J. Product. & Dev., 19(1):101 – 112 (2014)

BIOLOGICAL CONTROL ACTIVITY OF *Trichoderma* spp. AGAINST *Phytophthorainfestans in vitro*

M.F. Salem [1]*; Emara, H.A. [1]and Shalaby, M.A.[2]

1 Department of Environmental Biotechnology, Genetic Engineering and Biotechnology Research Institute, GEBRI, University of Sadat City, Egypt.*

2 Department of Plant Biotechnology, Genetic Engineering and Biotechnology Research Institute, GEBRI, University of Sadat City, Egypt.

ABSTRACT

The biocontrol potential of Trichoderma sppagainsttomatolate blight pathogen i.e. Phytophthorainfestans was studied in vitro. Antagonism test was carried out between Trichoderma spp and P. infestans and showed a radial growth inhibition of the pathogen, and a complete overgrowth ofTrichoderma sppon P. infestans.

Moreover, the biocontrol agentTrichoderma spp significantly reduced the disease.

In conclusion, from these results, it could be concluded that the biocontrol agents *Trichoderma*sppcould be used efficientlyfor the suppression of the late blight causer organism *P.infestans***.**

Keywords: Biological control, tomatolate blight, *Phytophthora infestans*, *Trichoderma spp*.

INTRODUCTION

Late blight disease caused by the pathogen *Phytophthorainfestans,* is probably the single most important disease of potatoes and tomatoes worldwide (Son *et al*., 2008).Disease was first recognized and became infamous in the mid-19th century, as it was responsible for many devastating epidemics, including the epidemic in Ireland which led to the great potato famine in 1845-1846, Worldwide losses due to late blight are estimated to exceed $5 billion annually and thus the pathogen is regarded as a threat to global food security (Latijnhouwers *et al.*, 2004). In the past few decades, the frequency and severity of the disease have increased in many parts of the world including Egypt and have been a serious

threat to Potato production. Despite the fact that much of the success in controlling the disease hasbeen due to the application of large quantities of chemical fungicides, theirextensive use is causing a serious pollution problem in the environment (Ragunathan and Divakar, 1996). Further, the chemical control of late blight is becoming more difficult due to the appearance of new and more aggressive *P. infestans*strains (Fernandez-Northcote *et al.,* 2000).

Thus, an alternative control strategy such as biological control should be sought (Ellis *et al.,* 1999). Biological control of crop disease is receiving increased attention as an eco-environmentally sound alternative to chemical pesticides. Some species of*Trichoderma* are among the major microorganisms that haveshown great potential for biological control of several plant pathogens. *Trichoderma* species have shown biocontrol potential against many plantpathogens including diseases caused by *Sclerotinia minor* (Jones and Stewart, 1997; Dolatabadi *et al*., 2011), *Botryosphaeriaberengeriana*f. spp. *piricola, Cladosporiumher barum* (Barbosa *et al.,* 2001), *Dioscorea* spp. (Okigbo and Ikediugwu, 2000) and *Pythiumultimum* (Naseby *et al.,* 2000). Besides, *Trichoderma* species have also shown efficacy against diseases caused by *Rhizoctoniasolani, Pythiumaphanidermatium, Fusariumoxysporum, Fusariumculmorum, Gaeumannomycesgraminis* var. *tritici, Sclerotiumrolfsii, Phytophthoracactorum, Botrytis cinerea* and by *Alternaria* spp. (Kucuk and Kivanc, 2003) and *Phytophthorainfestans.*

However, significant studies on biological control of late blight of tomato are scarce and hence the main aim of this study was to evaluate the efficacy of local isolates, *Trichoderma spp* against late blight of tomato *in vitro* .

MATERIALS AND METHODS

1-Phytophthora samples:

Sample of infected tomato leaves were collected from an open filed naturally infected for isolation of the causal pathogen. These infected tomato samples were mainly collected from open fields during fall and winter seasons, 2012 .These infected tomato samples were collected from the open fields and kept in plastic bags until arrival to the laboratory in the same day.

