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RESEARCH ARTICLE

BIOLOGICAL CONTROL OF *BIPOLARIS SPICIFERA*, *CURVULARIA LUNATA*, *FUSARIUM* SPP., *NIGROSPORA ORYZAE, EXSEROHILUM ROSTRATUM, ALTERNARIA ALTERNATE* AND *THANATEPHORUS CUCUMERIS* ON IRAQI RICE UNDER LABORATORY AND GREENHOUSE CONDITIONS

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ABSTRACT

This research dealt with findings that included laboratory and greenhouse studies on the efficacy of our isolates of *Trichoderma* spp. in controlling *Fusarium* spp., *Nigrospora oryzae, Exserohilum rostratum, Bipolaris spicifera*, *Curvularia lunata*, *Alternaria* spp., and *Thanatephorus cucumeris*. Ten isolates of *Trichoderma* spp. were pre-screened on chitin plates to determine its ability to digest chitin therefore indicating the presence of chitinase which is a good disease resistance candidate. In vitro test conducted with *Trichoderma* isolate T.7 against 23 isolates of pathogens via the dual culture technique showed that isolates exhibited high antagonistic activity and reduced radial growth for each pathogen. As a consequence of dual culture assay, greenhouse study was conducted to determine the effectiveness of *Trichoderma* isolate T.7 to suppress major phytopathogens by using local rice variety cv. *Forat Anbar* as the model plant in autoclaved soil. Four parameters were tested; antagonistic activity, disease incidence, disease severity and population density parameter of *Trichoderma* isolate T.7 for each pathogen. The greenhouse experiment exhibited *Trichoderma* isolate T.7 greater efficiency of reducing disease severity in majority of pathogens e.g. T.7+*T.cucumeris* R2, T.7+*F.solani* R11, T.7+*N. oryzae* R9 and T.7+*A.alternata* R20 which reduced disease severity by approximately 2.2 %. *Trichoderma* isolate T.7 addition had significant effect on growth parameters of rice plant, fresh and dry weight as well as nitrogen and protein concentration.

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INTRODUCTION

Sheath blight, rot root and blast diseases are a major problem as the causal agents of rice due to produce complex secretomes (Dean *et al.,* 2005; Maryam *et al.,* 2013, Sousa *et al.,* 2013). Therefore resistance towards these pathogens can be provided through the development of resistant genotypes (Najeeb *et al.,* 2008; Hamdia, 2014). However the resistance reported by any cultivar is short-lived as there is resistance breakdown three to four years into use (Kim and Kim 2009; Liang-fen *et al.,* 2010; Cai-Lin *et al.,* 2011; Ali and Alwan, 2012). Plant microbe interactions that are beneficial to the plant system would include the use of biological control agents that have the ability to reduce the devastating impact of plant pathogens such as *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* (Yanjun and Shiping 2010; Nandani *et al.,* 2012). Rice diseases have been caused by various plant pathogens which have extremely broad host range and the high survival rate of resistant forms such as chlamydospores under different environmental conditions (Khosravi *et al.,* 2011; Kamaluddeen and Abhilasha, 2013; Schwarz *et al.,* 2012; Maryam *et al.,* 2013; Zhang *et al.,* 2012). These pathogens which are the most important disease influencing rice production and food safety in many rice-growing countries in the world (Salih *et al.,* 2000; Sami, 2006; Shabana *et al.,* 2008; Filippi *et al.*, 2011; Nur Ain *et al.,* 2011; Sharma *et al.,* 2014). Plant growth promoting microorganisms (PGPM) and biological control agents (BCA) have secondary beneficial effects that may be exploited for utilization as bio-inoculants (Tyler *et al.*, 2008; Nenkateswarlu *et al.,* 2015). PGPM approaches can be used to control rice diseases, such as *Trichoderma* spp. have been reported to have strong

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antagonistic activity that may be capitalized to reduce soil borne pathogen populations. *Trichoderma* strains as a biomass, as of their variety of activities displayed have a large range of applications which developed the antagonistic potential which consider a basis of the effective control, and their mode of action is antagonists of most post-harvest as biocontrol agents of wide sets of phytopathogenic fungi (Gao, 2016; Nakazawa *et al.,* 2016). And the biodegradative efficiency is a reach source of useful enzymes, their antagonists selected as evidence of direct their interactions or after the demonstration that substances or antibiotics toxic to the potential pathogens were secreted into the growth medium (Nadia and Aliaa, 2014). *Trichoderma* is a fungal genus that was described in 1794 including many species is initially isolated from soil and decomposing organic matter. Strains within this genus include a wide spectrum of evolutionary solutions that range from very effective colonizers with high biodegradation potential, to hard plant symbiosis or colonize to the rhizosphere area (Behdani *et al.,* 2012). Moreover, many strains of *Trichoderma* spp. are able to antagonize plant pathogens (Manandhar and Yami, 2008), using competition for the substrate, antibiosis, and parasitism as main antagonistic strategies (Hamdia *et al.,* 2015). The antagonistic potential is the base for effective applications of different *Trichoderma* strains as an alternative to the chemical control against a wide set of fungal plant pathogens (Harman *et al.,* 2004). Overall, the mycoparasitism of *Trichoderma* spp. according to Hamdia and Kalaivani (2014) is the most widely studied. However, *Trichoderma* strains mycoparasitism are difficult to demonstrate and still not clear to understand in situ until recent time due to the technical difficulties in making microscopic observations e.g. fluorescence imaging and differential staining to the soil–root interface (Thornton *et al.,* 2002).

