INFLUENCE OF *Trichoderma* SPECIES ON MYCOTOXINS PRODUCTION AND PATHOGENIC CAPABILITY OF *Fusarium moniliforme* ASSOCIATED WITH LENTIL SEEDS

Hassan. A. H. Hasan[a] and Nashwa M. A. Sallam[b]

[a]Botany Department, Faculty of Science, Assiut University, Assiut, Egypt

[b]Plant Pathology Department, Faculty of Agriculture, Assiut University, Assiut, Egypt

Abstract: Different fungal species were isolated from seeds of lentil (*Lens esculenta* L.) cv. Giza 9. *Aspergillus flavus, A. niger, Fusarium moniliforme* and *Penicillium chrysogenum* were the principal fungal species isolated. *Trichoderma aureoviride, T. harzianum* and *T. polysporum* examined for their antagonistic activity against the toxigenic fungi. *T. aureoviride* expressed the highest reducing percentage for *F. moniliforme* mycotoxins (zearalenone by 60% and diacetoxyscirpenol by 45% reduction) in liquid medium as compared with other tested *Trichoderma*. However, *Trichoderma* species have not any reduction effect on aflatoxin production by *A. flavus* in liquid medium. *T. aureoviride* was selected for treatment lentil seeds infected by *F. moniliforme*. The results show that *T. aureoviride* at 18% moisture content (MC) reduced zearalenone by 20-30% and this reduction increased to 38% reduction at 25% MC. Germination of lentil seeds was reduced by *F. moniliforme* at 18% MC and this effect changed by *T. aureoviride* application from 83% into 97% after 2 weeks and from 33% into 77% after 4 weeks of treatment. Application of the antagonistic fungus *T. aureoviride* to infested soil with *F. moniliforme* caused 50% reduction in percentage of lentil root rot disease severity.

Key words: lentil, *Trichoderma*, *Fusarium*, mycotoxins, wilt disease**.**

Introduction

Lentil (*Lens esculenta* L.) is one of the most important seed crops grown worldwide. World production was 2.875 million MT on about 3.36 million hectares and increased by about 65% over the past 25 years (FAO, 1996).

Fusarium moniliforme is one of the most commonly reported soil-borne and seed-borne fungal pathogens infecting seeds and the most prevalent *Fusarium* isolated from lentil (Abd-Elkader *et al*., 1978, Abdel-Hafez, 1984 and EL-Maraghy, 1988). *Fusarium moniliforme* produces secondary metabolites such as zearalenone and diacetoxyscirpanol (Nelson *et al*., 1983), which affect human and animal health. *Fusarium mniliforme*

was reported to cause root rot disease to different plant crops especially lentil and causing significant economic losses (Belabid *et al*., 2000).

It is very important to control such disease in the agriculturally important commodity. Recognizing the hazards of pesticides to man and his environment, many countries in the world today are considering biological control as the best alternative to chemical control of plant pests and diseases (Davide, 1990).

Some species of *Trichoderma* had potentials for controlling root rot, wilt and foliar pathogens of vegetable crops. Seed treatment with biocontrol agents is an appropriate method for biocontrol of soil-borne plant pathogens in the rhizosphere (Mao *et al*, 1998 and Kerry, 2000).

The present study was carried out to identify fungal species associated with seeds of lentil (*Lens esculenta* L.) cv. Giza 9. Also, to investigate the effect of *Trichoderma* species (*T. aureoviride, T. harzianum* and *T. polysporum*) on mycotoxin production and the pathogenic capability of *Fusarium moniliforme* associated with lentil seeds.

Materials and methods

Isolation of fungal flora from lentil seed.

Samples of lentil seeds (*Lens esculenta* L. cv. Giza 9.) were collected from the retail markets and transferred immediately to the Laboratory. Dilution plate method was used for isolation the common fungi associated with seeds on glucose-Czapek's medium. The developing colonies after 1 week of incubation at 28^{o}C were examined microscopically and identified using the following references: Raper and Fennell (1965) for *Aspergillus*, Pitt (1985) for *Penicillium* and Booth (1977) for *Fusarium*. The average number of colonies per dish was multiplied by the inverted dilution factor to obtain the number of colonies per g of seeds.