2-Trichoderma samples:

Different samples were collected from newly reclaimed areas in Egypt, Nobaria, Behera Governorate, Egypt, from rizhosphere of tomato, by digging up to 15 cm depth during October to December, 2012. These samples were obtained and collected in sterile plastic pots. The samples were transported to the laboratory in aseptic conditions. *Trichoderma spp* were isolated and maintained in PDA slant.

3-Pea agar medium(PAM):

Pea Agar Medium was consisted from 120 g frozen peas that were autoclaved for 15 min at 120 c in 1 liter distilled water then pea broth were filtered using 4 layers of cheese cloth then squeezing gently to remove all excess liquid from peas. The volume was adjusted to 1 liter with distilled water and 20 g of agar was added and 10 g sucrose sugar then the medium was autoclaved again for 20 min. at 15 psi.

4-Potato dextrose agar medium (PDA):

Potato Dextrose Agar medium (PDA) prepared as follows by cutting2oo g potato to small pieces and autoclaved for 20 min. at 120 c in 1 liter water then filtered using 4 layers of cheese cloth to remove all excess liquid from Potato then volume was adjusted to 1 liter and 12 g of agar was added and 10 g dextrose then the medium was autoclaved again for 30 min. at 15 psi then allow pea and PDA medium to cool down to 40 c before adding the mix of Antibiotics under sterile conditions mix of antibiotics added to culture medium (per liter).

- ***Ampicillin***:100 mg dissolve in ml sterile water .
- ***Mycostatin***: 50 ml dissolve in 1 ml sterile water .
- ***Pentachloronitrobenzene (PCNB)****:* 10 mg dissolve in ml Dimethyl sulfoxide (DMSO).
- ***Rifampicin****:* 10 mg dissolve in 1 ml (DMSO).

5-Isolation and identification of four Trichoderma spp

Fungal isolates of *Trichoderma spp* were isolated from soil samples by using Potato Dextrose Agar (PDA) medium. Samples were inoculated over plates by Multiple Tube Dilution Technique (MTDT) and the plates were incubated at 23°C for 5 days. The fungal colonies which were picked up and purified by inoculating and incubated at 23°C for 9 days. Green conidia forming fungal bodies were selected and identified through microscopic

observation to be *Trichoderma spp*. The culture was maintained on PDA slantsA 4mm disc of inoculum from sub cultured plates of *Trichoderma spp* were transferred to Potato Dextrose Agar(PDA) slants and maintained as pure culture.

The plates were then incubated at room temperature (26±2°C) for ten days. After complete sporulation, conidia from the medium were harvested by washing them thoroughly with sterilized water containing Tween-20 (0.2%) for immediate useHarvested conidia were air dried under laminar air flow and stored in a small air tight screw cap vials (10 cm with 2.5 cm diameter) in refrigerator at 4°C before using for further studies. Colony Forming Units (CFU)were estimated by plating technique Suspension of spores was made using sterilized distilled water with Tween-20 (0.2%) and filtered through a double layered muslin cloth. Spore count was made using a double rolled Neuberger's Haemocytometer after necessary serial dilutions under phase contrast microscope. From the stock solution, dilutions were made to obtain the required concentrations.

6-Isolation of Phytophthora infestans from infected tomato leaves:

Isolation of the causal organism *Phytophthora infestans* was isolated from the infected leaf tissues according to the following protocol:

1. Sporulation lesions on leaf tissue from the field were washed in fresh water and placed in a humid chamber with the leaf `s abaxial side up.
2. Plates were incubated at 15-18 c with a 14 hour light period for 1 day or until sporulation appears.
3. Small pieces of infected tissue from the sporulating border of lesion were cut out and placed on top of a drop of water on the abaxial side of tomato leaves in a humid chamber (upturned Petri dish containing water agar).
4. Dishes were incubated at room temperature for 1Week or until there is abundant sporulation.
5. Sporangia were picked from the top of tomato leaves and transferred directly onto medium in a petri dish containing rye agar with antibiotics.

