the flow time of the CS solution and the pure solvent, the specific and intrinsic viscosity will be obtained through the Mark-Hauwink equation (Kasaai 2007) as follows:

$$[\eta] = K.M_v{}^{\alpha}$$

where: η = the intrinsic viscosity of the solution; Mv= molecular weight (kDa), K= coefficient (3.04×10^{-5}), α= coefficient (1.26).

To the CS-LMW solution, 0.25 ml of Tween 80 was added with constant stirring for 30 minutes, and then 0.1% Na-TPP solution with two different volume ratios (CS: TPP volume ratio [3:1] and [5:2]) was added dropwise while stirring at room temperature. Stirring was continued for one hour, then the solution was stored at 4°C until further testing (Mardliyati et al. 2012; Nadia et al. 2014).

Observation of CS-TPP particles with scanning electron microscope (SEM) and particle size analyzer (PSA)

The SEM was prepared by dripping the CS-TPP sample into the specimen holder. The sample was dried for 24 hours, and then coated with gold metal using an ion coater. Furthermore, the specimen samples were analyzed using SEM (Zeiss). The samples were also measured for particle size analysis using Particle Size Analyzer (Malvern Instruments Ltd. Co, UK).

Effect of CS-TPP to Colletotrichum spp. (in-vitro and in-vivo assays)

In the in-vitro assay, two mL of CS-TPP solution, with a ratio of [5:2] and [3:1], was added to a warm Potato Dextrose Agar (PDA) medium in a screw cap tube. The mixture was poured into a petri dish and then allowed to harden. PDA in a petri dish was perforated in the middle media with a cork borer, and then *Colletotrichum* spp. Isolate was put into the hole and incubated for 3 days. The study was arranged in a randomized complete design with five replications. The control treatments were prepared to contain only PDA media and fungi (Hamdayanti et al. 2017). The formula determines the fungal inhibition growth:

$$FI = \frac{(Gc - Gt)}{Gc} \times 100\%$$

where: FI= fungal inhibition, Gc = growth area of control fungus, Gt = growth area of treated fungus (Mahdizadeh et al. 2015)

In the in-vivo assay, the healthy chili fruit cv. 'Tanjung' with the same size and level of maturity was applied using the fruit coating method. In each

treatment, ten chili fruits were assigned in two replications. The fruit was washed with sterile water and dried. The fruit was soaked in CS-TPP solution and then dried again. The fruit was wounded with a sterile needle and re-soaked in a suspension of *Colletotrichum* spp. conidia and dried. A control treatment was carried out without CS-TPP immersion. Fruits were stored in sterile boxes and wrapped in clear plastic to prevent moisture. Observations were made periodically until the fruit rotted. Anthracnose severity score in chili refers to the procedure of James (1977) with a slight modification, namely the severity score 0 = 0 - < 1%; 1 = 1 - 20%; 2 = 21 - 40%; 3 = 41 - 60%; 4 = 61 - 80%; and 5 = 81 – 100%. The following formula calculates disease suppression (DS) against fungi:

$$DS = \frac{(Sc - St)}{Sc} \times 100\%,$$

where: DS=disease suppression, Sc=severity on control, St=severity on treatment.

RESULT AND DISCUSSION

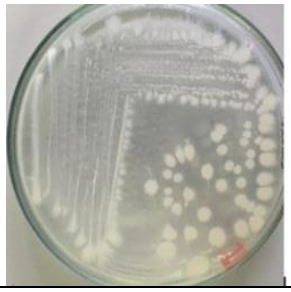
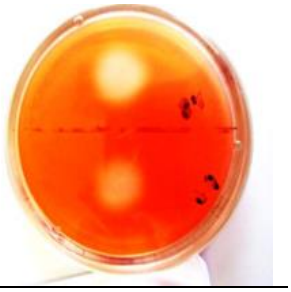
Chitinase Activity and CS-LMW

The results of bacterial isolation for chitinolytic propagation are shown in Table 1. The qualitative results of chitinase indicate that RB isolate is one of the chitin-degrading bacteria that produce chitinase. The enzymes produced by RB isolates had an average chitinolytic index of 1.60 $\pm$ 0.2 cm. The diameter of the clear zone depends on the amount of Glc-NAc monomer produced from the hydrolysis of chitin by breaking the -1,4-N-acetylglucosamine bonds produced. The greater the amount of Glc-NAc monomer produced, the larger the clear zone formed (Fadhil et al. 2014).