Cell wall of *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp.and *T. cucumeris* structure from chitin, glucan and other oligosaccharide compounds, and these compounds are exist inside each hyphal apex, the glucanase and chitinase produced by *Trichoderma* strains will play a crucial role in the degradation of cell walls pathogens, and these enzymes were induced by provided chitin to the media as a carbon source (Nadarajah *et al.,* 2014). As a consequence of the variety of activities displayed by the *Trichoderma* strain conglomerate, a large range of applications have been developed: the antagonistic potential is the basis for the effective control of a wide set of phytopathogenic fungi (Hamdia and Kalaivani, 2013)**.** The aim of this study is to determine the effectiveness of *Trichoderma* isolate T.7 as biocontrol agent against six isolates of *T. cucumeris*, eight isolates of *Fusarium* spp., one isolate of *N. oryzae*, two isolates of *C. lunata*, one isolate of *B.spicifera*, one isolate of *E.rostratum* and four isolates of *Alternaria* spp. in rice plant.

MATERIALS AND METHODS

Preparation of chitin assay

Ten (10) different *Trichoderma* isolates (T.1, T.2, T.3, T.4, T.5, T.6, T.7, T.8, T.9 and T.10) were cultured on media chitin agar plates to screen for chitinase activity is listed based on their chitinase activity. A colloidal suspension of chitin was prepared according to the Hamdia (2014) with some modified as following: Chitin (16.0g) was digested in 150 mL of H_3PO_4 and completed the volume to 100 mL by 1L of deionized distilled water (ddH_2O) at temperature 5°C. The colloidal suspension passed through a whatman #1 filter paper and pH 7.0. Chitin plates were prepared per 1L as following: 15.0g of Agar, 10.0g precipitated chitin; 2.0g of $NaNO_3$, 1.0g of KH_2PO_4, 0.5g of $MgSO_4.7H_2O$, 0.5g of KCl, and pH adjusted to 5.0. Spores of *Trichoderma* isolates were dissolved in 10 mL of distilled water, and completed the method as mentioned in Hamdia (2014). One hundred (100) µl of dilutions 1×10^9 spore/mL was applied aseptically on potato dextrose agar (PDA) plates via spread plate technique, and incubated for 4 days at (28±2 ^{0}C) to observe fungal growth over a period of four days. Four days post culturing, the colony forming units (CFU) for each isolate was determined according to Hafedh (2006), *Trichoderma* isolates were screened using the chitin plate repeated twice.

In vitro Study of Antagonistic Ability between *Trichoderm*a sp. T.7 and Rice Phytopathogens

One isolate was chosen among 10 *Trichoderma* isolate determined for antagonistic activity against plant pathogens via the dual plate method and greenhouse study based on chitinase activity study. Antagonistic study was conducted using dual culture technique, twenty-three pathogens (*B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris*) were selected for these studies under the laboratory conditions based on the virulence assay was conducted under greenhouse conditions by Hamdia *et al.* (2016). In this study one week old fungal culture of the rice pathogens were placed individually on one half of the plates that were divided into two equal portions and the other had a 5 mm disk of *Trichoderm*a sp. T.7, the plates were incubated for 5 days at (28±2 ^{0}C). The antagonistic activity was scored according to the scale of Alfredo and Aleli (2011), mean of three plates (9 cm diameter) were used as replicates for each treatment.

Greenhouse Experiment

Antagonistic Ability between *Trichoderm*a sp. T.7 and Rice Phytopathogens

The experiment was carried out under greenhouse conditions with various rice pathogens were isolated from rice plant in the laboratory as it has been mentioned previously. Seeds of *Oryza sativa* cv. *Forat Anbar* obtained from Agriculture Research Directorate were used in this study as it is the most widely grown rice variety in Iraq. The rice seeds were placed on sterilized plastic dishes and then incubated at 40 ^{0}C for 48 hours to stimulation their germination. The filter paper was then moistened by using 3 mL ddH_2O and the seeds were left for 3 days at (28±2 ^{0}C). The soil used in this experiment was sterilized at 121 ^{0}C /1.5 Kg / cm^2 for 1.5 hours and prepared into pots, and filled with 4 kg of soil (Clay: 25%, Sand; 47.15%, Silt: 27.85%), the soil was then infested with *T.cucumeris* R1,*T.cucumeris* R2, *T.cucumeris* R4,*T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F. oxysporum* R6, *F.solani* R8,

F.solani R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7,*C.lunata* R21, *B. spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 besides control without microbes, watered and mixed daily to keep moistened for one week. Once these seeds had germinated, the seedlings roots of cv. *Forat Anbar* were inoculated with each pathogen suspension as mentioned below by dipping them for 15 minute before transplantation into pathogen infested soils. Every experimental pot was allocated 3 seedlings. After paddy plants growth had stabilized, transplantation seedlings were visually examined for wilt symptoms, later the disease score was estimated for disease incidence and severity after 4 months from planting. The data for disease incidence and severity is evaluated according to (Hamdia, 2014). The data from observations were statistically analysed using a randomized complete design (RCD), via a Duncan's Multiple Rating Test (Duncan 1955), the experiments were applied in triplicate.

Preparation of Organisms

For greenhouse experiment, all fungal isolates were cultured on potato dextrose agar (PDA) media and incubated at (28±2 ^{0}C) for seven days. For this experiment which was involving rice pathogens, the inoculum size was set at (9×10^7) of *C. lunata* R7, *C.lunata* R21(21×10^6), *N. oryzae* R9 (6×10^5), *F.solani* R3 (1×10^7), *F. oxysporum* R6 (15×10^5), *F.solani* R8 (23×10^8), *F.solani* R11 (17×10^8), *F.solani* R13 (5×10^8), *F.solani* R16 (11×10^9), *F.verticillioides* R17 (23×10^{11}), *A.alternata* R18 (45×10^4), *A.alternata* R20 (14×10^4), *Bipolaris spicifera* R15 (3×10^5), *E.rostratum* R19 (9×10^4), *A.tenuissima* R23 (12×10^4) and *A.tenuissima* R24 (23×10^4) spore /mL. *T.cucumeris* R1 was counted about (25×10^6), *T.cucumeris* R2 (23×10^5), *T.cucumeris* R4 (32×10^5), *T.cucumeris* R10 (9×10^5), *T.cucumeris* R12 (13×10^5) and *T.cucumeris* R14 (19×10^5) basidia in 4 fields/dish.