7-Antagonistic test between Trichoderma spp and P. infestans:

Dual culture method was employed to analyze whether *Trichoderma spp* inhibits the growth of *P. infestans*as described in Sivakumar *et al.* (2000). Briefly, 1 cm diameter mycelial plug of *P. infestans* (7 days old) was placed on one side of a petri-dish (9 cm diameter) containing pea agar and preincubated at 23C for 2 days to initiate growth. Later, 1 cm diameter disc of *Trichoderma spp* (5 days old) was placed 6 cm away from the pathogen on

the dual plates; whereas sterile PDA disc was placed in the control plates. The assay was done in five replicatesand the radial growth of the pathogen was measured 4 days after incubation at 23C.

The percent radial mycelial growth inhibition (I) was calculated as follows:

$$I= [(C–T)/C] \times 100$$ according to Sivakumar*et al.,* 2000;

Where, C is radial growth measurement of the pathogen in the control plates and T is radial growth of the pathogen in the dual plates.

8- Assay for volatile metabolites of *Trichoderma*spp:

Bottoms of two Petri dishes containing PDA were individually inoculated with a disc of *P. infestans* and *Trichoderma* spp. The bottoms were adjusted (one base placed over the other one) and attached by Para film. The control sets did not contain the antagonist. The cultures were incubated at room temperature (23$\pm$ 2°C), and diameter of radial growth of fungi was measured at 24 and 48 hours. The percent inhibition was obtained using the formula as follows:

$$\text{Percent inhibition (I)} = C – T/ C \times 100$$

Where, C: Diameter of radial growth of *P. infestans* in control; T :Diameter of radial growth of *P. infestans* in treatment.

9-Extracted metabolites of T.viride and T.harzinum against P.infestans:

The purified sterile metabolites of *Trichoderma* spp. were diluted four times, and their inhibitory effect was tested. 1 ml of the original and dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of the extracts were mixed with 20 ml of molten PDA separately and then poured into Petri dishes. PDA supplemented with sterile distilled water served as control. A mycelia disc of *P. infestans* was transferred on the centre of both test and control plates and incubated for 24 hours at room temperature. The linear growth of mycelia was measured and the percentage of inhibition was calculated. Same procedures were applied for metabolites extracted from *P. infestans* against *Trichoderma* spp.

Statistical analysis:

Statistical significance of antagonistic effect of *Trichoderma* spp. was tested by 't' test (P = 0.05), results produced by the *Trichoderma* spp. were expressed as mean ± SD of three experiments, and were subjected for analysis of variance and Tukey test at P = 0.05 using statistical software SPSS Windows version 13.0.

RESULTS

The results of dual culture of *Trichoderma* antagonists and the causal pathogen *Phytophthora infestance* demonstrated that the antagonists were significantly different from control as well as within them. Moreover, both *T. viride*and *T. harzianum* had an antagonistic effect on *P. infestance. Whereas, T. harzianum* highly inhibited the growth of test pathogen compared *to T. viride*, and the percentage of inhibition increased about two times *to T. viride* (Table1). Moreover, we have to mention that *T. hamatum* has the highest percentage of inhibition after 3 days of interactions compared with other biocontrol agents (Table 2).

Table 1: Effect of Four*Trichoderma* spp. on the radial growth of *P. infestans* in dual culture method after 48 hrs.

Antagonists	**Radial growth at 48 hours**		**Inhibition, %**
	Control, mm	**Test, mm**	
T.virde	79.34	62.42	21.32
T.atrovride	82.15	59.98	26.98
T.harzianum	79.19	50.21	36.59
T.hamatum	72.10	50.16	30.42

Table 2: Effect of four *Trichoderma* spp. on the radial growth of *P. infestans* in dual culture method after 72 hrs**.**

Antagonists	**Radial growth at 72 hours**		**Inhibition, %**
	Control, mm	**Test, mm**	
T.virde	88.63	51.13	42.31
T.atroviride	89.40	49.11	45.06
T.hariznuim	90.20	55.78	38.15
T. hamatum	88.70	37.24	58.01

The volatile metabolites of *T. harzianum*and *T. hamatum* showed the highest significant growth inhibition against *P. infestance* at 24 hours incubation. The inhibition produced by the *T. viride* was significantly the lowest among the tested biocontrol agents (Table 3).

