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Effect of a Fungal Pathogen, *Trichoderma hamatum*, on Growth and Germination of *Ciboria carunculoides* Under Laboratory Conditions

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Abstract.- *Ciboria carunculoides* is one of the major fungal pathogens that attack mulberry fruits and cause huge lose of mulberry production. Many *Trichoderma* spp. produce a wide variety of toxic and antibiotic metabolites that are active against plant pathogens. This study evaluated the biological control activity of *Trichoderma hamatum* against *C. carunculoides.* The results showed that *C. carunculoides* was not detected on mulberry leaves through out the collection period, whereas *C. carunculoides* was detected in mulberry fruits. *T. hamatum* spore, fermentation broth and ethylacetate extracts caused high radial growth inhibition and germination inhibition of *C. carunculoides*, with the inhibition value up to 80% and 90%, respectively. The LC_{50} of *T. hamatum* ethylacetate extract in radial growth inhibition of *C. carunculoides* was 778.4 µg/ml. The use of *T. hamatum* offered a promising, safe and effective alternative to fungicides in treatment against popcorn disease of mulberry.

Key words: *Trichoderma hamatum*, growth, germination, *Ciboria carunculoides*

INTRODUCTION

Mulberry is grown worldwide as a crop for silkworm rearing. Apart from this many parts of mulberry tree have been used as uncooked or processed foods for health care (Hong *et al*., 2007). Due to increase in fruit demand, growing area of the tree has increased remarkably (Kishi, 1998). According to the index of plant diseases, mulberries are susceptible to many pathogens (Anonymous, 1960). *Ciboria carunculoides* (Siegler and Jenkins) Whetzel, *C. shiraiana* (Henn.) Whetzel and *Scleromitrula shiraiana* (Henn.) Imai have been reported separately as causal agents of the fruit diseases (Kishi, 1998; Kohn and Nagasawa, 1984; Whetzel and Wolf, 1945). *C. carunculoides* is one of the major fungal pathogen that attack mulberry fruits by causing disease named as "popcorn disease". Popcorn diseased mulberry fruits have greatly enlarged ovaries with small and succulent calyx lobes in comparison with fleshy lobes of normal fruits (Gray and Gray, 1987). As a result, normal drupelets formation and maturation are prevented, thereby destroying the mulberries as edible fruit (Siegler and Jenkins, 1922).

The use of chemical fungicides like methamphetamine sodium, iprodione, carbendazium, procymidone, glyphosate, vinclozolin and dicloraz is one of the main tactics being used for the management of *C. carunculoides* (BenYephet *et al*., 1986; Hubbard *et al*., 1997; Ye *et al*., 2014), but it is more and more difficult to control disease with chemical fungicides. It is one of the important managements to control disease by using biological control methods. These contain application-specific fungal antagonists as well as vaccination to rhizosphere fungi antagonists such nonspecific *Trichoderma* and *Gliocladium* species (Mishike, 1998). *Trichoderma* species are common in soil adhering to root surface as well as in root surface plants (Mishra, 1996). These are reported as endophytic saprophytes as they readily colonies the root surface or cortex of host plant (Harman *et al*., 2004). Species of this genus are well-reported as biocontrol agents against several fungal pathogens through mechanisms such as mycoparasitism (mycelial coiling), antibiosis, cell wall degrading enzymes and induced resistance in host plant against diseases by altering plant gene expression (Pandya and Saraf, 2010; Alfano *et al*., 2007). Studies showed that *T. hamatum* can reduce the occurrence

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of foliar diseases of several vegetable crops by altering genes involved in stress and protein metabolism (Al Dahmani *et al*., 2005; Khan *et al*., 2004; Horst *et al*., 2005).

Although many studies have been conducted to investigate the effects of *Trichoderma* species on the growth and development of a number of fungal pathogens, few studies have directly examined their ability to control *C. carunculoides*. Therefore, these investigations examine the effects of different *T. hamatum* extracts on growth and germination of *C. carunculoides* in soil and plant leaves.