Study of *Trichoderm*a sp. T.7 as Biological Control Agent against Rice Phytopathogens

The greenhouse experiment was carried out using *Trichoderm*a sp. T.7 as the best *Trichoderma* isolates among 10 isolates during their chitinolytic assays. *Trichoderm*a sp. T.7 that was tested against pathogens *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* under the laboratory conditions. Though the laboratory experiments provided us with the excellent antagonistic ability, we repeated the experiments with *Trichoderm*a isolate T.7 against all pathogens as there may be differences in reactivity shown on agar and in the soil. The inhibitory ability of *Trichoderm*a isolate T.7 against various pathogens isolates on rice plant was studied in pots. We chose to initially conduct with autoclaved soil so that we may see the interaction of *Trichoderm*a isolate T.7 on the above phytopathogens, inoculum size for *Trichoderma* spp. was containing about 1 x 10^9 spore /mL.

Fresh and Dry Weight Parameters

In this study fresh weight was calculated of shoot, root and Panicle for each treatment by using a sensitive balance type (Mettler pc 4000), dry weight of shoot, root and Panicle were estimated in the oven type (Memmert) under temperature at 65 ^{0}C for 72 hours. Kueldahl Apparatus was used to appreciate nitrogen and protein in the panicle in the end of season according to the method described in (Hamdia *et al.,* 2015).

RESULTS

Screening of *Trichoderma* spp. for Chitinolytic Assays

The result of the chitin plate assay is denoted in Fig.1 where typical plates with colony forming unit (CFU) on chitin agar plates as denoted in (Fig. 2), plates with good growth were yellowish in color and produced significant chitinolytic activity as it is seen on plates (Fig. 2). Differences were observed among isolates with respect to chitinase production after induction with colloidal chitin and chitin degradation. Among all the *Trichoderma* isolates, T.7 and *Trichoderma* isolate T.8 were showed high CFU ability (6.07×10^9 and 6.05×10^9 cfu/100µl respectively) were observed that have the most efficient chitinase production (Fig. 1 and 2), and chitinase production was observed within 48 hours of induction with colloidal chitin containing medium. One other hand, in isolates where chitinase production was not observed, growth of the isolates was very slow e.g. *Trichoderma* isolate T.2 and T.3 were gave lowest value chitinase enzyme activity 1.41×10^9 and 1.96×10^9 cfu/100µl respectively.

Table 1. Antagonistic activity between *Trichoderma* isolate T.7 and *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* under laboratory conditions

Treatments	**Degree of antagonism after 5 days
T.7+*T.cucumeris* R1	+++
T.7+*T.cucumeris* R2	++
T.7+*T.cucumeris* R4	++
T.7+*T.cucumeris* R10	++
T.7+*T.cucumeris* R12	++
T.7+*T.cucumeris* R14	++
T.7+*F.solani* R3	++
T.7+*F.oxysporum* R5	+++
T.7+*F. oxysporum* R6	++
T.7+*F.solani* R8	++
T.7+*F.solani* R11	+++
T.7+ *F.solani* R13	++
T.7+*F.solani* R16	++
T.7+*F.verticillioides* R17	++
T.7+*N. oryzae* R9	+++
T.7+*C. lunata* R7	++
T.7+*C.lunata* R21	++
T.7+*Bipolaris spicifera* R15	++
T.7+*E.rostratum* R19	++
T.7+*A.alternata* R18	+++
T.7+*A.alternata* R20	+++
T.7+*A.tenuissima* R23	+++
T.7+*A.tenuissima* R24	+++

**According to scale by Alfredo and Aleli (2011) that involve four degrees:

+++ The antagonistic fungus was able to grow over the pathogen and pathogen growth completely inhibited.

++ The pathogen growth completely inhibited, but antagonist was not able to grow over the pathogen.

+ Mutual inhibition initially, but antagonist was overgrown by pathogen.

- Pathogen growth not inhibited, antagonist was overgrown by pathogen.

Figure 2. Chitinase enzyme activity assay, chitin plates with the ten *Trichoderma* isolates, besides cultured each isolate on plates minus chitin as control

