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Isolation of Tomato Fruits Mycoflora and Evaluation *in vitro* and *in vivo* by *Trichoderma harzianum*

Mokhtar Hamitou[1,2*], Laid Dehimat[2] and M. Mourad Senoussi[1]

[1]Laboratory of Bimolecular and Plant Amelioration, Larbi-Ben-M'hidi University, Oum El Bouaghi, BP 358, Constantine Road, 04000, Algeria
[2]Laboratory of Mycology, Biotechnology and Microbial activity, University of Mentouri, Constantine, 25000. Algeria
**Corresponding author*

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A B S T R A C T

The present investigation aimed is to isolate and identify the mycetes accompanying the tomato fruits (*Lycopersicon* esculentum), and to evaluate *in vitro* and in *vivo* the ability of *T.harzianum* to control the isolated mycetes. Some infected tomato fruits by mycetes were brought from Oum-elbouaghi market. The results of isolation allowed the identification of *Stemphylium sp.* and *Aspergillus niger*. One isolate of *T.harzianum* / *Hypocrea lixii* was utilized in this study. The results of direct confrontation (*in vitro*) of *T.harzianum* against *Stemphylium sp.* and *A.niger* on PDA medium indicated the inhibition of mycelium growth in variable degrees; it was equal in the fourth day of the experiment to 54.54 % and 52.17% for *Stemphylium sp.* and *A.niger* respectively. However, it did not show any growth of the tested fungus when re-planting a disk from the interaction hyphal area between *T.harzianum* and *Stemphylium sp.* or *A.niger* from dual cultures, while *T.harzianum* grew alone in the plates. The microscopic observations of mycelia of dual culture in slide methods showed that the mycelia of *T.harzianum* induced degradation and aggregated the spores and analyzed the mycelia of *A.niger* , overgrowing the mycelia of *Stemphylium sp* and coiled around of them and degrading them. In *vivo* screening showed after 10 days of incubation an antagonistic activity of *T.harzianum* against the tested fungus on tomato fruits, with inhibition equal to 100 % and 95% in *Stemphylium sp.* and in *A.niger* respectively, compared with controls. Beside we found that the treated fruits with *T.harzianum* stayed saints as compared with control, when *Stemphylium* and *A.niger* soft rot infected all surface the test fruits. This strain of *T.harzianum* may offer potential for biological control of tomato *Stemphylium* and *A.niger* soft rot.

Introduction

Tomato plant (*L.esculentum* L.) originated South America belongs to Solanaceae family is a widely grown vegetable in the world. It is the most popular vegetable world-wide. The leading producer of tomato in the world is USA followed by China, Italy, Turkey, Egypt, Spain, Romania, Brazil and Greece (Wani, 2011). In Algeria, the

tomato crop is grown over an area of 292 000 hectares, it accounts for 51% of the total vegetable production (Nechadi *et al.,* 2002). Tomato crop suffered every year from a number of pathogenic diseases (Wani, 2011). The fungus *Stemphylium solani* causes leaf blight of tomato in Brazil (Mehta, 1998). Most the Stemphylium species on record as plant pathogens in Japan (Daisuke *et al.,* 2015). *A.niger* caused a disease called black mold on certain fruits and vegetables such as grapes, tomato, onions and peanuts (Sharma, 2012). Mallek *et al.,* (1995) reported that the *A.niger* was a one of the most common pathogens and caused loss of 25% in tomato fruit in Egypt. *A.niger* was responsible for the post harvest rot of Tomato fruits in Nsukka (Nigeria) and the pathogenesis tests confirmed that, the fungal isolate is one of the causal agents of the rot (Jude and Nneka, 2012). Chemical compounds have been used to control plant diseases, this has no doubt increased crop production but with the attendant deterioration of the environment and human health. In addition to killing target pathogens, pesticides may also kill various beneficial organisms and their toxic effects can persist in the soil but abuse in their employment has favored the development of pathogens resistant (Kamala and Indira, 2012 ; Nneka and Uken, 2013). The Algerian farmer used the chemical fungicides to control the fungal diseases of tomato plants, but in more time the treatment traces are observed in tomato fruits, because they usually aren't rinsed fruits before marketing (fig.1.1). Biological control using potential microorganisms having strong antifungal activity is coming up as an alternative strategy for disease management, which is also ecology-conscious and environment friendly (Kamala and Indira, 2012). Several biocontrol strategies have been proposed for controlling the plant pathogens, but practical applications are still limited (Hibar *et al.,* 2007). *Trichoderma* species are common soil-inhabiting fungi that have been developed as effective biocontrol agents against various phytopathogenic micro-organisms (Bel Haj Khethr *et al.,* 2008).