Table 3: Effect of volatile metabolites of four *Trichoderma spp.* on the radial growth of *P. infestans* after 24 hours.

Antagonists	**Radial growth at 24 hours**		**Inhibition, %**
	Control, mm	**Test, mm**	
T.viride	60.67	43.71	27.95
T.atroviride	60.20	40.45	32.80
T.harzianum	60.67	31.14	48.67
T. hamatum	64.30	42.32	34.18

Trichoderma spp showed no significant difference between the radial growths of *P. infestans* in test and in control plates at 48 hours incubation and 0% inhibition. But *T.atroviride* significant 0.29% (Table 4).

Table 4: Effect of volatile metabolites of *Trichoderma spp.* on the radial growth of *P. infestans* after 48 hours.

Antagonists	**Radial growth at 48 hours**		**Inhibition, %**
	Control, mm	**Test, mm**	
T. viride	89.24	89.24	0.0
T. atroviride	89.20	88.98	0.29
T. harzianum	89.24	89.24	0.0
T. hamatum	89.24	89.24	0.0

Radial growth of P. infestans in control 70.24 mm.

Trichoderma spp. extracted metabolite's was revealed the inhibitive bioactivity on the growth of *P. infestans*. However, the inhibitory effect produced by the *T. viride* is slightly higher than that produced by *T. harzianum*. and, both extracts showed inhibition even at 10000 time dilution. the results of extracted metabolites of *P. infestans* did not show any inhibition against the growth of *Trichoderma* spp. (Table 5).

DISCUSSION

Late blight, caused by the oomycete pathogen *Phytophthorainfestans*, is probably the single most important disease of potatoes worldwide and Egypt as well. We suffer from this fatal potato disease all over Egypt. It is destructive

Table 5. Effect of extracted metabolites of *T.viride* and *T.harzianum*.on the radial growth of *P. infestans.*

Antagonists	Dilutions	Diameter of radial growth	Inhibition %
T. harzianum	10	36.9	47.46
	10-1	39.25	44.12
	10-2	41.9	40.43
	10-3	40.63	42.15
	10-4	44.49	36.66
T. viride	10	30.0	57.28
	10-1	35.25	49.81
	10-2	36.33	48.27
	10-3	37. 73	46.28
	10-4	41.31	41.18

wherever potatoes are grown without the use of fungicides, except in hot, dry, and irrigated areas (Thurston and Schultz, 1981). Biological control is a good alternative for sustainable agriculture to overcome the problems of public concern associated with pesticides and pathogens resistant to chemical pesticides and to become eco-friendly (Akhtar and Siddiqui, 2008). In this study *Trichoderma spp*has been found to retard the radial growth of *P. infestans*. This antagonistic mode of action of *Trichoderma* could be attributed to the production of antibiotics and fungal cell wall degrading enzymes (Chutrakul*etal.,* 2008; Sharma *et al.,* 2009). A similar inhibitory action of *Trichoderma* strains (TH1, N47 and T12) against a related oomycete pathogen, *Pythiumultimum*, was reported earlier (Naseby *et al.,* 2000). The observed mycoparasitic action of *Trichoderma* in this study suggests that it has a good potential in controlling *P. infestans.* Similar mycoparasitic action of *Trichoderma* strains were also reported against related pathogens, *Phytophthoracinnamomi* (Pugeg and Ian, 2006) and *Phytophthoracapsici* (Ezziyyani *et al.,* 2007*)* *Trichoderma* species were found to give better control of *Botryosphaeriaberengeriana*when inoculated three days in advance than when the two are co-inoculated. Germination of fungal spores on the leaf surface is a critical stage in the development of the host-pathogen interface, and one in which the pathogen is often vulnerable (Campbell, 1989). The motile zoospores of *P. infestans*have no cell wall and hence are probably extremely vulnerable to adverse conditions, and are also