Trichoderma species source.

The isolates of *Trichoderma aureoviride, T. harzianum* and *T. polysporum* were obtained from the stock culture of Plant Pathology Department, Faculty of Agriculture, Assiut University.

Effect of *Trichoderma* species on mycotoxins production by toxigenic fungi in liquid medium.

Fifty ml medium (g/L: sucrose 20, yeast extract 5, $NaNO_3$ 2, KH_2PO_4 1, KCl 0.5, $MgSO_4$ 0.5) were added in 250 ml Erlenmeyer conical flask. After autoclaving, discs of 7-day old culture of *F. moniliforme* inoculated half flasks and the others inoculated by *A. flavus. Trichoderma* species (*T. aureoviride, T. harzianum* and *T. polysporum*) were inoculated in all flasks without the controls and then

incubated at 28°C for 7 day, in rotatory shaker at 120 rpm. Three flasks were used for each toxigenic fungal species and control without *Trichoderma* species. The growth mass of different treatments was determined after drying the mycelia in oven at 80°C for 24h. Mycotoxins were extracted from different cultures by chloroform and determined as mentioned below.

Effect of *T. aureoviride* on *F. moniliforme* mycotoxin and germination of seeds.

The seeds in sterile polyethylene bags were surface sterilized by 3% NaOCl solution for 2 min and rinsed in 3 changes of sterile distilled water. Thereafter, discs of 7-day old culture of *F. moniliforme* inoculated the samples. Then, *T. aureoviride* was selected for treatment lentil seeds infected by *F. moniliforme*. Sterile distilled water; sufficient to raise the moisture contents of the seeds to 18 and 25% were added. The samples were placed in a refrigerator for 2 h and shaken thoroughly. The inoculated seeds were incubated at 28°C for 4 weeks. Periodically, the water contents were readjusted and after 2 and 4 weeks samples were taken for assaying germination and mycotoxin production. Treated seeds were placed in plastic pots irrigated with tap water and incubated in dark at 28°C. The length of shoot system and percentage of germination were assayed after 7 days. The viability of seedlings was determined by calculating the vigour index (VI) of the seeds. VI= length of shoot (cm) x germination percentage.

Mycotoxin analysis.

The culture of each treatment was extracted with chloroform, evaporated and separated by TLC using chloroform-methanol (97:3 v/v) as the developing solvent. Aflatoxin B was determined according to Nabney and Nesbitt (1965), zearalenone was determined according to Mirocha *et al.* (1974) and diacetoxyscirpenol was determined according to Thrane (1986) with using reference standards.

Pathogenicity test.

Pathogenicity test of *Fusarium moniliforme* was carried out under greenhouse condition on Lentil (*Lens esculenta* L.) cv. Giza 9. Inoculum of the tested isolate was prepared by growing the fungus in one liter glass bottles containing barley grains medium (100 gm of barley grains+ 50 ml water) and autoclaved at 121°C for 20 min., then incubated for 15 days at 25°C. Soil infestation was conducted by mixing fungal inocula to sterilized clay soil at the rate of 2% (w/w). Infested soil was placed in 30 cm. autoclaved pots. Non infested soil was used as control. Four pots were used as replicates for each treatment (4 plants per pot). After 2 months planting date, root rot disease

severity percentage was recorded according to Allam (1990).

Effect of *T. aureoviride* on lentil root rot disease.

The biocontrol agent *T. aureoviride* was prepared by growing in (250 ml) bottles each containing 50 ml of liquid potato dextrose medium and incubated at 25°C. Ten days after incubation the obtained culture was centrifuged for 5 min. at 3000 rpm. Culture filtrate was blended in 50 ml distilled sterilized water by using Waring Blender. Propagules of the fungus were resuspended in sterile distilled water to give concentration of $7x10^7$ CFU/ml. Five milliliters from *T. aureoviride* suspension were added to previously infested soil with *F. moniliforme* at the time of planting (with Giza 9 lentil cultivar) as mentioned above. Four pots were used as replicates for each treatment (4 plants per pot). Disease severity percentage was recorded 2 months after planting.

Statistical analysis.

All data were subjected to statistical analysis and means were compared using L.S.D. test (Gomez and Gomez, 1984).

