SCIENCE LETTERS 2013 | Volume 1 | Issue 1 | Pages 21-24

Volatile and non-volatile antifungal compounds produced by *Trichoderma harzianum* SQR-T037 suppressed the growth of *Fusarium oxysporum* f. sp. *niveum*

Waseem Raza*[1], Muhammad Faheem[1], Sohail Yousaf[3], Faheem Uddin Rajer[1], Muhammad Yameen[2]
[1]College of Resources and Environmental Sciences, Nanjing Agricultural University, 210095, Nanjing, China
[2]Department of Applied Chemistry & Biochemistry, Government College University, Faisalabad, Pakistan
[3]Department of Environmental Sciences, Quaid-i-Azam University, Islamabad, Pakistan

Abstract
Trichoderma is widely used in agricultural biotechnology and have been already used as biocontrol agents against numerous plant pathogens and quite a few have been developed for commercial use. We isolated a new strain of *Trichoderma harzianum* SQR-T037 and evaluated the antifungal potential of its volatile and non-volatile compounds against *Fusarium oxysporum in vitro*. The results demonstrated that strain SQR-T037 produced volatile compounds that can inhibit the growth of *F. oxysporum* up to 40%, while the non-volatile antifungal compounds extracted from the liquid culture significantly inhibited the growth of *F. oxysporum*. In the dual culture assay, the strain SQR-T037 overgrew the strain *F. oxysporum*. The incubation time of six days with a temperature of 30°C and pH of 6 were found optimum for the maximum production of antifungal compounds by strain SQR-T037. This research showed the great potential of strain SQR-T037 as an antagonist to control the *Fusarium* wilt of watermelon under greenhouse and field conditions.
Key words: Antagonism, biocontrol, *Trichoderma harzianum*, volatile compounds.

Received June 15, 2013; **Revised** July 11, 2013; **Accepted** July 18, 2013
***Corresponding author:** Dr. Waseem Raza; Email: waseem@njau.edu.cn; Phone: +86-15161459593; Fax: +86-2584432420

Introduction

Watermelon (*Citrullus lanatus* Thumb.) is one of the important cash crops in many areas of the world but its production is under threat because of the emergence of soil borne diseases. Fusarium wilt of watermelon caused by *Fusarium oxysporum* f. sp. *niveum* is one of those threatening diseases [1]. An intensive farming practice aggravated wilt disease in China. The pathogen can exist in the soil for several years through the production of chlamydospores [2] and is difficult to control. Watermelons are susceptible to Fusarium wilt at all growing stages and infected seedlings stunted, the older leaves drop or may turn yellow and plants normally wilt and die [3]. Conventional methods, such as crop-rice rotation, cultivars resistance and moisture regulation were all useless. The application of fungicides might be the useful method to control *Fusarium* wilt, but it would cause huge environmental problems and ecological disaster [4]. Biological control agents (BCAs) provide a suitable alternative to control *F. oxysporum* in an environment friendly manner [5]. Several BCAs, *Ulocladium atrum* [6], *Bacillus* spp. [7] band *Pseudomonas* spp. [8], have been reported effective to control Fusarium diseases. As antagonists, *Trichoderma* spp. have also been reported to be effective for the biocontrol of multiple soil-borne plant diseases [9, 10]. The fungus *Trichoderma* contains many species and strains and is commonly present in nearly all soils. Some strains of Trichoderma are saprophytic while others are pathogenic to other fungi such as *Pythium* [11, 12].

Trichoderma is widely used in agricultural biotechnology and have been already used as biocontrol agents against numerous plant pathogens and quite a few have been developed for commercial use [11, 13]. *Trichoderma* readily colonizes plant roots and some strains are rhizosphere competent i.e. able to grow on roots and form symbiotic associations with plants. *Trichoderma* species are resistant to most agricultural chemicals, including fungicides, although individual strains differ in their resistance [14]. We isolated a new strain of *Trichoderma harzianum* SQR-T037 and evaluated the antifungal potential of its volatile and non-volatile compounds against *F. oxysporum in vitro*.

Material and Methods

Microbial strains

An antagonistic strain *T. harzianum* SQR-T037 (SQR-T037) and pathogen strain *Fusarium oxysporum* f. sp. *niveum* from the Jiangsu Key Laboratory for Organic Solid Waste Utilization, Nanjing Agriculture University, China was cultured on potato dextrose agar (PDA) and maintained at 4°C.