the main infective propagules (Erwin and Ribeiro, 1996). Thus, zoospores can be a potential target in biocontrol of *P. infestans.* In conclusion, *Trichoderma spp* showed good potential in controlling potato late blight under *in vitro* conditions. In addition to that, the *Trichoderma spp* used in this study was isolated from local climatic conditions in Egypt and effectively proved that it has potential to be used as a biocontrol agent in the future. The non-volatile metabolites of *T. viride*and *T. atroviride* showed the highest significant ($P < 0.05$) growth inhibition against *P. infestance*at 24 hours incubation. The inhibition produced by the *T. harzianum* was significantly ($P < 0.05$)the lowest among the tested biocontrol agents and this leads us to its optimum way of application in greenhouse and open field conditions through producing its metabolites directly to the infected soil ,as both *T.viride and T.atroviride* worked as a biocontrol agents with a unique antagonism mechanism called antibiosis compared with *T.harzianum* which antagonize the causal pathogen with the mechanism of mycoparasitism. This is lead us to put into consideration its practical and efficient way during application either greenhouse or under open field conditions. Further research is needed to determine its field performance. In general, as any other biological control systems, biological control of late blight should be regarded as one of the safest technology of the integrated control program rather than a method to be used alone.

In conclusion, from these results, it could be concluded that the biocontrol agents *Trichoderma* spp could be used efficiently for the suppression of the late blight causer organism *P. infestans***.**

REFERENCES

Akhtar, M.S and Siddiqui, Z.A. (2008). Arbuscularmycorrhizal fungi as potential bioprotectants against plant pathogens.*Mycorrhizae: Sustainable Agriculture and Forestry*, 61–97.

Barbosa, M.A.G., Rehn, K.G., Menezes, M and Mariano, R.L. (2001). Antagonism of *Trichoderma* species on *Cladosporium herbarium* and their enzymatic characterization. Brazilian Journal of Microbiology, **32**: 98-104.

Chutrakul, C., Alcocer, M., Bailey, K and Peberdy, J.F. (2008). The production and characterization of Trichotoxinpeptaibols by *Trichoderma asperellum.* Chemistry and Biodiversity, **5**: 1694- 1706.

Dolatabadi, K.H., Goltapeh, E.M., Varma, A and Rohani, N. (2011).*In vitro* evaluation of arbuscularmycorrhizal-like fungi and *Trichoderma* species against soil borne pathogen. Journal of Agricultural Technology**, 7**(1): 73-84.

Campbell,R.(1989). *Biological Control Of Microbial Plant Pathogens*. Cambridge University Press, Cambridge, 232 pp.

Ellis, R.J., TimmsWilson, T.M., Beringer, J.E., Rhodes, D., Renwick, A., Stevenson, L and Bailey,M.J.(1999). Ecological busis for biocontrol of damping off disease by *pseudomonas fluorecens* 54/96. *Journal of Applied Microbiology*, **87**:454-463.

Erwin,D.C and Ribeiro, O. K., 1996. Phytophthora disease worldwide. American Phytopathological Society, Minnesota, 562 pp.

Ezziyyani, M.; Reguena, M. E.; Egea-Gilabert; Cand Candela, M. E. (2007). Biological control of phytophthora root rot of pepper using *Trichoderma harzianum* and *Streptomyces rochei* in combination. *Journal of phytopathology*, **155**:342-349.

Fernandez-Northcote, E.N., Navia, O and Gandarillas, A. (2000). Basis of strategies for chemical control of potato late blight developed by PROINPA in Bolivia. *Fitopatologia*, **35:** 137-149.

Jones, E. E. and Stewart, A. (1997). Biological control of *Sclerotinia minor* in lettuce using *Trichoderma* species. *Proc. New Zealand Plant Protection Conf.*, **50:**154-158.

Kucuk, C and Kivanc, M. (2003). Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. *Turkish Journal of Biology*, **27:** 247- 253.

Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G., VanWest, P and Govers, F. (2004). AGα subunit controls zoospore mobility and virulence in the potato late blight pathogen *Phytophthorainfestans. Molecular Microbiology*, **51:** 925-936.