Results and Discussion

Mycoflora isolated from lentil seeds.

Different fungal species were isolated from lentil seeds *Aspergillus flavus, A. niger, A. terreus, Fusarium moniliforme* and *Penicillium chrysogenum* (Figure, 1). *A. flavus* has potentiality to produce aflatoxin B_1 and B_2 and *F. moniliforme* produced zearalenone and diacetoxyscirpanol. El-Nagerabi and El-shafie (2001) found that *Aspergillus* was the most prevalent genus in lentil seeds followed by *Rhizopus, Penicillium* and *Fusarium*. *F. moniliforme* is one of the most commonly reported seed-borne fungal pathogens infecting seeds and the most prevalent *Fusarium* isolated from lentil (Abdel-Hafez, 1984 and EL-Maraghy, 1988). *A. flavus* was reported to produce aflatoxins (Shroeder and Boller, 1973) and *F. moniliforme* was reported to produce zearalenone and diacetoxyscirpanol (Nelson *et al.*, 1983), which affect human and animal health.

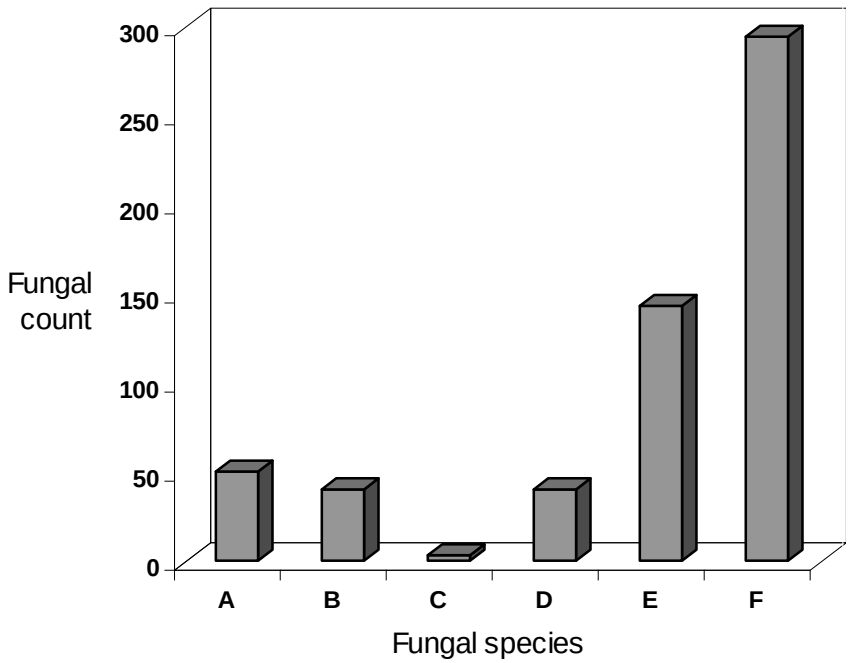


Figure (1): Common fungal flora (count of colonies/g seeds) isolated from lentil seeds on glucose Czapek's medium by dilution plate method at 28°C and their mycotoxin potentiality. A= *Aspergillus flavus* (produced aflatoxin B), B= *A. niger*, C= *A. terreus*, D= *Fusarium moniliforme* (produced diacetoxyscirpenol and zearalenone), E= *Penicillium chrysogenum*, F= Total count.

Effect of *Trichoderma* species on mycotoxins production by toxigenic fungi in liquid medium.

Trichoderma species (*T. aureoviride*, *T. harzianum* and *T. polysporum*) were tested for their antagonism against the toxigenic fungi (*A. flavus* and *F. moniliforme*). *T. aureoviride* expressed the highest reducing percentage for *F. moniliforme* mycotoxins (zearalenone by 60% and diacetoxyscirpenol by 45% reduction) followed by *T. polysporum* (Table, 1). However,

they have no reduction effect on aflatoxin production by *A. flavus*.