Antifungal volatile compounds assay

Antifungal volatile compounds assay was conducted in dual plates. The modified MS medium (1.5% agar, 1.5% sucrose, and 0.4% TSA (w/v)) was used to inoculate strain SQR-T037 in one plate except control plates and the other plates containing PDA medium (potato infusion 200g, dextrose 20g, agar 15g in 1L of distilled water) was used for *F. oxysporum* to test the growth inhibition of the volatile compounds. The pathogen containing plates was placed over strain SQR-T037 containing plate and

both plates were sealed with parafilm and incubated at 28°C for three days and then the diameters of the fungal growth on the plates were measured up to 9 days.

Antagonism dual culture assay

The antagonism dual culture assay was conducted in PDA medium. An 8 mm plug of strain SQR-T037 was placed adjacent to the one side of plate while an 8 mm plug of *F. oxysporum* was placed adjacent to the other side of the plate and incubated at 28°C. After seven days, growth of both fungi was observed.

Nonvolatile compounds production by strain SQR-T037 and their optimization

For the optimum incubation time for maximum antifungal compounds production, a 8 mm plug from PDA plate of strain SQR-T037 was inoculated in PDB medium and inculcated at 28°C for 8 days. Each day, the biomass of strain SQR-T037 and antifungal activities of ethyl acetate extract of broth culture were evaluated. For collecting biomass and ethyl acetate extract, the biomass of strain SQR-T037 was collected by eight layers of sterilized cheese cloth, and then centrifuged at 12000 rpm for 10 min. The collected biomass materials were pooled together and dried at the 60°C before weighing. Cell free liquid culture was added with ethyl acetate and placed in a shaker over night at room temperature. Next day, ethyl acetate was collected, evaporated to dryness using rotary evaporator and the dried residues were dissolved in methanol. The antifungal activity of extract was determined by agar diffusion assay using *F. oxysporum* as test pathogen.

For the determination of optimum temperature for maximum production of antifungal compounds by strain SQR-T037, 8 mm plug of strain SQR-T037 was inoculated in PDB medium and incubated at different temperatures from 20-40 °C with the difference of 5 °C. Similarly, for the determination of optimum pH, PDB medium was prepared with different pH values from 2-7 with 1N HCl or 1N NaOH and after the inoculation of strain SQR-T037, incubated at 28 °C and 170 rpm. After five days, total biomass and cell free liquid culture was collected as described earlier. The liquid culture was extracted with ethyl acetate and evaluated for antifungal compounds production by agar diffusion assay using *F. oxysporum* as test pathogen.

The effect of different C sources on antifungal compound production by strain SQR-T037 was evaluated. The C sources used are listed in Table 4. After five days, biomass was determined and antifungal compounds from cell free liquid culture were extracted with ethyl acetate and antifungal activity was evaluated as described earlier.

Results and Discussion

Antifungal activity of volatile compounds

The results of antifungal volatile compounds assay produced by strain SQR-T037 showed that the volatile compounds were highly effective to suppress the growth of *F. oxysporum* up to 9 days. The continuous growth inhibition of *F. oxysporum* was reached from 18 to 40 % from first to nine days (Fig. 1). In addition, in the presence of volatile compounds produced by strain SQR-T037, F. oxysporum was not able produced pigment. Hundreds of volatile compounds are produced by *Trichoderma* spp. like Siddiquee et al. [15] reported the production of 282 volatile compounds by *T. harzianum*. Many biocontrol antagonistic strains have been reported to produce volatile compounds that inhibit the growth of pathogen fungi significantly [16]. Volatile compounds produced by *T. harzianum* decreased the growth of *Colletotrichum capsici* which causes leaf blight on basil, chickpea and pepper as well as dieback in pigeon pea [17]. In addition, Yang et al., [18] reported nematicidal activity of volatile compounds produced by *Trichoderma* spp. These volatile compounds are also helpful for producing strains as *Trichoderma* conidiation was induced by its own volatile compounds [19].

Table 1: Effects of volatile compounds produced by *Trichoderma harzianum* SQR-T037 on the growth of *Fusarium oxysporum.*

Days	Control (with out volatile compounds) (mm)	With volatile compounds (mm)
1	8.5	7
2	15	12
3	20	16
4	25	18
5	28	20
6	33	22
7	36.5	24
8	41	25.5
9	46	27.5

Antagonism dual culture assay

The results of antagonism dual culture assay results showed that the strain SQR-T037 out compete and overgrow *F. oxysporum* in a few days. This shows the excellent antagonistic potential of strain

SQR-T037 against *F. oxysporum*. Similar results of *T. harzianum* ATCC 74058 to over compete *Penicillium citreonigrum* and *Fusarium graminearum* were reported by Konakovsky [20].