Quantitatively, RB isolates produced a crude extract of chitinase obtained from binding the enzyme with chitin substrate in liquid chitin media. The Glc-NAc standard measurement was obtained with a high linearity equation ($y = 0.0062x - 0.0004$; $R^2 = 98.93$), and the average sample chitinase concentration was determined to be 9.84 $\pm$ 0.09 ppm. Chitinase can catalyze the hydrolysis of chitin completely to produce molecules of glucosamine and acetic acid, while the partial hydrolysis of chitin will produce molecules of chitobiose (Kim and Ji 2001). Chitinase belongs to a group of hydrolase enzymes that can degrade chitin directly into LMW products. This enzyme can degrade polymeric chitin into chitin oligosaccharides, deacetylchitobiose, and Glc-NAc (Kidibule et al. 2018).

Higher chitinase activity was used to degrade CS by mixing a ratio ($^{v}/_{v}$) of CS solution (20 ml) with chitinase (0.2 ml). The solution mixture was incubated at 37 ^{o}C for 24 hours. Incubation temperature greatly affects enzyme activity because enzymes can be easily denatured when exposed to high temperatures, which results in decreased enzyme performance (Suryadi et al. 2014). Incubation time affects the enzyme hydrolysis process, the longer the incubation time, the more the enzyme produced. To get CS-LMW, the hydrolysis process was carried out at a constant temperature. Breaking with a long hydrolysis time will cause these bonds to reunite to increase viscosity and MW values. Measurement of CS-LMW by the Ostwald viscometer using 1% acetic acid solvent resulted in a MW of 48.2 kDa.

Table 1 Morphological characteristics of RB isolates and chitinolytic activity on chitin media.

Characteristics of bacteria (morphology)	Single colony	Chitinolytic activity
Color white, small/medium size, smooth texture, convex elevation, entire edge, serrated, undulate		

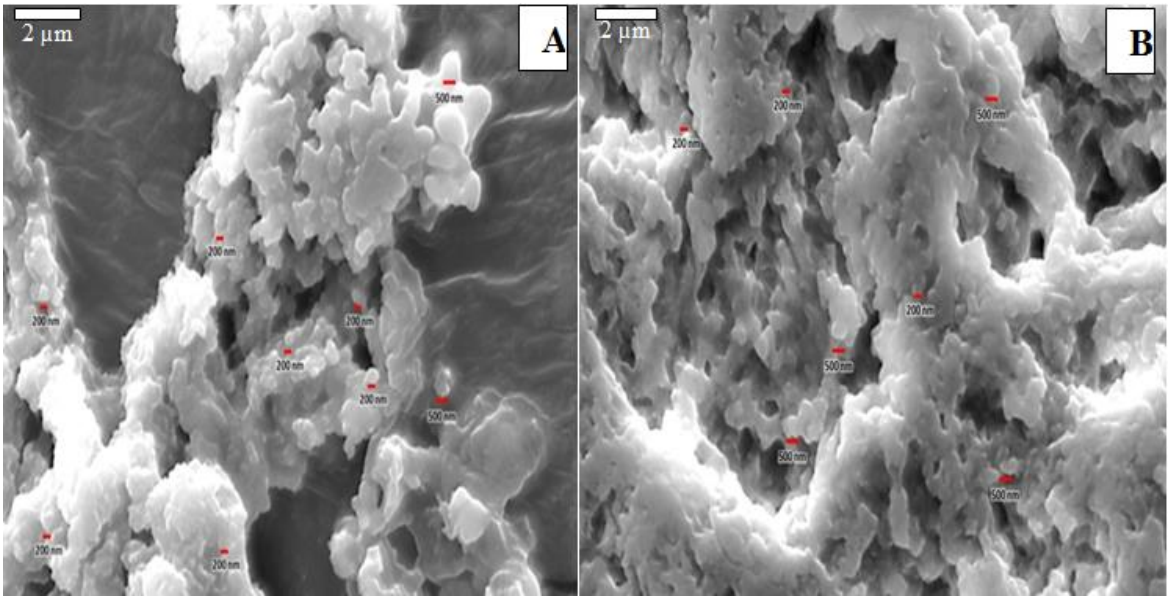


Figure 1 The particle morphology of CS-TPP [3:1] (A) and CS-TPP [5:2] (B)