Trichoderma species are known to produce extracellular cell wall degrading enzymes that are considered to be a major factor in the hyperparasitic mechanism (Elad *et al* 1982 and Gyorgy *et al* 1996). The lytic enzymes of hyperparasite's brake down cell wall polysaccharides and penetrate into the cytoplasm of the target fungi. No literatures are available on effect of *Trichoderma* on mycotoxin production. Most of researches are on effect of bacteria on aflatoxin production by *A. flavus* (Cuero *et al.*, 1988 and Gourma and Bullerman, 1995) and fumonisin production by *F. verticillioides* (Cavaglieri *et al.*, 2005).

Table(1): Effect of *Trichoderma* species on growth and mycotoxin production by toxigenic fungi in liquid medium.

Fungal species	***A. flavus***			***F. moniliforme***					
	Dry wt (mg/50 ml)	pH	Aflatoxin B(µg/50 ml)	Dry wt (mg/50 ml)	pH	Zearalenone (µg/50 ml)	Inhib-ition %	Diacetoxysc-irpenol (µg/50 ml)	Inhib-ition %
Control	204	8.3	150	154	8.2	135	-	83	-
T. aureoviride	215	8.0	150	180	8.4	53*	60	45*	45
T. harzianum	183	8.4	155	174	8.3	105	22	60	28
T. polysporum	241	7.5	153	171	8.4	75*	44	52*	37

*Mean significant decrease compared to control.

Effect of *T. aureoviride* treatment on mycotoxins production by *F. moniliforme* on lentil seeds.

T. aureoviride was selected for treatment lentil seeds infected by *F. moniliforme*. The results show that *T. aureoviride* at 18% MC of seeds reduced zearalenone by 20-30% and this reduction increased to 38% reduction at 25% MC (Table, 2). However, *T. aureoviride* had less effect to reduce diacetoxyscirpenol production. The inhibition in toxin amount may be attributed to the *Trichoderma* that may cause a change in biochemical environment affect the metabolic pathway available to the toxin formation and the capability of *Trichoderma* to degrade toxin following its formation.

The results obtained in the present study, *in vitro* (Table, 1) and *in vivo* (Table, 2), indicated that *T. aureoviride* was effectiveness in reducing mycotoxins production by

F. moniliforme in vitro (in liquid medium) than *in vivo* (on lentil seeds). Papavizas and Lewis (1983) and Gyorgy *et al* (1996) found a partial correlation between the *in vitro* physiological characteristics and the practical biocontrol ability. Bevivino *et al* (1998) obtained a poor correlation between in vitro and greenhouse studies when assessing *Burkholderia cepacia* antagonism against *F. moniliforme* and *F. proliferatum* on maize.

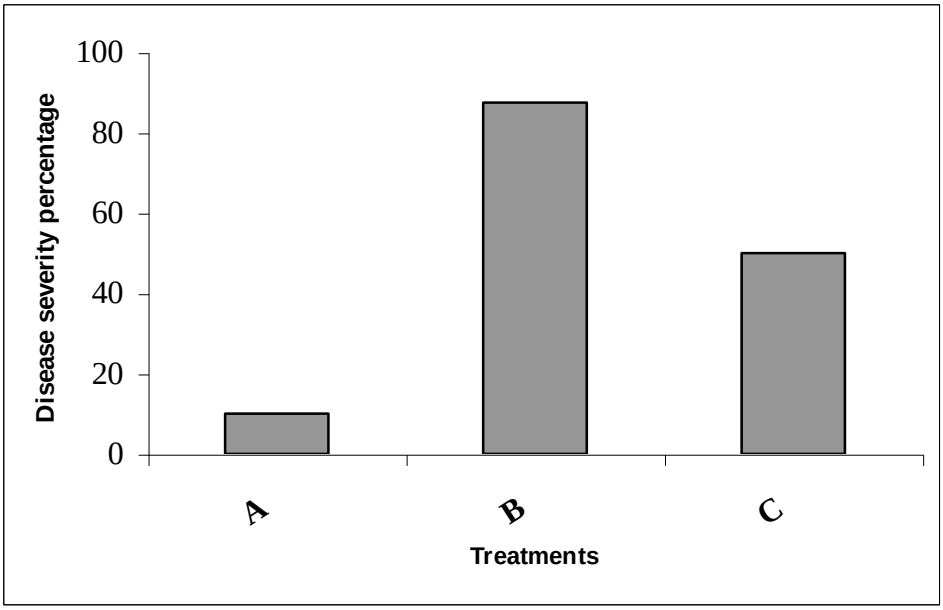
Pathogenicity test.