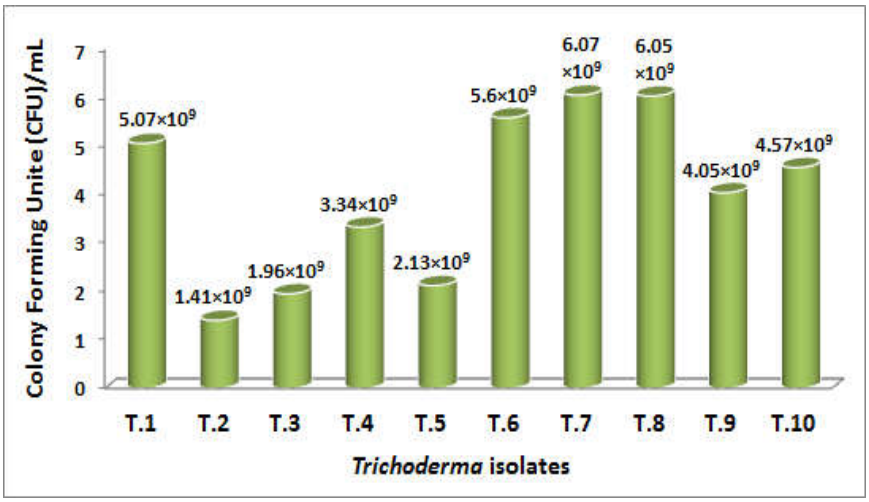

Figure 1. The colony forming unit of *Trichoderma* isolates

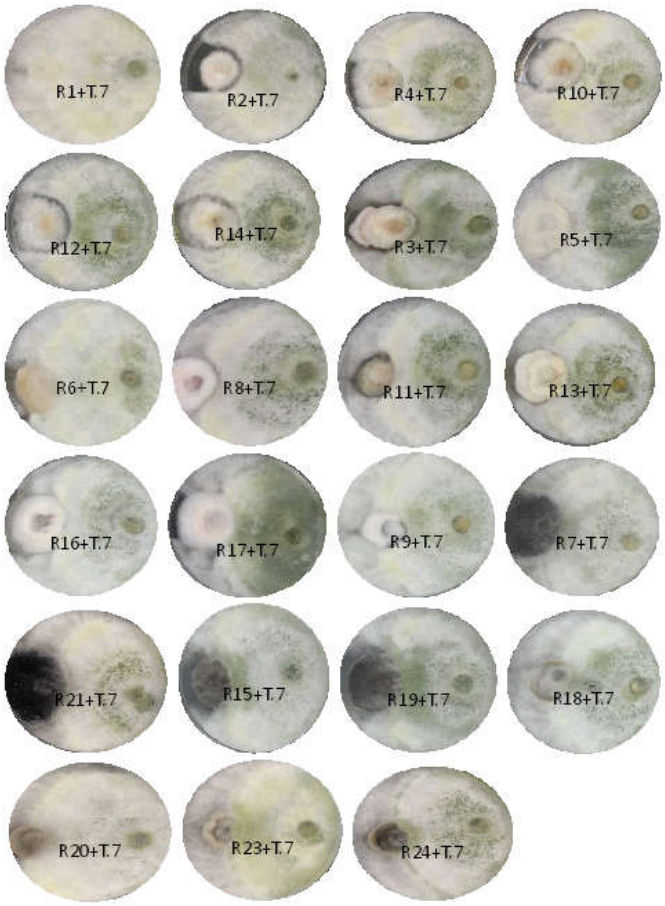

Figure 3. Antagonistic activity between *Trichoderma* isolate T.7 and *T.cucumeris* R1,*T.cucumeris* R2,*T.cucumeris* R4,*T.cucumeris* R10,*T.cucumeris* R12,*T.cucumeris* R14,*F.solani* R3,*F.oxysporum* R5,*F. oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7,*C.lunata* R21, *Bipolaris spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 via dual culture technique under laboratory conditions. As seen in plates the *Trichoderma* isolate T.7 (right side) appears to have covered the pathogens cultures (left side)

In vitro Study of Antagonistic Ability between *Trichoderma* sp. T.7 and Rice Phytopathogens

Trichoderma sp. T.7 was selected as the best isolate among ten *Trichoderma* isolates during their chitin plate assay. On the basis of preliminary screening of virulent pathogenicity test in greenhouse experiment according to Hamdia *et al*. (2016), *Trichoderma* sp. T.7 was used via the dual culture technique combination with *T.cucumeris* R1,*T.cucumeris* R2,*T.cucumeris* R4,*T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3,*F.oxysporum* R5, *F. oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7, *C.lunata* R21, *B. spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 to determine the isolates that showed virulence activity. *Trichoderma* sp. T.7 displayed high ability to reduce radial growth of all pathogens ability five days post-inoculation (Fig. 3), but was varied in antagonistic activity. The best antagonistic potential were displayed by *Trichoderma* sp. T.7 with *T.cucumeris* R1, *F.oxysporum* R5, *F.solani* R11, *N. oryzae* R9, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R22 and *A.tenuissima* R23 (Table 1). All these isolates scored (+++ degree) inhibition of target. The *Trichoderma* sp. T.7 inhibited the growth of pathogens by covering 80 to 100 % growth of the 9 cm petri dishes (Fig. 3). *Trichoderma* sp. T.7, showed with the rest pathogens moderate antagonistic activity (++ degree).

Table 2. Antagonistic activity between *Trichoderma* isolate T.7 and *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* under greenhouse conditions

Treatments	*Disease incidence	**Disease severity
Control	0.0 g	0.0 I
Trichoderma T.7 alone	0.0 g	0.0 I
T.7+*T.cucumeris* R1	11.1 f	8.9 fgh
T.7+*T.cucumeris* R2	11.1 f	2.2 hi
T.7+*T.cucumeris* R4	22.2 e	14.3 f
T.7+*T.cucumeris* R10	11.1 f	4.4ghi
T.7+*T.cucumeris* R12	44.4 c	23.3 e
T.7+*T.cucumeris* R14	33.3 d	24.4 e
T.7+*F.solani* R3	66.7 a	66.7 a
T.7+*F.oxysporum* R5	22.2 e	22.2 e
T.7+*F. oxysporum* R6	22.2 e	22.2 e
T.7+*F.solani* R8	22.2 e	22.2 e
T.7+*F.solani* R11	22.2 f	2.2 hi
T.7+ *F.solani* R13	55.6 b	55.6 b
T.7+*F.solani* R16	22.2 e	8.9 fgh
T.7+*F.verticillioides* R17	55.6 b	37.8 d
T.7+*N. oryzae* R9	11.1 f	2.2 hi
T.7+*C. lunata* R7	33.3 d	33.3 d
T.7+*C.lunata* R21	44.4 c	35.6 d
T.7+*Bipolaris spicifera* R15	55.6 b	46.7 c
T.7+*E.rostratum* R19	11.1 f	11.1 gf
T.7+*A.alternata* R18	11.1 f	4.4ghi
T.7+*A.alternata* R20	11.1 f	2.2 hi
T.7+*A.tenuissima* R23	65.1 a	60 ab
T.7+*A.tenuissima* R24	33.3 d	24.5 e

*Disease incidence and **Severity of rice plants, the column that have same letters do not differ significantly from each other at p ≤0.05 according to Duncan's multiple range tests. The experiments were conducted in triplicate for each pathogen. *Disease incidence and **Severity were scored after 3 months from sowing according to Hamdia (2014).