Naseby, D.C., Pascual, J.A and Lynch, J.M. (2000). Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythiumultimum*populations, soil microbial communities and soil enzyme activities. *Journal of Applied Microbiology*, **88**: 161-169.

Okigbo, R.N and Ikediugwu, F.E.O. (2000). Studies on biological control of post-harvest rot in yams (*Discorea*spp.) using *Trichoderma viride*. *Journal of Phytopathology*, **148**: 351-355.

Pugeg, I.N.A and Ian, D.G. (2006). Mycoparasitic and antagonistic inhibition on *Phytophthoracinnamomi*rands by microbial agents isolated from manure composts. Plant Pathology 5: 291- 298. *Journal of Agricultural Technology* 2011, **7** (6): 1589-1602 1602

Ragunathan, V and Divakar, B.J. (1996).*Integrated pest management strategies. In: Molecular biology of the biological control of pests and disease of plants.* ed. by M. Gunasekaran and D. J. Weber, pp.191-194. CRC Press, Florida.

Sharma, K., Kumar, M and Misra, R.M. (2009). Morphological, biochemical and molecular characterization of *Trichoderma harzianum* isolates for their efficacy as biocontrol agents. *Journal of Phytopathology*, **157:** 51-56.

Sivakumar, D.; Wijeratnam, W.R.S.; Wijesundera, R.L.C.; Marikar, F.M.T and Abeyesekere, M. (2000). Antagonistic effect of *Trichoderma harzianum* on post-harvest pathogens of Rambutan (*Napheliumlappaceum*). *Phytoparasitica* 28: 240-247.

Son, S.W., Kim, H.Y., Choi, G.J., Lim, H.K., Jang, K.S., Lee, S.O., Sung, N.D and Kim, J.C. (2008). Bikaverin and fusaric acid from *Fusariumoxysporum*show antioomycete activity against *P. infestans*.Journal of Applied Microbiology 104: 692-698.

Thurston, H.D. and Schultz, O. (1981). Late blight. In: *Compendium of potato diseases*, pp. 40-42, (Hooker, ed). American Phytological Society, U.S.A.

دراسة تأثير التضاد الحيوي لفطر تريكوديرما ضد فطر الفيتوفثرا انفيستنس فى المعمل

الاسماء واماكن العمل

أجرى هذا البحث فى معمل امراض النبات بقسم التكنولوجيا الحيوية النباتية بمعهد الهندسة الوراثية و التكنولوجيا الحيوية- جامعة مدينه السادات

تم خلال هذه الدراسه عزل المسبب المرضى لمرض الندوه المتاخره من عينات نباتات بطاطس مصابه بالندوة المتاخرة ثم تم عزل فطر التريكوديرما من عينات من التربه الملاصقه لجذور نباتات الطماطم بعمق ١٥ سم ثم تم تعريف هذه العزلات الى اربع اجناس مختلفه من الفطر التريكوديرما وقد اجريت تجارب التضاد الحيوى بين الاجناس الاربعه المعزوله وفطر الفيتوفثرا وكانت النتائج على النحو التالى

اظهرت الانواع الاربعه من فطر التريكودرما قدرات مختلفة على تثبيط الفطر الممرض ولكن اعطى النوع هرزيانم نسبه تثبيط اعلى من باقى الانواع حيث سجل ٣٦.٥٩% تثبيط بينما سجل النوع فريدى ٢١.٣٢% وسجل النوع هماتم ٣٠.٤٢% بينما سجل النوع اتروفريدى ٢٦.٩٨% وذلك بعد فتره تحضين ٤٨ ساعه

وفى تجارب المستخلصات الايضيه المتطايره اعطى تريكوديرما هريزانيم اعلى نسبه تثبيط ٤٨.٦٧% بينما اعطى فيردى اقل نسبه تثبيط ٢٧.٩٥% وذللك بعد فتره تحضين ٢٤ ساعه.وفى تجارب المستخلصات الأيضية أظهرت العزلتين تأثيراً حتى على تخفيف ١ : ١٠٠٠٠.

التوصية: