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Evaluation of three species of *trichoderma* as potential bio-control agent against *colletotrichum gloeosrioides.*, a casual agent of anthracnose disease in onion

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ABSTRACT

The research was conducted to screen and evaluate the effect of three *Trichoderma* species as a potential bio-control agent against *Colletotrichum gloeosrioides* isolated from onion under *in vitro* condition. In in vitro test, antagonistic effect of the three *Trichoderma* species against *C. gloeorioides* was evaluated using dual culture test and the presence of active compounds of the three antagonists was determine while mycoparasitism interaction was also observed under microscope using slide culture technique. Pathogenicity testing was also determined using onion bulb as test crop. Results showed in the percent growth inhibition of the three Trichoderma species during 14 days of incubation, *T. longibrachiatum* obtained the highest percentage inhibition of 64.68% compared to *T. harzianum* and *T. asperellum* with 59.16% and 47.73% inhibition respectively. It was also observed that hypal coiling was common on the three *Trichoderma* species against *C. gloeosrioides*. The presence of active compounds among the three *Trichoderma* were also tested and it showed at 500 µl/ml crude extract of *T. longibrachiatum* obtained the highest inhibition of 15.58% which make it more active against a wide range of microorganism. Pathogenicity test of C. gloeosrioides against the three Trichoderma showed the onion bulbs inoculated with the pathogen only was highly pathogenic. However, bulbs treated with the antagonist showed that *C. gloeosporioides* was observed as weak and mild pathogenic. This could be concluded that the presence of Trichoderma species could lessen the pathogenic effect of *C. gloeosporioides* and could be used as natural biocontrol agent of some plant diseases.

KEYWORDS: *Trichoderma*, Bio control, Anthracnose, Onion

INTRODUCTION

Onion (*Allium cepa* L.) under the Liliaceae family is either a biannual or perennial plant, bearing semi-cylindrical leaves which emerged from a subterranean bulb and having fascicled, short, branched roots. During the cultivation process of onion in the farm, onions are very susceptible to pathogens especially when the environmental conditions favored their growth. Once infected by fungi or bacteria, it can lead to reduce quality and productivity of onion [1]. In the past year, *Colletotrichum* species, the causal agent of anthracnose seriously damaged several onion fields in Nueva Ecija as well as neighbouring provinces. Anthracnose was responsible

for severe leaf blighting in some fields [2]. This resulted to yield loss, high price and shortage of onion supply in the market.

Biological control is a component of an integrated pest management strategy. It is defined as the reduction of pest populations by natural enemies and typically involves an active human role. This is referred to as natural control or biological control which includes the use of predators, parasitoids, and pathogens. Biological control agents of plant diseases are most often referred to as antagonists [3].

The genus *Trichoderma* is very popular in organic farming. It is employed widely in agriculture because it releases a variety of compounds that induce systematic resistance against soil- borne pathogen, and enhances crop productivity [4].

Thus, this study is designed to determine if the three species of *Trichoderma* can be a potential bio-control agent against *C. gloeosporioides*. in onion. Further, the effect of these natural extracts in onion subjected to *C. gloeosporioides* was also determined.

MATERIALS AND METHODS

Collection and isolation of C. gloeosporioides causing disease in onion:

Prior to isolation, advancing disease portion was cut into 1 cm² section, disinfected and washed with sterile water for 3 minutes. The tissues were blot dried on sterile filter paper, then plated on water agar and incubated for 7 days at room temperature.

Morphological and cultural characterization of pathogen:

The culture of the pathogen was identified morphologically and culturally. The colony of the culture of the pathogen grown in previously plated Potato Dextrose Agar (PDA) was described and also the spore was observed under the compound microscope. Then, it was submitted to the Laboratory Service Division, Philippine Center for Postharvest Development and Mechanization, Science City of Muñoz, Nueva Ecija for verification of the identification made.

Antagonistic fungi:

Different species of *Trichoderma* such as *T. longibrachiatum*, *T. asperellum* and *T. harzianum* were used as antagonist against fungal pathogen causing disease in onion. Pure cultures of the three species of *Trichoderma* were obtained from the microbiology laboratory of RM-CARES.