The aim of the present investigation was to isolate and to identify the mycetes accompanying the tomato fruits (*Lycopersicon esculentum*), and to evaluate the *in vitro* and *in vivo* ability of *T.harzianum* to control that isolated mycetes.

Materials and Methods

Fungal strains

Stemphylium sp. and *Aspergillus niger* were isolated from infected tomato fruits, which were brought from Oum-elbouaghi market, and identified based on the microscopic observations of their reproductive and colony characteristics in laboratory of microbiology, university of Oum-elbouaghi (Algeria) (Botton *et al.,* 1990; Rémi, 1997; Robert *et al.,*, 1981). A local strain of *T.harzianum* / *Hypocrea lixii* , was identified in the same laboratory and verified in Walloon Center of Biology Industrial, University of Liege, Belgium.

In vitro Evaluation of the antagonistic capability of *T.harzianum* against *Stemphylium sp.* and *A. niger*, on PDA medium (direct confrontation)

To study the direct confrontation between *T.harzianum* and *Stemphylium sp.* or *A.niger* Two plugs of mycelium (8mm diameter) were cut from the margins of actively cultures growing on PDA medium, one carrying the stock of *T.harzianum* and the other of *Stemphylium sp.* or *A.niger.* Then they placed at the periphery of Petri plates

(9cm in diameter) at the same distance on PDA medium (dual cultures). One plug of *Stemphylium sp.* or *A.niger* were maintained as controls (alone cultures). Each replicate has three plates. Both the dual and alone cultures were incubated at 25°c for four days, and measurement of colony diameters (in millimeters) was taken every 24 hours. The percentage of inhibition growth (I) was calculated by using the formula given below: [I (%) = (1 –T /C) x 100]. Where: I=Percentage inhibition of pathogen growth by antagonists. C=Radial growth in control. T=Radial growth in the treatment (Berber *et al.,* 2009; Hamitou and Dehimat, 2015).

Evaluation of dual culture using slide method

For each pathogen (*Stemphylium* or *A.niger*)-*T.harzianum* interaction, a clean slide was placed in 9 cm diameter plates and sterilized. Following that, a small amount of PDA medium was spread over the slide to make a thin PDA film on the slide. The 5 mm discs of one week old of each pathogen and *T. harzianum* isolates were placed on the opposite sides of the slide 3 cm apart on the PDA surface.

Then 5ml of distilled water was added to the plate to prevent drying and then incubated at 25°c for a week. At the end of incubation period, region of contact between *T. harzianum*–Pathogen hyphae was stained with lacto phenol and cotton blue and examined under a light microscope (Al-Saeedi and Moqdad, 2014).

Preparation of tomato fruits

Intact red tomatoes (*L.esculentum* Mill.), uniform in size and color, were obtained from the market of Oum-Elbouaghi city. The fruits were surface-sterilized by soaking in 2% aqueous sodium hypochlorite for 5 min, they were thoroughly rinsed with sterile distilled water, dried using sterile filter papers, and then wounded by removing a rectangular area at the equator of each fruit, (3cmx4cm) in diam. and 3 mm in depth, from the surface, using a sterile scalpel (Berrada *et al.,* 2012).

In vivo. Evaluation of the antagonistic capability of *T.harzianum* against *Stemphylium sp.* and *A.niger* on tomato fruits

Fresh cultures of *Stemphylium sp., A.niger* and *T.harzianum* were used for each experiment to evaluate the antagonistic activity. Two plugs of mycelium (8mm diameter) were cut from the margins of actively cultures growing on PDA medium, one carrying the stock of *T.harzianum* and the other of *Stemphylium sp.* or *A.niger* were then placed one beside of the other at the center of the rectangular area of tomato fruits.

As control, fruits were either inoculated with *Stemphylium sp.* or *A.niger* alone. The fruits were then stored at 20°C± 2.for 10 days in autoclaved glass jars with hermetic covers. The percentage of disease reduction of *Stemphylium* or *A.niger* rot on tomato fruits, was calculated using the following formula:

(%) = (A-B)/A×100

where A is the lesion diameter recorded in tomato fruit inoculated with the *Stemphylium sp.* or *A.niger* alone

B is the lesion diameter recorded in infected tomato fruits treated with *T.harzianum*. All *in vivo* antagonism assays were made in triplicate (Berrada *et al*., 2012; Hamitou and Dehimat, 2015).