MATERIALS AND METHODS

Fungal strains

Ciboria carunculoides isolate (Cc01), originally isolated from Mulberry, deposited at Sericulture and Agri-food Research Institute of Guangdong Academy of Agricultural Sciences, was used in this study. The fungal isolate was identified as the method described by Hu *et al*. (2011). *Trichoderma hamatum* isolates originally from soil, deposited at Sericulture and Agri-food Research Institute of Guangdong Academy of Agricultural Sciences under 4°C condition. For inoculum production, *C. carunculoides* and *T. hamatum* were cultured on potato dextrose agar (PDA) medium (Potato infusion 200 g/L; Dextrose 20 g/L and Agar 20 g/L dissolved in deionized water 1000 ml and sterilized at 121℃ at 15 psi for 25 min) and incubated at 20±1°C and 26±1°C for 10 days, respectively. Conidia of *C. carunculoides* were harvested using the method as Liu and Robert (1993). Conidia of *T. hamatum* were harvested with distilled water containing 0.1% Tween-80 (Liu and Robert, 1993). Conidia were counted in a Fuchs-Rosenthal hemocytometer and a suspension of 1×10^7 conidia/ml was prepared.

Detection of the dynamics of Ciboria carunculoides *in mulberry leaf and soil*

Branch and soil near mulberry tree were collected one time per two weeks from March to September in mulberry. Mulberry leaves and fruits were detached from the branch samples and washed with deionized water three times before extracting DNA, respectively. *Ciboria carunculoides* was identified by PCR with primer sets (Cc-F: TCCTCCGCTTATTGATATGC and Cc-R: GGAAGTAAAAGTCGTAACAAGG) using the method as described by Hu *et al*. (2011). The specific fragment clone (about 500 bp) were amplified in a 50-ul reaction system (1 ul DNA solution as template, 0.2 uM each primer, 0.2 mM each dNTP, 2.5mM $MgCl_2$, 1×*Taq* Polymerase Buffer, and 2.5 U *Taq* Polymerase) by denaturation at 94℃ for 5 min, followed by 30 cycles of 30 s at 94°C, 1 min at 55°C, 1 min at 72°C and terminating with a final extension at 72°C for 10 min. The amplified fragments were sequenced at Invitrogen Com. (China) to verify the sequence. The soil sample contained the surface and 5 cm depth of the soil near mulberry tree. The sclerotia was obtained from the soil sample (300g soil) and then was put into sterile plastic Perti dish with wet filter paper inside and incubated at 20±1°C for germination examination.

Fermentation suspension and metabolic extract of Trichoderma hamatum

T. hamatum liquid culture was prepared by adding spores to Czapek liquid medium (composed of peptone 0.5%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, KCl 0.05%, $FeSO_4\cdot7H_2O$ 0.001%, sucrose 3% (w/v)). The liquid medium was incubated at 200 rpm and 26±1°C for 7 days. Fermentation suspension was prepared by filtering the liquid culture solution through Ø 0.22μm filter membrane. The solution together with mycelium was extracted by petroleum ether, ethyl acetate (EtOAC), acetone and methanol sequentially. Each of the metabolic extract was evaporated under reduced pressure, yielding 100 g of residue, which was stored at 4 ° C as the metabolic extract preparation for next experiments. All chemicals were purchased at Qianghui Bio-chemical Company.

Effects of fermentation suspension and different metabolic extract on radial growth of Ciboria carunculoides

Freshly prepared fungal suspension of *C. carunculoides* (100 μl of 1×10^6 conidia.ml^{-1}) was inoculated in the centre of plates with PDA medium using a micro applicator and was spread to cover the whole plate. The plates were incubated for mycelial

preparing at 20±2°C, 80±5% R.H., and L14:D10 h for 2-3 days. Mycelial discs together with medium (Ø1 cm) were removed and cultured on PDA medium having fermentation suspension and different metabolic extracts of *T. hamatum* (Table III), respectively, and incubated at 20±1°C. The same medium without fermentation suspension and metabolic extract served as a control. There were 10 Petri dishes for each fermentation suspension and different metabolic extract. Colony diameters were measured after 4-5 days. All the treatments were replicated three times. The average diameter of every colony was calculated as (long diameter + short diameter) / 2 (Ali *et al*., 2009). Percent mycelia growth inhibition was calculated as (the mycelium diameter in control minus that in treatment) divided by the mycelium diameter in control and then multiply 100%.