Pathogenicity test of *Fusarium moniliforme* was carried out under greenhouse condition on Lentil (*Lens esculenta* L.) cv. Giza 9. The tested isolate of *F. moniliforume* was able to infect lentil plants and causing roor rot disease symptoms. Percentage of infection was 87.5% compared with control. Belabid *et al.* (2000) reported that *Fusarium* root rot is one of the most important disease affecting lentils and causing significant economic losses.

ffect of *T. aureoviride* treatment on lentil seeds germination and root rot disease.

F. moniliforme caused reduction in germination percentage and seedling viability of lentil after 2 weeks at 18% moisture content This reduction increased by increasing incubation periods, after 4 weeks, and moisture content, 25%. The germination improved with *T. aureoviride* application from 83% into 97% after 2 weeks and from 33% into 77% after 4 weeks of treatment (Table, 2). The application of the antagonistic fungus *T. aureoviride* to infested soil with *F. moniliforme* caused 50% reduction in percentage of disease severity (Figure, 2).

There are a number of ways by which antagonistic organisms suppress growth of pathogens and control diseases. Their action could be through parasitism, production of toxic compounds and induction of host resistance. The microorganisms inducing resistance generally compete with pathogens for nutrients in host tissues and form barriers between the host and the pathogen to prevent penetration and infection (Cook, 1990). Some *Trichoderma* isolates can compete and colonize potential infection courts (Hjeljord and Tronsmo, 1998). Cotxarrera *et al* (2002) found that *T. asperellum* has the potential to be biocontrol of *Fusarium* wilt.



Figure(2): Effect of *T. aureoviride* on lentil plant root rot disease caused by *F. moniliforme*. A= Control (plants without treatment), B= *F. moniliforme* only, C= *T. aureoviride* + *F. moniliforme.*

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تأثير أنواع التريكوديرما على إنتاج السموم الفطرية والقدرة المرضية لفطر فيوزاريوم مونيليفورمى المصاحب لبذور العدس

حسن أحمد حسن حسان [1] و نشوى محمد عاطف سلام [2]

قسم النبات – كلية العلوم – جامعة أسيوط – مصر [1] ، قسم أمراض النبات – كلية الزراعة – جامعة أسيوط – مصر [2]

تم في هذا البحث عزل الأنواع الفطرية الشائعة من بذور العدس وهى اسبرجيللس فلافس و اسبرجيللس نيجر و فيوزاريوم مونيليفورمى و بنسيلليوم كريزوجينوم.

تم اختبار تأثير ثلاثة عزلات تريكوديرما هى (تريكوديرما اوريوفيردى و تريكوديرما هارزينيوم و تريكوديرما بوليسبوروم) على انتاج سموم الفطريات اسبرجيللس فلافس و فيوزاريوم مونيليفورمى. ولقد تبين أن تريكوديرما اوريوفيردى هى أفضل العزلات المضادة في اختزال إنتاج سمى الزيراليتون بنسبة 60 % و الدياسيتوكسى سكيربينول بنسبة 45 % بواسطة فيوزاريوم مونيليفورمى فى البيئة السائلة بينما لم تظهر أي من العزلات المضادة الفطرية الثلاث تأثيرا مثبطا لإنتاج الأفلاتوكسين بواسطة اسبرجيللس فلافس.

وبدراسة تأثير عزلة تريكوديرما اوريوفيردى على إنتاج السموم الفطرية في بذور العدس وعلى إنبات البذور المعاملة بفطرة فيوزاريوم مونيليفورمى، تبين تثبيط الزيراليتون بنسبة أقل من البيئة السائلة وتنشيط إنبات البذور من 83 % إلى 97 % بعد أسبوعين من المعاملة عند 18 % محتوى مائي.

أدت إضافة المضاد الفطرى تريكوديرما اوريوفيردى الى التربة المعاملة بفطرة فيوزاريوم مونيليفورمى الممرضة إلى خفض نسبة الإصابة بمرض عفن جذور العدس بنسبة 50 %.