Evaluation of the antagonists against Colletotrichum sp. in onion:

The antagonistic effect of the test antagonist on the growth of fungal pathogen causing diseases in onion was evaluated using dual culture method. Five mm mycelial plugs of each antagonist and fungal pathogen was aseptically inoculated equidistantly in previously plated petri dishes containing PDA. Plates inoculated only with the test pathogen served as control. Four plates for each interaction were incubated at room temperature. The percent growth inhibition (GI) was computed after 14 days of incubation as follows based on the study of Korsten et al. [5] as cited by Alvindia and Natsuaki (2008):

$$\mathrm{Gl} = \frac{\mathrm{Kr} - \mathrm{rl}}{\mathrm{Kr}} \mathrm{x}\ 100$$

Where:

Kr- distance (measured in mm) of fungal growth from the point of inoculation to the colony margin of the control plates;

rl- distance of fungal growth margin in the direction of the antagonist

Mycoparasitism interaction of each antagonist against fungal pathogen was determined using slide culture techniques. Agar blocks of the antagonists and fungal pathogens were grown equidistantly with a distance of 1 cm in a glass slide containing a very thin water agar. The slides were placed in sterile petri dish line with moist sterile filter paper. Slides were observed under the compound microscope after two weeks to determine the interaction of the antagonists and pathogens.

Test for the active compounds of the antagonists against fungal pathogen:

The active compounds of the antagonists were determined following the method of Talubnak and Soytong, [6]. The antagonist was cultured in potato dextrose broth (PDB) for 14 days. Biomass of the antagonist was filtered and collected for extraction method using rotary vacuum evaporator. One ml of different concentration of crude extracts (0, 100, 500 and 1,000 µl/ml) of antagonist were tested in petri dishes. Four mm mycelial disk of the pathogen was inoculated in the center of petri dish containing PDA and different concentration of crude

extracts of microbial antagonist and incubated at room temperature. Colony diameter was recorded daily. Growth inhibition was also recorded and computed using the formula:

$$Gl = \frac{(Kr - rl)}{Kr} x\ 100$$

Where:

Kr- Distance (measured in mm) of fungal growth from the point of inoculation to the colony margin of the control plates.

rl- Distance of fungal growth margin in the direction of the antagonist; and

Gl- Percent inhibition growth

Statistical Analysis:

All the data collected were analyzed with Analyses of Variance (ANOVA) using Completely Randomized Design (CRD) with four replications. Duncan's Multiple Range Test (DMRT) was used in comparing means at 5% level of significance.

Pathogenicity test (onion bulb):

Four onion bulbs were washed with sterile water and disinfected with 10% sodium hypochlorite solution for 3 minutes and then rinsed again with sterile water and air dried. Four mm of 7 day old fungal pathogen and antagonist were attached side by side to the surface of the onion bulb. The control was inoculated only with agar disk. The bulb was kept inside the moist chamber for 4-5 days; the diameter of the lesions was recorded at 5th day of incubation. Disease severity of the onion bulb was determined using the grading scale of Villa Nova et al. [7].

RESULTS AND DISCUSSION

Morphological and cultural characteristics of the pathogen:

The color of *C. gloeosporioides* varied from white to grey. The growth pattern was either circular with the mycelia showing a uniform growth pattern and radial in a ring like pattern. The cultures on PDA grew well with growth rate of 80.93 mm after 7 days of incubation. The mycelium was hyaline, brown or both, sometimes abundant, at times sparse with floccose, loose or compact growth. In this study, based on cultural and morphological identification the isolate was identified as *C. gloeosporioides.*

Dual culture:

The result of the dual culture of antagonist and pathogen showed that the three species of *Trichoderma* namely *T. longibrachiatum, T. harzianum* and *T. asperellum* had antagonistic effect against *C. gloeosporioides* (Table 1).