Effect of fermentation suspension and ethylacetate extracts on germination of C. carunculoides

A basal liquid medium was prepared with KNO_3 10 g, KH_2PO_4 5 g, $MgSO_4$ 2.5 g, $FeCl_3$ 0.02 g, Sucrose 50 g dissolved in deionized water into 1000 ml. The basal medium was sterilized at 121°C at 15 psi for 25 minutes. Fermentation suspension and ethylacetate extracts were added to the basal medium at different concentrations (Table V) while the basal medium without fermentation suspension and ethylacetate extract served as control. *C. carunculoides* (1×10^4 conidia.ml^{-1}) was added to each flask. The flasks were incubated at 20±1°C. Percent germination was calculated after shaking for 36 - 48 h at 200 rpm by placing one ml of suspension on cavity slides. Five separate fields were observed for germination at 400x magnifications for each treatment and over 500 conidia were observed randomly in each treatment or control. Conidia with germ tubes equal to or greater than the width were considered to have germinated. The entire experiment was replicated three times on different dates.

Effect of Trichoderma hamatum *and ethylacetate extract against* C. carunculoides *on leaf surface*

Mulberry leaf washed with deionized water was dipped into *C. carunculoides* with the concentration of 1×10^6 conidia ml^{-1} and then was put into clean plastic Petri dish. Once the treated leaves dried out, different concentrations of *T. hamatum* solution and ethylacetate extract (Table IV) were sprayed to the treated leaves surface, separately, while the treated leaf without *T. hamatum* and ethylacetate extract served as control. The plastic petri dish were incubated at 20±1 °C for 16-36 h and then the leaves were washed with deionized water for germination rate examination. The difference of spore between *C. carunculoides* and *T. hamatum* was easy to be identified by the morphological feature of spore that the spore of *C. carunculoides* is reniform with the size in 6.4-9.6×2.4-4 µm (Hu *et al*., 2011), and the spore of *T. hamatum* is ovoid and shortly ellipsoid with the size in 1.7-3.2×1.3-2.5µm average 2.4×1.9µm. Inhibitory percentage on germination was calculated as (the germination in control minus that in treatment) divided by the germination in control and then multiply 100%. The entire experiment was replicated three times on different dates.

Statistical analysis

All experiments were replicated three times and the results were expressed as average of three times determinations. Radial growth, inhibition growth and inhibition germination data were analyzed by Analysis of variance (ANOVA) and treatment means were compared by using Tukey's HSD test for mean comparisons at 5% level of significance. All statistical analysis was performed using SAS 8.01(2000).

RESULTS

Dynamics of Ciboria carunculoides *in mulberry leaf and soil*

Total 120 leaves were tested by PCR with the primer sets (Cc-F/Cc-R) to identify infection of *Ciboria carunculoides*, the result showed that the specific DNA fragment was detected from all infected fruit sample and was identified with 100% similarity to the sequence of HQ833459.1 in NCBI (Fig. 1).

The data regarding percentage detection of *C. carunculoides* on mulberry leaf and fruit at different collection times has been shown in Table I.

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Query 1     CCTGATTCGAGGTCATCCGTAGAATTGATAGGCGGAAGCCGTCGAGGCCCGTAACGAGAG   60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 486   CCTGATTCGAGGTCATCCGTAGAATTGATAGGCGGAAGCCGTCGAGGCCCGTAACGAGAG   427

Query 61    GTTACTACGTTCAGGACCCAACGGCGCCGCCACTGATTTTAGAGCCTGCCATCGCTGACA   120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 426   GTTACTACGTTCAGGACCCAACGGCGCCGCCACTGATTTTAGAGCCTGCCATCGCTGACA   367

Query 121   CGGCTCAATACCAAGCTGGCTATACCCTGTTCCCAGGCTACGCCTCTTGATGGCTATATG   180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 366   CGGCTCAATACCAAGCTGGCTATACCCTGTTCCCAGGCTACGCCTCTTGATGGCTATATG   307

Query 181   ACGCTCGAACAGGCATGCCCTTCGGAATACCCAAGGGCGCAATGTGCGTTCAAAGATTCG   240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 306   ACGCTCGAACAGGCATGCCCTTCGGAATACCCAAGGGCGCAATGTGCGTTCAAAGATTCG   247

Query 241   ATGATTCACTACTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCC   300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 246   ATGATTCACTACTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCC   187

Query 301   AGAACCAAGAGATCCGTTGTTGAAAGTTTTAATGTTATATAGTGCTCAGACGATAAAAAA   360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 186   AGAACCAAGAGATCCGTTGTTGAAAGTTTTAATGTTATATAGTGCTCAGACGATAAAAAA   127

Query 361   TAGTGTGTGATGAGATTGGCCTGCGCCAAAGCAACGTGTGCATAACCGAGGGTGGTGATC   420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 126   TAGTGTGTGATGAGATTGGCCTGCGCCAAAGCAACGTGTGCATAACCGAGGGTGGTGATC   67

Query 421   TTGTCACTCTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACG           471
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 66    TTGTCACTCTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACG           16
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Fig. 1. The partial sequence of *Ciboria carunculoides* amplified from sample with primer set.