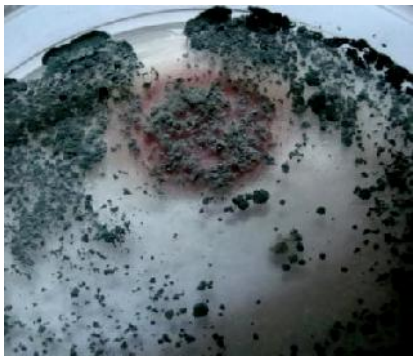

Fig. 2: Over growth of *Trichoderma harzianum* SQR-T037 on *Fusarium oxysporum* in dual antagonism assay**.**

Antifungal activity of non-volatile compounds

The results of antifungal activity of nonvolatile or diffusible compounds produced by strain SQR-T037 showed that the extracted antifungal compounds significantly inhibited the growth of *F. oxysporum* in agar diffusion assay as shown in Fig. 2. The *Trichoderma* strains have been reported to produce different antimicrobial compounds like 6-pentyl-α-pyrone, with a strong, coconut-like aroma, was produced by *T. harzianum* in liquid culture [21]. This compound showed broad-spectrum antimicrobial characteristics against species such as *Rhizoctonia solani*, *F. oxysporum* f. sp. *lycopersici* [22], *Botrytis cinerea* [23] and *F. moniliforme* [24]. The antifungal compounds production is most important mechanism of action for the effective biocontrol of soil borne diseases under green house or field conditions as has been reported by Thomashow and Weller [25].

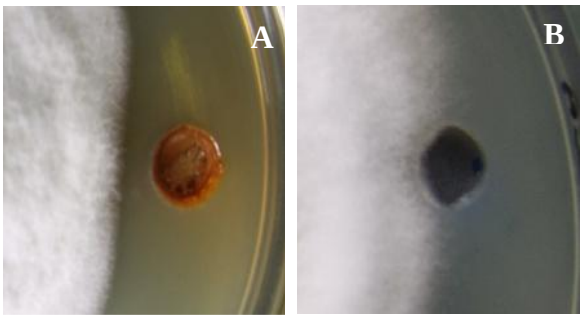

Fig. 3: Effect of antifungal compounds produced by *Trichoderma harzianum* SQR-T037 against *Fusarium oxysporum***. A**: antifungal compounds application; **B**: Control (methanol).

Effect of growth conditions and C sources on antifungal compounds production

The results of incubation time effect on antifungal compounds production showed that the maximum biomass of strain SQR-T037 was obtained after five days while maximum antifungal activity was observed after six days. The temperature of 30^{o}C with the pH of 6 was found optimum for the maximum biomass production and maximum antifungal compounds production by strain SQR-T037. The results also showed that the lower pH values were more effective than higher pH values in promoting antifungal compound production.

Table 2: Effect of incubation time on antifungal compounds production by *Trichoderma harzianum* SQR-T037.

Days	Dry biomass (g/L)	Antifungal activity (mm)
1	1.56	5
2	2.71	9
3	3.68	13
4	6.30	14
5	8.29	17
6	7.66	18
7	7.00	11
8	6.85	4.4

Table 3: Effect of temperature on antifungal compounds production by *Trichoderma harzianum* SQR-T037.

Temperature (^{o}C)	Biomass (g/L)	Antifungal activity (mm)
20	2.5	1.8
25	5.0	5.5
30	8.5	11
35	6.5	7
40	4.0	3

Table 4: Effect of pH on antifungal compounds production by *Trichoderma harzianum* SQR-T037.

pH	Biomass (g/L)	Antibiotic activity (mm)
2	3.5	3
3	5.9	7.5
4	7.6	8
5	8.5	9
6	9.2	10.5
7	8.4	4

The results of the effect of C sources showed that maltose was optimum for maximum biomass production while maximum antifungal activity of ethyl acetate extract was found in the presence of glycerol. Different results were reported by Konakovsky [20] that maximum antifungal compound production was found at 28°C after 7 days and at the pH of 5 or lower. They also reported that the input of oxygen during fermentations appeared to have a major influence on secondary metabolite

production since the best yields were obtained with a slight overpressure in the system. Therefore, in future more research is required to increase the production of antifungal compounds by providing optimum conditions.

Table 4: Effect of pH on antifungal compounds production by *Trichoderma harzianum* SQR-T037.