Table 1: Types of interaction of the antagonists and pathogen

Non-pathogenic Fungi	Pathogenic Fungi	Grade	Interaction
1. *T. longibrachiatum (+)*	*C. gloeosporioides (-)*	Grade 2	Mutual intermingling
2. *T. harzianum(+)*	*C. gloeosporioides (-)*	Grade 2	Mutual intermingling
3. *T. asperellum(+)*	*C. gloeosporioides (-)*	Grade 2	Mutual intermingling

Note: (+)= aggressor (-)= victim; Grade 1= Mutual intermingling without any microscopic sights of interaction; Grade 2= Mutual intermingling growth where the growth of the fungus is ceased and bring over growth by the opposed fungus; Grade 3= Intermingling growth where the fungus under observation is growing into the opposed fungus either above or below; Grade 4= Sight inhibition of both the interacting fungus with narrow demarcation line (1-2); Grade 5= Mutual inhibition of growth at a distance of >2 mm

The bio control activity by *Trichoderma* can occur by means of several antagonistic mechanisms such as nutrient competition, antibiotic production, and mycoparasitism [8, 9].

Mycoparasitism has been proposed as the major antagonistic mechanism displayed by *Trichoderma* species [10].

Table 2: Percent growth inhibition of the three *Trichoderma* species against *C. gloeosporioides*

Antagonist	Percent inhibition after 14 days of inoculation (%)
T. longibrachiatum	64.68^{a}
T. harzianum	59.16^{b}
T. asperellum	47.73^{c}

*Means with different superscript are significantly different at 5% level of significance using DMRT

Based on the dual culture test, all of *Trichoderma* isolates inhibited the mycelia growth of *C. gloeosporioides* with varying efficiencies (Table 2). *T. longibrachiatum* obtained the highest percent growth inhibition after 14 days of incubation with a mean value of 64.68% while the lowest growth inhibition was rated in *T. asperellum*.

A microbial biological control agent may express different mechanisms against pathogen during their antagonistic activity. It weakens or destroys the pathogen, parasitize the pathogen directly, produce antimicrobial compounds, compete for space and nutrients and produce enzymes that attack the cell components of the pathogen [11]. Antibiosis, production of antibiotic compounds and inhibition of other microbes, is the most important mechanism expressed by the antagonistic bacteria [12]. In this study, the antagonistic activity expressed by *Trichoderma* species in dual culture method might be due to the one or combination of all above mechanisms.

Figure 2 shows the interaction exhibited by *Trichoderma* species against the pathogen *C. gloeosporioides.* Hyphal coiling of three *Trichoderma* species on the hyphae of *C. gloeosporioides* was observed.

The *Trichoderma* species are useful avirulent plant symbiots that act as bio control agent against phytopathogenic fungi via mechanisms of competition, rhizophere competence, mycoparasitism, antibiotic and enzyme production, induced resistance, and promoting plant growth [13, 14,15]. The majority of *Trichoderma* species are antagonist of phytopathogenic fungi and have been broadly used as the most important bio control agent [16].

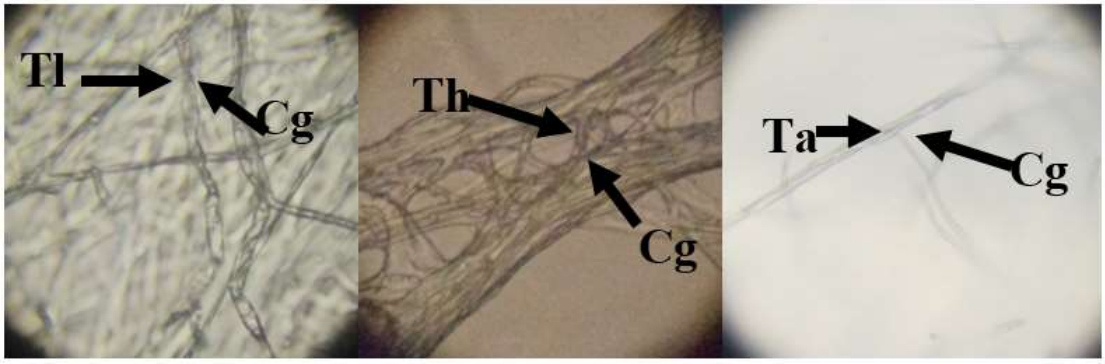

Fig. 2: Hyphal coiling of (A)*T. longibrachiatum* (Tl) on *C. gloeosporioides*(Cg) hyphae (B)*T. harzianum* (Th) on *C. gloeosporioides* (Cg) (C)*T. asperellum* (Ta) on *C. gloeosporioides* (Cg) hyphae.

Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host [17]. After host recognition, *Trichoderma* species attaches to the host hyphae via coiling and penetrate the cell wall by secreting cell wall-degrading enzymes [18].

Test for active compounds:

The percent inhibition obtained by the different concentrations of crude extracts from *Trichoderma* was evaluated as shown in Table 3. *T. asperellum* at 500 µl/ml crude extract concentration (TA 500) significantly obtained the highest percentage with a mean value of 24.40%. on the other hand, the lowest growth exhibited was noted in TA 1000 with a mean of 8.62%. Statistical analysis recorded significant differences among treatments evaluated.

Table 3: Percent growth inhibition of crude extracts of *Trichoderma* species

Crude extract concentration (µl/ml)	Growth inhibition (%)
TA100	9.23^{e}
TA500	24.40^{a}
TA1000	8.69^{e}
TL100	9.00^{e}
TL500	14.49^{c}
TL1000	12.00^{d}
TH100	11.22^{d}
TH500	15.58^{b}
TH1000	11.53^{d}

*Means with different subscript are significantly different at 5% level of significance

Increasing the concentration of *T. harzianum* (TH1000) to 1000 µl/ml showed no significant differences with TH 100 with the recorded inhibition rate of 11.53% and 11.22%, respectively. However, the result was

significantly comparable with the result obtained from *T. harzianum* at a lower concentration of 100 µl/ml with 11.22% inhibition rate.

Trichoderma species produce various volatile and non-volatile antimicrobial compounds which are active against a wide range of microorganisms [19]. Growth inhibition of the pathogens by the *Trichoderma* metabolites has been reported by several researchers [14, 18].

Pathogenicity test (onion bulb):

Pathogenicity test was performed to determine the ability of *Trichoderma* to inhibit the growth of *C. gloeosporioides* in vivo. Table 4 shows the disease severity of the onion bulb.

The inoculated onion bulb without antagonist recorded the highest lesion with a mean of 26.27 mm and highest disease rating of 4 which is highly pathogenic. On the other hand, onion bulb exhibited the smallest lesion with a mean of 3.89 mm and disease rating of 1.

Table 4: Disease severity of the onion bulb

Treatments	Mean of Lesions (mm)	Grading Scale	Indication
Control (uninoculated and untreated)	0	0	Non- pathogenic
C. gloeosporioides	26.27	4	Highly pathogenic
T. longibrachiatum vs. *C. gloeosporioides*	5.04	2	Mildly pathogenic
T. harzianum vs. *C. gloeosporioides*	3.89	1	Weakly pathogenic
T. asperellum vs. *C. gloeosporioides*	4.05	1	Weakly pathogenic

Trichoderma grows fast and compete with disease causing fungi for food, space as well as developing mycotoxin system for many soil or foliar pathogen [19], as well as stimulatory effect on growth of onion seedlings, seed germination, vigour index and fresh weight of seedlings [20]. It is well documented that the interaction of *Trichoderma* species with the plant may promote growth, improves crop yield, increase nutrient availability and enhance disease resistance [21,22]. In addition, some species of *Trichoderma* can colonize root surfaces, interact with the plant and exchange compounds that can cause substantial changes in plant metabolism [23]. Gajera et al. [24] reported that *Trichoderma* are develop exactly on other fungi's hyphae, coils around them and degrades the cell's walls. The action of parasitism restricts the development and activity of pathogenic fungi as well as produce secondary metabolites like cellular exochitinases [25,26] with antibiotic activity [13,27,28].

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