C. carunculoides was not detected on mulberry leaves through out the collection period whereas *C. carunculoides* was detected in all the infected mulberry fruits from March 15 to May 25 before the total fruit harvesting from May 25th to 30th. The appearance of *C. carunculoides* on no-infected fruits showed some interesting findings having different rates of disease appearance on different collection dates. Disease appearance rates increased during March 15th to April 30th and decreased afterwards. Maximum disease appearance on infected fruit (61.3%) was observed on April 30th (Table I).

Table I.- Percentage of detection of *Ciboria carunculoides* in mulberry leaf and fruit (%)

Collection time	Infected leaf	Infected fruit	Appearance no infection fruit
March 15	0	100	28.5± 3.0 c
March 30	0	100	41.6 ± 3.8 b
April 15	0	100	46.1 ± 3.7 b
April 30	0	100	61.3 ± 4.5 a
May 15	0	100	27.9 ± 2.3 c
May 24	0	100	5.8 ± 1.5 d
F, df, P			93.54, 5, <0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

The number of *C. carunculoides* sclerotia and their germination rates in soil during collection period from March 15th to September 30th has been shown in Table II. The number of *C. carunculoides* sclerotia differed at different collection period with a gradual increase in sclerotia production from March 15th to April 30th and decrease afterwards. The highest number sclerotia (432) were produced on 30th April whereas the lowest number of *C. carunculoides* sclerotia (38) was observed on

September 30th. The germination of *C. carunculoides* in soil remained constant during March 15th to May 15th and decreased afterwards. Maximum rate of *C. carunculoides* germination in soil (100%) was observed from March 15th to May 15th while the lowest germination (19.4%) was observed for September 30th (Table II).

Table II.- Examination the number of fungal sclerotia in soil and its germination rate

Collection time	Number of fungal sclerotia	Germination rate
March 15	114	100.0± 0.0 a
March 30	132	100.0± 0.0 a
April 15	211	100.0± 0.0 a
April 30	432	100.0± 0.0 a
May 15	307	100.0± 0.0 a
May 30	214	79.2 ± 4.5 b
June 15	174	71.8 ± 3.9 b
June 30	101	59.5 ± 3.1 c
July 15	84	40.3 ± 2.6 d
July 30	72	31.6 ± 3.2 e
August 15	65	26.2 ± 3.5 ef
August 30	53	21.9 ± 2.2 f
September 15	45	20.6 ± 2.7 f
September 30	38	19.4 ± 1.9 f
F, df, P		105.3, 13, <0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

Table III.- Effects of fermentation broth and different metabolic extracts on radial growth of *C. carunculoides*

Treatment	Conc.	Radial growth (mm)	Inhibition rate (%)
Fermentation broth	Crude broth	8.4±2.3 d	87.7±4.9 a
Petroleumether extract	2000 µg/ml	32.8±3.6 b	52.0±3.7 c
ethylacetate extract	2000 µg/ml	10.1±1.6 d	85.2±6.1 a
Acetone extract	2000 µg/ml	23.1±2.4 c	66.2±5.3 b
Methanol extract	2000 µg/ml	21.2±3.1 c	69.0±48 b
CK (distilled water)		68.3±4.5 a	0.0±0.0 d
F, df,		63.7, 5,	81.5, 5,
P		<0.0001	<0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

Effects of fermentation broth and different metabolic extract

Radial growth of Ciboria carunculoides

The radial growth and the percentage germination inhibition of *C. carunculoides* by *T. hamatum* fermentation broth and different metabolic extracts differed significantly among different treatments (Table III). The highest radial growth (68.3 mm) was observed in control whereas the lowest radial growth (8.4 mm) was observed in fermentation broth. Similarly, the highest inhibition (87.7%) was observed for *T. hamatum* fermentation broth and the lowest inhibition rate (52.0%) was observed for petroleum extract. The inhibition rates for *T. hamatum* fermentation broth and ethylacetate extracts were similar with the inhibition rates of 87.7 and 85.2%, respectively.