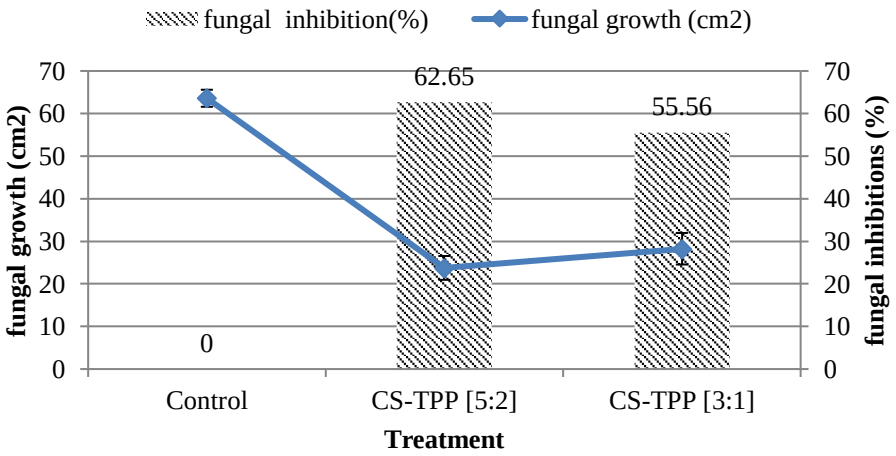


Figure 2 Effect of CS-TPP formula hydrolyzed by RB chitinase isolate on fungal growth and their inhibition under in-vitro assay

Formulation of CS-tripolyphosphate (CS-TPP) and particle analysis

Na-TPP is used as a crosslinker because it is non-toxic and has a lot of negative charges so that it can interact more strongly (Chattopadhyay and Inamdar 2012). Ionic interactions that occur between positively charged amine groups in CS and negatively charged polyanions (Na-TPP) will form a three-dimensional intermolecular or intramolecular network structure (Chookhongkha et al. 2013, Mardliyati et al. 2012).

The results of observations with SEM on CS-TPP particles are presented in Figure 1. The results of the observations show that the obtained CS-TPP has a nano-dimensional spherical shape. The particle size is determined by comparing the particle size with the image scale. Based on the SEM analysis of the CS-TPP particle image, the measurement results with SEM ranged from 200-500 nm. However, the particles still agglomerate in the form of aggregates, which indicates that the particles are still not stable, even though a stabilizer has been added in the formula. The measurement results of CS-TPP using PSA showed almost a similar particle size with nano dimension (671.1 nm).

Theoretically, adding an emulsifier (Tween 80) stabilizes the particles in the solution by preventing the occurrence of agglomeration between the particles (Suptijah et al. 2011). The decreased viscosity led to a smaller particle size in LMW, Thus, affecting better solubility of chitosan

in distilled water or acetic acid solution. According to Katas and Alpar (2006), the more amino groups on CS protonated, the more efficient interaction between negatively charged chitosan and polyanion (Na-TPP).

Inhibition of CS-TPP against *Colletotrichum* spp. in-vitro and in-vivo

Results of in vitro assay showed that CS-TPP could inhibit fungal growth. The area of the fungus growth in the control (without treatment) reached 63.59 cm^2. The area of fungal growth colonies in each CS-TPP treatment [5:2] (23.75 cm^2) and [3:1] (28.26 cm^2) was smaller than that of the control treatment (Figure 2). This indicates an inhibitory effect on fungi. However, the DS in each treatment as indicated by representative treatment formula on PDA plates did not show much difference (Figure 3).

In the in vivo assay, the infection process of *Colletotrichum* spp. begins with the attachment of spores to the surface of the fruit skin, then the spores germinate and form appressoria and hyphae to infect and remain dormant in the cell layer on the skin in a latent condition. Fruit's ethylene induces dormant conidia and appressoria activation during the ripening physiological process (Flaishman and Kulattukudy 1994). DS was calculated by comparing the area of chili rot in the control with each treatment. The results of in-vivo assay observations showed different DS (Figure 4). The difference in DS is thought to be due to differences in particle size and concentration of CS-TPP used.

The ratios between CS and NaTPP [5:2] and [3:1] was previously used in field trial, where the ratio [5:2] showed higher efficacy (Suryadi et al., 2019). In this study, the CS-TPP ratio [5:2] also had a lower disease severity (43.5%) compared to CS-TPP [3:1] (63%). Therefore, the ratio [5:2] was more effective in inhibiting the growth of anthracnose disease in chili. According to Sarwono et al. (2013), CS can suppress the anthracnose disease development in chili because it has an active group that will bind to *C. capsici*.

The mechanism by which CS affects the growth of some phytopathogenic fungi has not been fully elucidated, but several hypotheses have been proposed. Due to its polycationic properties, CS is thought to interfere with negatively charged macromolecular residues exposed to the surface of fungal cells. CS nanoparticles can destroy microbial genetic material, whereas CS, as a chelator, can bind metal ions into intracellular solutions, which play an important role in the survival of microbial cells. In addition, CS inhibits fungi by damaging cell walls, which are generally composed of layers of peptidoglycan and lipopolysaccharide. The fungus growth will be inhibited due to the host penetration failure by the fungi's appressorium. The positively charged acetylamino groups (NH_2COCH_3) and glucosamine ($C_6H_9NH_2$) in CS can bind to negatively charged macromolecules on the surface of fungal cells, causing inhibition of fungal growth (Gomes et al. 2017, Hassan and Chang 2017, Kumar et al. 2014).