The Effect of *Trichoderma* sp. T.7 as Biological Control Agent against Rice Phytopathogens in Greenhouse Conditions

Disease incidence and severity prameters of a local rice variety (cv.) Forat Anbar against causal agents *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* showed a significant differences ($p<0.05$) level of stimulation and increasing rice plant growth when treated with *Trichoderma* sp. T.7 (Fig. 3). The greenhouse experiment showed that *Trichoderma* sp.T.7 was effective in inducing significant decrease ($p<0.05$) in disease incidence e.g. *Trichoderma* T.7 alone, T.7+*T.cucumeris* R1, T.7+*T.cucumeris* R2, T.7+*T.cucumeris* R4, T.7+*T.cucumeris* R10, T.7+*N. oryzae* R9, T.7+*E.rostratum* R19, T.7+*A.alternata* R18 and T.7+*A.alternata* R20 were gave lowest value 11.1%. However, T.7+*F.solani* R3, T.7+*A.tenuissima* R23, T.7+ *F.solani* R13, T.7+*F.verticillioides* R17 and T.7+*B. spicifera* R15 were gave disease incidence at approximately 66.7, 65.1, 55.6, 55.6 and 55.6 % respectively.

Table 3. The effect of Pathogens on percentage of the shoot weight rate, root weight, dry weight and fresh weight of rice plant

Treatments	Average*of plant growth parameters				
	fresh weight of Shoot (g)	fresh weight of Root (g)	dry weight of Shoot (g)	dry weight of Root (g)	dry weight of Panicle (g)
Control	28.03 de	10.20 cd	9.64 cdefghi	1.66 efg	0.82 ghi
Trichoderma T.7 alone	44.43 b	13.05 b	13.13 bcd	2.46 cde	1.92 def
T.7+*T.cucumeris* R1	38.34 bc	4.37 fghi	7.23 efghij	1.83 efg	2.04 cdef
T.7+*T.cucumeris* R2	33.097 cd	8.27 de	12.12 bcde	2.33 cdef	1.23 fgh
T.7+*T.cucumeris* R4	14.71 hi	3.31 ghij	10.51 bcdefgh	0.98 fg	0.91 ghi
T.7+*T.cucumeris* R10	27.99 de	10.89 bc	9.04 cdefghi	3.17 bcd	1.57 efgh
T.7+*T.cucumeris* R12	26.15 def	2.99 ghij	7.98 defghij	1.54 efg	2.14 cdef
T.7+*T.cucumeris* R14	21.74 efgh	4.36 fghi	6.99 efghij	1.15 efg	1.82 def
T.7+*F.solani* R3	10.00 i	1.91 ij	4.78 ij	0.76 g	1.35 efgh
T.7+*F.oxysporum* R5	20.87 efgh	4.04 ghi	5.92 fghij	1.07 fg	1.55 efgh
T.7+*F. oxysporum* R6	36.88 bc	2.66 hij	11.20 bcdef	1.75 efg	2.10 cdef
T.7+*F.solani* R8	27.95 de	1.86 ij	8.95 cdefghi	0.87 g	0.73 hi
T.7+*F.solani* R11	40.99 bc	11.42 bc	15.11 ab	3.45 bc	2.88 abc
T.7+ *F.solani* R13	27.48 de	4.93 fgh	8.90 cdefghi	1.14 efg	1.68 defg
T.7+*F.solani* R16	16.74 ghi	6.73 ef	5.08 hij	1.04 fg	2.22 bcde
T.7+*F.verticillioides* R17	24.25 defg	2.45 hij	7.91 defghij	1.29 efg	2.24 bcde
T.7+*N. oryzae* R9	40.67 bc	10.10 cd	13.59 bc	2.34 cde	3.08 ab
T.7+*C. lunata* R7	13.85 hi	1.11 j	4.39 ij	0.81 g	1.46 efgh
T.7+*C.lunata* R21	18.50 fghi	2.50 hij	6.91 efghij	1.15 efg	2.12 cdef
T.7+*Bipolaris spicifera* R15	11.85 i	3.39 ghij	5.24 hij	1.99 defg	1.53 efgh
T.7+*E.rostratum* R19	32.49 cd	5.33 fg	10.93 bcdefg	2.28 cdef	1.93 def
T.7+*A.alternata* R18	59.69 a	17.99 a	18.67 a	4.80 a	3.68 a
T.7+*A.alternata* R20	44.02 b	10.89 bc	15.06 ab	3.82 ab	2.60 bcd
T.7+*A.tenuissima* R23	10.67 i	4.52 fghi	3.07 j	1.04 fg	0 .70 i
T.7+*A.tenuissima* R24	15.98 ghi	3.88 ghi	5.57 ghij	1.56 efg	0.88 ghi

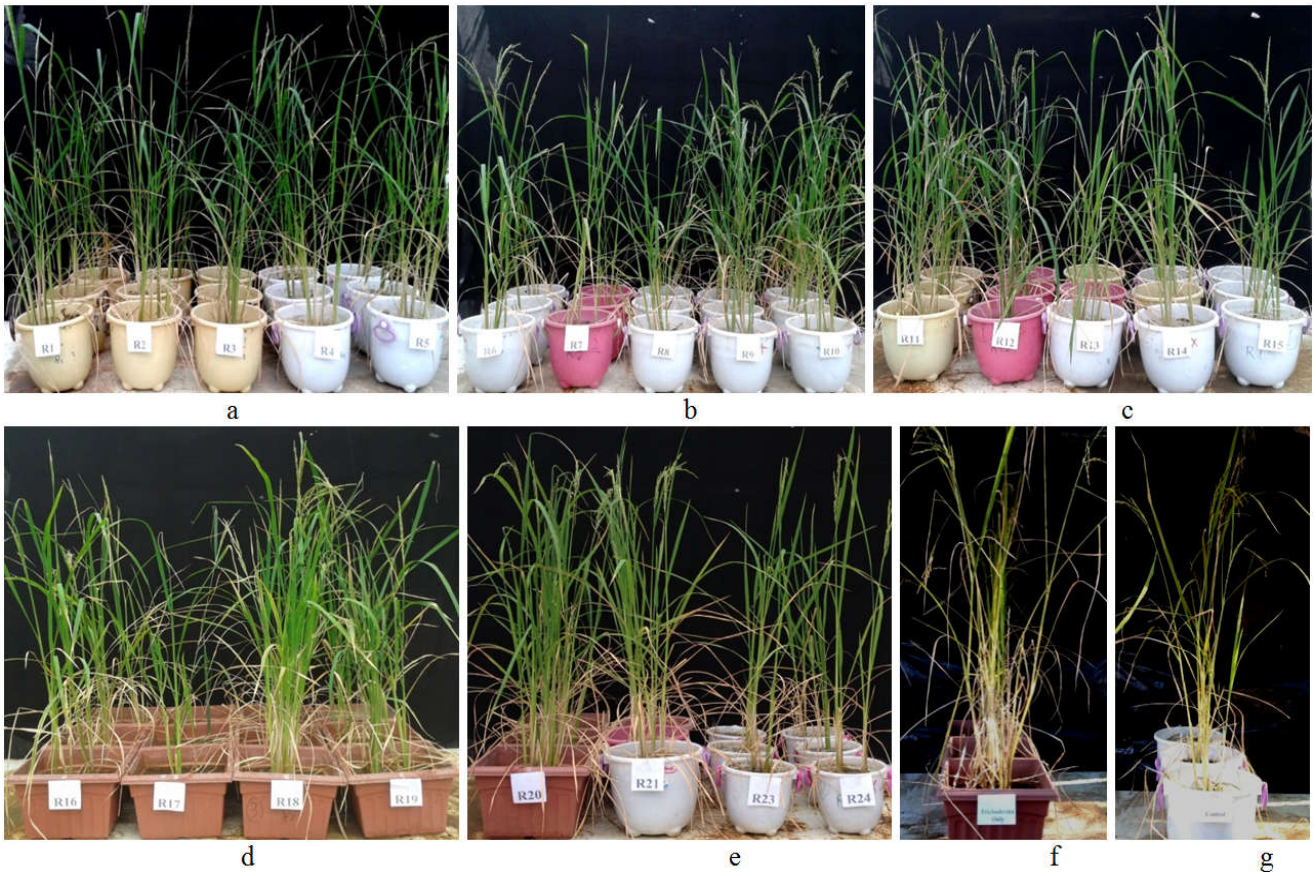

Figure 4. The effect of *T.harzianum* in combination with *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* infections on rice plants in the end of growth,under greenhouse conditions. (a) Control untreated. (b) *T.cucumeris* R1, *T.cucumeris* R2, *F.solani* R3, *T.cucumeris* R4, *F.oxysporum* R5. (c) *F. oxysporum* R6, *C.lunata* R7, *F.solani* R8, *N.oryzae* R9, *T.cucumeris* R10. (d) *F.solani* R11, *T.cucumeris* R12, *F.solani* R13, *T.cucumeris* R14, *Bipolaris spicifera* R15. (e) *F.solani* R16, *F.verticillioides* R17, *A.alternata* R18, *E.rostratum* R19. (f) Plants inoculated with *Trichoderma* isolate T.7. (g) Control (natural infection)

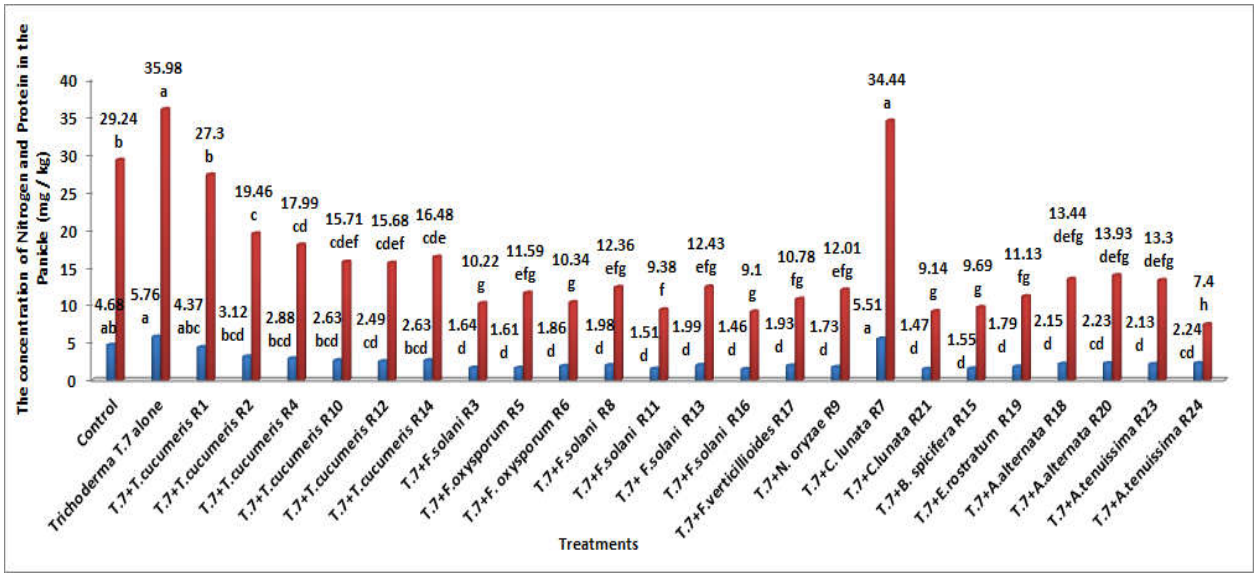

Figure 5. The concentration of nitrogen and protein in the rice panicle (mg / 0.25 gm of seeds), as comparison between rice plant pathogens treated with *Trichoderma* isolate T.7 under greenhouse condition. Numbers in each column that have same letter do not differ significantly from each other at p<0.05 according to Duncan's multiple range test. The column blue color indicates to the nitrogen according to the Mohammed (2002), and column red color indicates to the protein according to the Merrill and Watt (1973)