Table IV.- Effects of fermentation broth and ethylacetate extract on radial growth of *C. carunculoides*

Treatment	Concentration	Radial growth (mm)	Inhibition rate (%)
Fermentation broth	Crude broth	8.5±1.3 de	87.5±4.4 a
	2×crude dilution	16.5±2.1 d	75.7±3.6 b
	5×crude dilution	28.5±2.6 c	58.1±3.1 c
Ethylacetate extract	500 µg/ml	42.5±3.9 b	37.5±2.7 d
	100 µg/ml	31.7±2.5 c	53.4±3.5 c
	2000 µg/ml	10.3±2.1 d	84.9±4.1 ab
	3000 µg/ml	7.3±1.8 de	89.3±3,5 a
	4000 µg/ml	1.0±1.7 e	98.5±3.2 a
CK (Dist. water)	0 µg/ml	67.6±4.2 a	
F, df, P		65.8, 8, <0.0001	87.9, 7, <0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

The effects of *T. hamatum* fermentation broth and ethylacetate extracts used at different concentrations on radial growth and percentage inhibition have been shown in Table IV. Different concentrations of *T. hamatum* fermentation broth and ethylacetate extracts influenced the radial growth of *C. carunculoides* when compared to control. In case of *T. hamatum* fermentation broth, lowest radial growth (8.5 mm) was observed when crude extract was applied and higher radial growth was observed for 2X and 5X dilution of *T. hamatum* fermentation broth. For *T. hamatum* ethylacetate extracts the lowest radial growth (1.0 mm) was observed for 4000 µg/ml concentration whereas the lowest radial growth was shown by 500µg/ml concentration with mean radial growth of 42.5 mm.

The percentage inhibition of *C. carunculoides* by different concentrations of *T. hamatum* fermentation broth and ethyl acetate extracts differed among different concentrations (Table IV). For *T. hamatum* fermentation broth highest inhibition (87.5%) was observed for whereas the lowest inhibition rate was shown by 5X fermentation broth with average inhibition of 58.1%. In case of *T. hamatum* ethylacetate extract, lowest inhibition rate (37.5%) was observed for 500 µg/ml concentration and highest inhibition rate was observed for 4000 µg/ml. The LC_{50} of *T. hamatum* ethyl acetate extract in radial growth inhibition of *C. carunculoides* was 778.4 µg/ml, with the regression equation of Y=-2.5016+2.5946X (R^2=0.9665, X^2=4.8126, $P_{0.05}$ (658.93, 919.51), LC_{95}=3350.90 µg/ml).

Germination of C. carunculoides

The germination and growth inhabitation rates of *C. carunculoides* differed significantly under different concentrations of *T. hamatum* fermentation broth and ethylacetate extracts when compared to control (Table V). Highest *C. carunculoides* germination (97.6%) was observed in control. For *T. hamatum* fermentation broth, the lowest germination rate (11.4%) was observed when crude fermentation broth was applied showing (88.3%) inhibition over control while in case of *T. hamatum* ethylacetate extracts the lowest germination rate (0%) was observed for 5000 µg/ml concentration having 100% inhibition over control.

Table V.- Effects of fermentation broth and ethylacetate extract on germination of *C. carunculoides*

Treatment	Concentration	Germination rate (%)	Inhibition rate (%)
Fermentation broth	Crude broth	11.4±2.7 d	88.3±4.5 b
	2×crude dilution	24.5±2.8 c	74.9±3.7 c
	5×crude dilution	42.5±2.3 b	56.5±3.4 d
Ethylacetate extract	2000 µg/ml	14.1±1.8 d	85.6±4.1 b
	4000 µg/ml	1.8±1.2 e	98.1±4.3 a
	5000 µg/ml	0.0±0.0 e	100.0±0.0 a
CK (Dist.water)		97.6±5.2 a	
F, df, P		127.3, 5, <0.0001	53.7, 4, <0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

Effect of T. hamatum *and ethylacetate extract against* C. carunculoides *on leaf surface*

The effects of *T. hamatum* conidia and ethylacetate extracts used at different concentrations on germination inhibition on mulberry leaf surface have been shown in Table-6. Significantly different rates of growth inhibition were observed for *T. hamatum* crude fermentation broth and the concentrations of 1×10^6 conidia*ml^{-1} of *T. hamatum.* Highest germination inhibition (86.1%) was observed for 1×10^6 conidia*ml^{-1} of *T. hamatum*. In case of *T. hamatum* ethylacetate extracts, the lowest germination inhibition (74.5%) was observed for 2000 µg/ml concentration whereas the highest germination inhibition was observed for 5000 µg/ml concentration with average inhibition of 100%.