C sources	Dry biomass (g/L)	Antifungal activity (mm)
Sucrose	10.45	8.0
Lactose	7.35	6.0
Ribose	9.55	5.0
Maltose	12.25	9.6
Mannose	10.05	8.0
Starch	15.5	10.4
Glucose	11.0	9.0
Fructose	11.45	8.0
Galactose	9.95	**7.0**
Glycerol	18.5	**12.0**

Conclusions

This research showed the great potential of strain SQR-T037 as an antagonist to control the Fusarium wilt of watermelon under greenhouse and field conditions. We explored the potential of volatile and nonvolatile antifungal compounds that are major players in the control of pathogens; however, hydrolytic enzymes production and induction of systematic resistance should also be addressed in future research to explore the whole mechanism of action.

References

[1] Wu H, Yang X, Fan J, Miao W, Ling N, Xu Y, Huang Q and Shen Q (2009) Suppression of Fusarium wilt of watermelon by a bio-organic fertilizer containing combinations of antagonistic microorganisms. BioCont, 54(2): 287-300

[2] Garrett SD (1970) Pathogenic root-infection fungi. London, UK: Cambridge University Press

[3] Jones JB, Jones JP, Stall RE and TA Zitter (eds.) (1991) Compendium of tomato diseases. APS Press, St. Paul, Minn

[4] Boland GJ and Hall R (1987) Epidemiology of white mold of white bean in ontario. Can J Plant Pathol, 9: 218-224

[5] Mao W, Lewis JA, Hebbar PK and Lumsden RD (1997) Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium*. Plant Dis, 81: 450–454

[6] Huang HC and Erickson RS (2007) Ulocladium atrum as a biological control agent for white mold of bean caused bySclerotinia sclerotiorum. Phytoparasitica, 35: 15-22

[7] Sharma RR, Singh D and Singh R (2009) Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biol Cont, 50: 205-221

[8] Savchuk S and Dilantha Fernando WG (2004) Effect of timing of application and population dynamics on the degree of biological control of Sclerotinia sclerotiorum by bacterial antagonists. FEMS microbiol ecol, 49: 379-388

[9] Inbar J, Menendez A and Chet I (1996) Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. Soil Biol Biochem, 28: 757-763

[10] Yedidia I, Benhamou N and Chet I (1999) Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 65: 1061–1070

[11] Harman GE, Howell CR, Viterbo A, Chet I and Lorito M (2004) *Trichoderma* species-opportunistic avirulent plant symbionts. Nat Rev Microbiol, 2(1): 43–56

[12] Bae H, Roberts DP, Lim HS, Strem MD, Park SC, Ryu CM, Melnick RL and Bailey BA (2011) Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms, 24(3): 336-51

[13] Azin M, Moravej R and Zareh D (2007) Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Proc Biochem, 34(6–7): 563–566

[14] Harman GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. Phytopathol, 96(2): 190–194

[15] Siddiquee S, Cheong BE, Taslima K, Kausar H and Hasan MM (2012) Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. J Chromatogr Sci, 50(4): 358-367

[16] Yuan J, Raza W, Shen Q and Huang Q (2012)Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. Appl Environ Microbiol, 78(16): 5942-5944

[17] Dubey SC, Suresh M and Singh B (2007) Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. Biol Cont, 40(1): 1118–127

[18] Yang Z, Yu Z, Lei L, Xia Z, Shao L, Zhang K and Guohong L (2012) Nematicidal effect of volatiles produced by *Trichoderma* sp. J Asia-Pacific Entomol,15(4): 647–650

[19] Nemčovič M, Jakubíková L, Víden I and Farkaš V (2008) Induction of conidiation by endogenous volatile compounds in *Trichoderma* spp. FEMS Microbiol Lett, 284(2): 231–236

[20] Konakovsky V (2012) Optimization of fungal fermentation for the preparation of bioactive metabolites. (Master thesis) The institute for environmental biotechnology, Department for agrobiotechnology, Boku - University of natural resources and life sciences, Vienna, Austria

[21] Bonnarme P, Djian A, Latrasse A, Feron G, Ginies C, Durand A and Le Quere JL (1997) Production of 6-pentyl-alpha-pyrone by *Trichoderma* sp. from vegetable oils. J Biotechnol, 56: 143–150

[22] Scarselletti R, Faull JL (1994) *In vitro* activity of 6-pentyl-α-pyrone, a metabolite of *Trichoderma harzianum*, in the inhibition of *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici*. Mycol Res, 98 (10): 1207-1209

[23] Merlier, O.A.M., Boirie, M.J., Pons, B.J. and Renaud, C.M., 1984. European Patent Application. EP 84-400545

[24] El-Hasan A, Walker F and Buchenauer H (2008) *Trichoderma harzianum* and its metabolite 6-pentyl-alpha-pyrone suppress fusaric acid produced by *Fusarium moniliforme*. J Phytopathol, 156: 79-87

[25] Thomashow LS and Weller DM (1996) Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. Plant-microbe inter, 1: 187-235