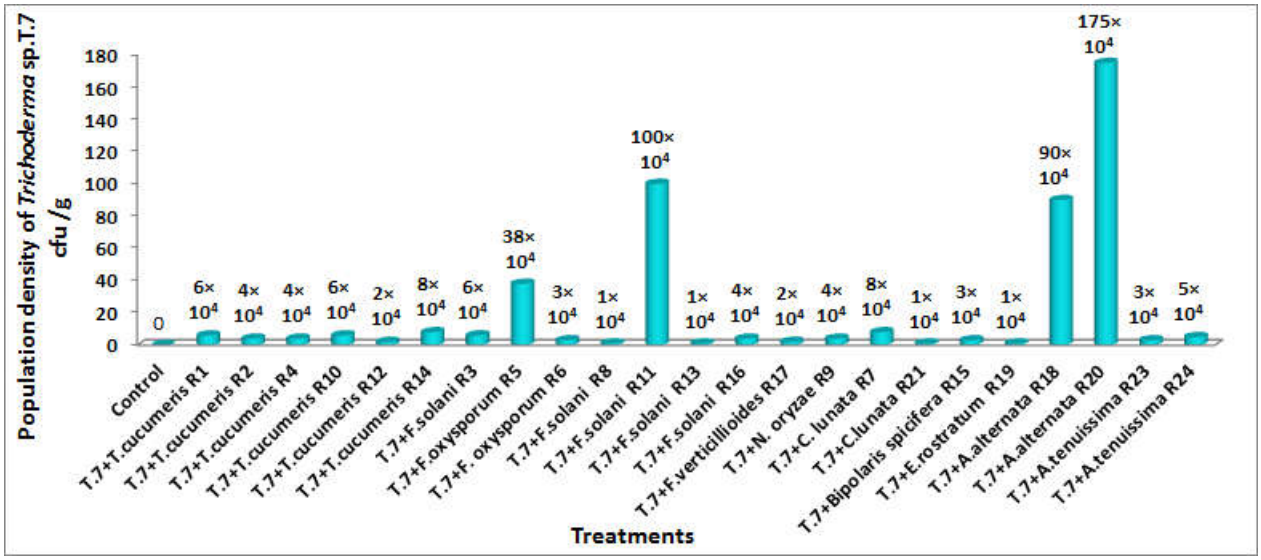

Figure 6. Population density of *Trichoderma* spp. in the end of experiment under greenhouse condition

Visual ratings of disease severity parameter decreased significantly in T.7+*T.cucumeris* R2, T.7+*F.solani* R11, T.7+*N. oryzae* R9 and T.7+*A.alternata* R20 were gave 2.2% respectively as comparison with T.7+*F.solani* R3, T.7+*A.tenuissima* R23 and T.7+ *F. solani* R13 which resulted in significant effect of pathogens at approximately 66.7, 60 and 55.6 % respectively (Table 4).

The Efficiency of *Trichoderma* isolate T.7 on Plant Growth Parameters (Morphological) Fresh Plant Root Weight, Plant Shoot Weight, and Dry Weight

Table 3 shows the results of the effect of *T.harzianum* was added to the soil on fresh shoot weight of the rice plant after the end of the season. We can see the *Trichoderma* isolate T.7+*A.alternata* R18, *Trichoderma* isolate T.7 alone, T.7+ *A.alternata* R20, T.7, T.7+*F.solani* R11 and T.7+*N. oryzae* R9 as they gave high value 59.69, 44.43, 44.02, 40.99 and 40.67 g respectively, as compared to T.7+*B. spicifera* R15, T.7+*A.tenuissima* R23 and T.7+*F.solani* R3 which gave lowest value 11.85, 10.67 and 10 g respectively compared to the control treatment that achieved 28.03 g (Table 3). Fresh root weight results showed increased in T.7+*A.alternata* R18, *Trichoderma* isolate T.7 alone, T.7+*F.solani* R11, T.7+*A.alternata* R20, T.7+*T.cucumeris* R10, control and *N. oryzae* R9 were gave 17.99, 13.05, 11.42, 10.89, 10.89, 10.20 and 10.10 g as comparison with T.7+*F.solani* R3, T.7+*F.solani* R8 and T.7+*C. lunata* R7 which gave 1.91, 1.86 and 1.11 g respectively. Dry shoot weight of the rice plant with T.7 was increased in T.7+*A.alternata* R18, T.7+*F.solani* R11, T.7+*A.alternata* R20, T.7+*N. oryzae* R9 and *Trichoderma* isolate T.7 alone were gave 18.67, 15.11, 15.06, 13.59, 13.13

and 13.05 g respectively than T.7+*A.tenuissima* R23, T.7+*F.solani* R3 and T.7+*C. lunata* R7 which exhibited 3.07, 4.78 and 4.39 g respectively. Dry weight of Root revealed that T.7+*A.alternata* R18, T.7+*A.alternata* R20, T.7+*F.solani* R11 and T.7+*T.cucumeris* R10 were gave 4.80, 3.82, 3.45 and 3.17 compared to T.7+*T.cucumeris* R4, T.7+*F.solani* R3, T.7+*F.solani* R8 and T.7+*C. lunata* R7 which gave 0.98, 0.76, 0.87 and 0.81 g respectively. Dry weight of Panicle was showed a significant increase in T.7+*A.alternata* R18 and T.7+*N. oryzae* R9 were exhibited 3.68 and 3.08 g than T.7+*T.cucumeris* R4, T.7+*A.tenuissima* R24, control, T.7+*F.solani* R8 and T.7+*A.tenuissima* R23 which revealed 0.91, 0.88, 0.82, 0.73 and 0 .70 g respectively (Table 3).