Table VI.- Effect of *T. hamatum* **and** ethylacetate **extract against** ***C. carunculoides*** **on leaf surface**

Fungicide	Concentration	Inhibition germination (%) (Mean± S.E)
Trichoderma hamatum	Crude fermentation broth	81.7 ± 3.4 a
	1×10^6 conidia*ml^{-1} spore	65.9 ± 4.1 b
	1×10^8 conidia *ml^{-1} spore	86.1 ± 3.2 a
		F=63.8, df=2, P<0.0001
Ethylacetate extract	2000 µg/ml	74.5 ± 4.7 c
	4000 µg/ml	91.4 ± 3.1 b
	5000 µg/ml	100.0 ± 0.0 a
		F=97.3, df=2, P<0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

DISCUSSION

The use of synthetic chemicals for plant disease management can harm the environment as well as human health (Vinale *et al.*, 2008). Therefore many physical and biological tools are being used as a safer alternative to the use of chemical fungicides. These alternate tools include the use of biological control agents, plant bioactive compounds and physico-chemical methods (El-Hassan *et al.*, 2013). Many Trichderma spp. have been used to protect commercially important crops (Verma *et al.*, 2007).

The present study addresses the biological control activity of *T. hamatum* against *C.*

carunculoides. The questions to be answered are what are the factors involved in this inhibition. Varying interactions and degrees of inhibition in growth and development of *C. carunculoides* with *T. hamatum* on agar plates and plant leaves were studied to investigate the mechanisms of control. Understanding the mechanism(s) of action involved in the bio-control processes is of primary importance in establishing these characteristics. Such an understanding can provide much insight about where and when the interaction occurs and how the pathogen will be affected. In order to survive and compete, *Trichoderma* produces a wide variety of toxic and antibiotic metabolites that are active against plant pathogens and extracellular hydrolytic enzymes which were involved in the inhibition, competition, and mycoparasitism of phytopathogenic fungi. (Thrane *et al*., 2000; Eziashi *et al*., 2006; Vinale *et al*., 2008; Andrabi *et al*., 2011). Our results showed that *T. hamatum* fermentation broth and extracts caused inhibition in radial growth and germination of *C. carunculoides* on agar plates. The inhibition of radial growth and germination that occurred on agar plates is considered as antibiosis and competition for nutrients and/ or space, as defined by Cook and Baker (1983). The antibiotic metabolites may inhibit the pathogen activities by diffusing toxic chemical substances from the antagonist in the medium or due to its direct effect on the target pathogen by occupying the whole area of growth. On the other hand, the toxicity of antibiotic compounds released in the culture filtrate by *T. hamatum* which completely inhibited the growth of the pathogen mycelium may be similar to the metabolites produced by other Trichoderma species.

The biocontrol potential and growth promotion of Trichoderma has been widely studied and used. Trichoderma saprophytic and endophytic ability to colonize above ground parts has received quite a bit of attention in the past few years (Metcalf and Wilson, 2001; Hohmann *et al*., 2011). Although *T. hamatum* has an ability to grow more rapidly on complex carbon, cellulose and nutrient substrates, typical of those found on root surfaces, El-Hassan *et al*. (2013) showed that *T. hamatum* can also be an efficient endophytic colonizer of the above ground parts. Our results also showed that different conidial concentrations of *T. hamatum, T. hamatum* fermentation broth and ethylacetate extracts caused germination inhibition of *C. carunculoides* and this inhibition increased with increase in concentration. Another reason for studying the efficacy of *T. hamatum* and ethylacetate extract against *C. carunculoides* on leaves was that the microenvironment of leaf surface is similar to that surrounded mulberry flower, so we imitated the microenvironment to test the effect of *T. hamatum* and ethylacetate extract against *C. carunculoides* during the flower period. The flower period is a short and sensitive time to be infected by *C. carunculoides*.

In conclusion, our data show that the *T. hamatum* have potential biocontrol activity against popcorn disease of mulberry caused by *C. carunculoides*. Therefore, the use of *T. hamatum* offered a promising, safe and effective alternative to fungicides in treatment against popcorn disease of mulberry. However, further studies are required to render these isolates technically and economically for efficient use as biocontrol agents on agronomic scale.

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