The results of this study indicate the potential use of CS: TPP as a coating application for anthracnose disease management on chili. In addition to being environmentally friendly and biodegradable, CS: TPP showed an excellent growth inhibition against the fungus *Colletotrichum* spp. in postharvest chili fruit than without the application. However, the effectiveness of the formula in longer periods of storage still needs to be further tested, specifically for other postharvest chili cultivars in a storage environment.

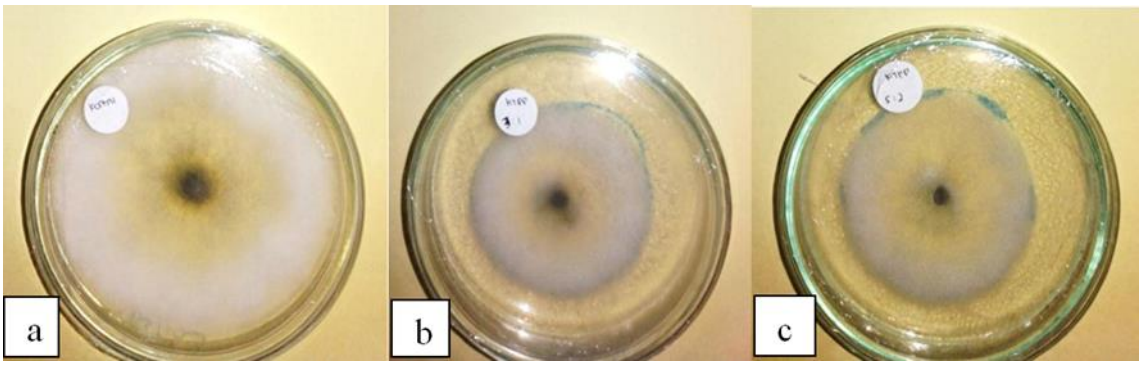


Figure 3 Representative of fungal growth inhibition on PDA media plates containing CS-TPP formula (in-vitro assay). (a) control, (b) CS-TPP [3:1], and (c) CS-TPP [5:2]

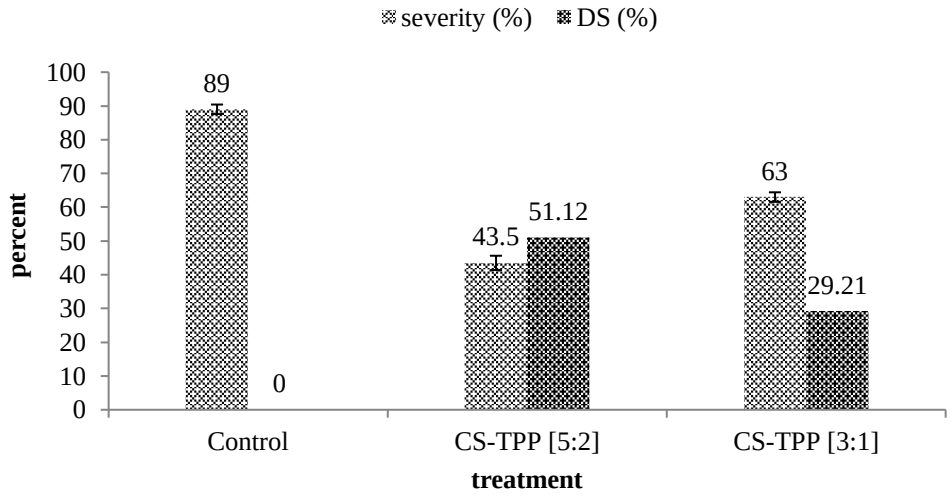


Figure 4 Effect of CS-TPP [5:2] and [3:1] extracted using chitinase from RB isolate to severity and anthracnose disease suppression on chili fruits cv. Tanjung (in-vivo assay). DS= disease suppression

CONCLUSION

CS-TPP ratios [3:1] and [5:2] affected the growth of *Colletotrichum* spp. under in-vitro assay while using in-vivo assay (on chili fruit), CS-TPP ratio [5:2] showed higher inhibition of anthracnose disease compared to ratio [3:1], with the DS of 51.12%, and 29.21% respectively.

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