The results had been shown in Figure 6 exhibited concentration of nitrogen and protein were evaluated in panicle. The concentration of nitrogen was increased in treatments *Trichoderma* T.7 alone, control, T.7+*T.cucumeris* R1 and T.7+*C. lunata* R7 which gave 5.76, 4.68, 4.37 and 5.51 mg / kg respectively, as in comparison with lowest value in T.7+*C.lunata* R21 and T.7+*F.solani* R16 were gave 1.47 and 1.46 mg / kg. The concentration of protein according to the Merrill and Watt (1973)was found high value in *Trichoderma* T.7 alone, T.7+*C. lunata* R7, control (treatment without pathogen), T.7+*T.cucumeris* R1 and T.7+*T.cucumeris* R2 were gave 35.98, 34.44, 29.24, 27.30,19.46, 17.99 mg / kg respectively. However, there was a significant decreased of protein value found in T.7+*A.tenuissima* R24 which was gave 7.40 mg / kg as we can see in (Fig. 5). In this study, Fig. 6 shows that population density (CFU) of *Trichoderma* spp. in the end of experiment under greenhouse condition found the highest colonization in *A.alternata* R20, *F. solani* R11 and T.7+*A.alternata* R18, they were gave 175×10^4, 100×10^4, and 90×10^4 cfu /g respectively. However as we can see from (Fig. 6) the lowest value were found with T.7+*F.solani* R8, *F.solani* R13, *C. lunata* R21 and *E.rostratum* R19 which they gave approximately 1×10^4 cfu /g.

DISCUSSION

The colony forming unite was used in this study as a parameter of the ability of the isolate to degrade chitin and utilize it as substrate, chitin breakdown would involve the production of chitinase enzyme. The growth ability of *Trichoderma* isolate T.7 on colloidal chitin plate was highest value 6.07×10^9 cfu/100µl as compared to the other nine isolates (Fig. 1 and 2), and evaluated as the best source for chitinolytic assays based on laboratory assay. This finding is similar with many authors that found several species of Trichoderma have been documented to prevent wilt disease by the highest chitinolytic enzyme activity (Susana, 2006; Hamdia, 2014). *Trichoderma* isolate T.7 with pathogens *B. spicifera*, *C. lunata*, *Fusarium* spp., *N. oryzae, E. rostratum, Alternaria* spp. and *T. cucumeris* were the most antagonistic scoring +++ score on the dual culture test. Although we had identified the best isolates via dual culture assays we used all isolates as there have been incidences where different results were obtained on plates then in soil. Trichoderma sporulation could overcome the heavy sporulation of pathogens and eventually outgrow the pathogen as we can see in (Fig. 3). Here we believe that Trichoderma parasitized pathogens as the pathogen could not be re-isolated from these plates, *Trichoderm*a sp. T.7 that scored (+++) was stopped the growth of the pathogen prior to contact. *Trichoderm*a sp. T.7 was considered as highly antagonistic and was able to colonize pathogens within 4 days as reported by several researchers (Sundaramoorthy *et al.,* 2012; Hamdia, 2013 and 2014, Samuelian, 2016).The interaction between *Trichoderm*a sp. T.7 and pathogens is the best example of the influence of metabolites produced by both the organisms on each other as seen in (Fig. 4) where a zone of interaction between antagonists and pathogen is clearly visible (Poornima,2011; Rawat *et al.,*2015).

When we refer to the antagonistic activity shown on dual culture techniques in Table 1 and Fig. 3, the best antagonistic potential were displayed by *Trichoderm*a sp. T.7 with *T.cucumeris* R1, *F.oxysporum* R5, *F.solani* R11, *N. oryzae* R9, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23, *F.solani* R3 and *A.tenuissima* R24 (Table 1). Here however, when applied *Trichoderm*a sp. T.7 in the soil, the only *Trichoderm*a sp. T.7 showed identical results in the dual culture technique and provided better antagonistic values to suppress disease under greenhouse with *F.solani* R11, *N. oryzae* R9 and *A.alternata* R20 which reduced disease severity in certain pathogens by approximately 2.2 % Table 2, which indicated a possibility of effective mycoparasitism in soil against pathogens as compared to T.7+*F.solani* R3 and T.7+*A.tenuissima* R23 which showed significant increasing in disease severity 66.7 and 65.1%. There have been previous reports that showed that *Trichoderm*a spp. was able to induce growth under different condition (Ashraf *et al.,* 2005; Shalini and Kotasthane, 2007; Schuter and Schmoll 2010; Alfredo and Aleli 2011). Also, the results had been shown in Fig. 5 and 6 exhibited significant differences ($p<0.05$) in nitrogen and protein concentration such as T.7+*C. lunata* R7 which gave 5.51 and 34.44 mg/gm as in comparison with rest of pathogens. This however, certain researchers stated protein concentration of seeds infected by *C. lunata* was not significantly correlated with vegetative aggregates as we can see in Fig. 5 (Seshu *et al.,* 1988). The population density parameter of fungal biomass *Trichoderm*a isolate T.7 (Fig. 6) had been decreased disease severity when applied with *A.alternata* R20 under greenhouse conditions, which typically gave high percentage of colony forming unit CFU at approximately 175×10^4 cfu /g in the end of season. Fungal population density were correlated significantly with growth parameter (Yoshioka *et al.,* 2012) may be due to the production of chitinase and glucanase (Nadarajah *et al.,* 2014; Shahbazi *et al.,* 2014) that are thought to be involved in mycoparasitism presented in (Fig. 4).

Conclusion

This study was conducted using rice as the host plant and tests were conducted in the laboratory and greenhouse by using *Trichoderm*a isolate T.7 to select for suitable candidate to be further studies at the field level against these pathogens. The beneficial effect of biological agent was shown in the lower degree of disease infected and severity demonstrated. This study has been identified *Trichoderm*a isolate T.7, future work should be directed towards obtaining more methods that may result in excellent inhibition of rice pathogens.

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