Results and Discussion

In vitro Evaluation of the antagonistic capability of *T.harzianum* against *Stemphylium sp.* and *A.niger* on PDA medium (direct confrontation)

The results of the direct confrontation between *T.harzianum* against *Stemphylium sp.* and *A.niger* on PDA medium, showed that when the mycelium of the both cultures came due to the contact together, the hyphal growth of *Stemphylium sp.* and *A.niger* were found to be inhibited by hyphae of *T.harzianum* fig.(2.1 and.2.4).That inhibition in the third day of the experiment was: 41.18 % and 29.41% and in the fourth day the amounts were: 54.54 % and 52.17% for *Stemphylium sp* and *A.niger* respectively (table1) and (fig.3). Besides, showed no growth of mycelia of *Stemphylium sp.* or *A.niger* when re-planting the disks from the interaction hyphal area between *T.harzianum* and *Stemphylium sp.* or *A.niger* from dual cultures, while *T.harzianum* grew alone in the plates Fig (2.3 and 2.6).

Evaluation of dual culture using slide methods

The microscopic observations of mycelia of dual culture in slide methods showed that the mycelia of *T.harzianum* overgrowing the mycelia of *Stemphylium sp* and coiled around of them and degrading them fig.(4.2, 4.3); induced degradation and aggregated the spores and analyzed the mycelia of *A.niger* fig (4.5,4.6), compared with controls fig(4.1 and 4.4).

In vivo Evaluation of the antagonistic capability of *T.harzianum* against *Stemphylium sp.* and *A.niger* on tomato fruits

After 10 days of incubation the *T.harzianum* showed an inhibition activity with a different ratios against *Stemphylium sp.* and *A.niger* on tomato fruits. The latter was equal to: 75 % and 91.66% in the seventh day and the amount in the tenth day reached 100 % and 95% for *Stemphylium sp* and *A.niger* respectively (fig.5).

Beside we found that the treated fruits with *T.harzianum* stayed saints (fig. 6.d) and (fig.7.d) compared with controls when *Stemphylium* and *A.niger* soft rot infected all surface the fruits (fig.6.c) and (fig.7.c).

Table.1 In vitro. Effect of *T.harzianum* on the mycelia growth of *Stemphylium* sp. and *A.niger*, on PDA medium.

		Radial growth rate (mm) after:			
	Fungus species	**24 hour**	**48 hour**	**72 hour**	**96 hour**
Dual culture	***T.harzianum***	30	78	130	130
	Stemphylium sp	6	16	20	20
Alone culture	***Stemphylium sp***	10	20	34	44
% inhibition of mycelia growth	***Stemphylium sp***	40	20	41.18	54.54
Dual culture	***T.harzianum***	34	80	130	130
	A.niger	10	20	24	22
Alone culture	***A.niger***	12	22	34	46
% inhibition of mycelia growth	***A.niger***	16.16	9.1	29.41	52.17

Fig.1 Treated tomato fruits with fungicide, 1. Infected tomato fruits with *A.niger* rot,2., Infected tomato fruits with *Stemphylium* rot,3., Some tomato fruits utilized in the *in vivo* test, 4.

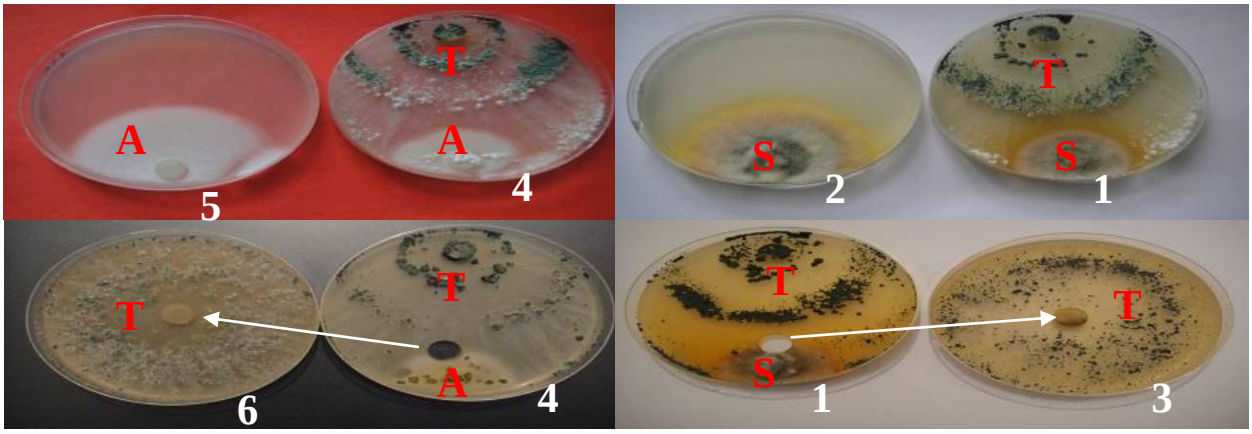

Fig.2 *In vitro* effect of *T.harzianum* against *Stemphylium sp.* and *A.niger*. dual cultures(1)and (4)., controls (2) and (5)., re-planting plates (3) and (6). S= *Stemphylium* , A= *Aspergillus*, T=*Trichoderma*.

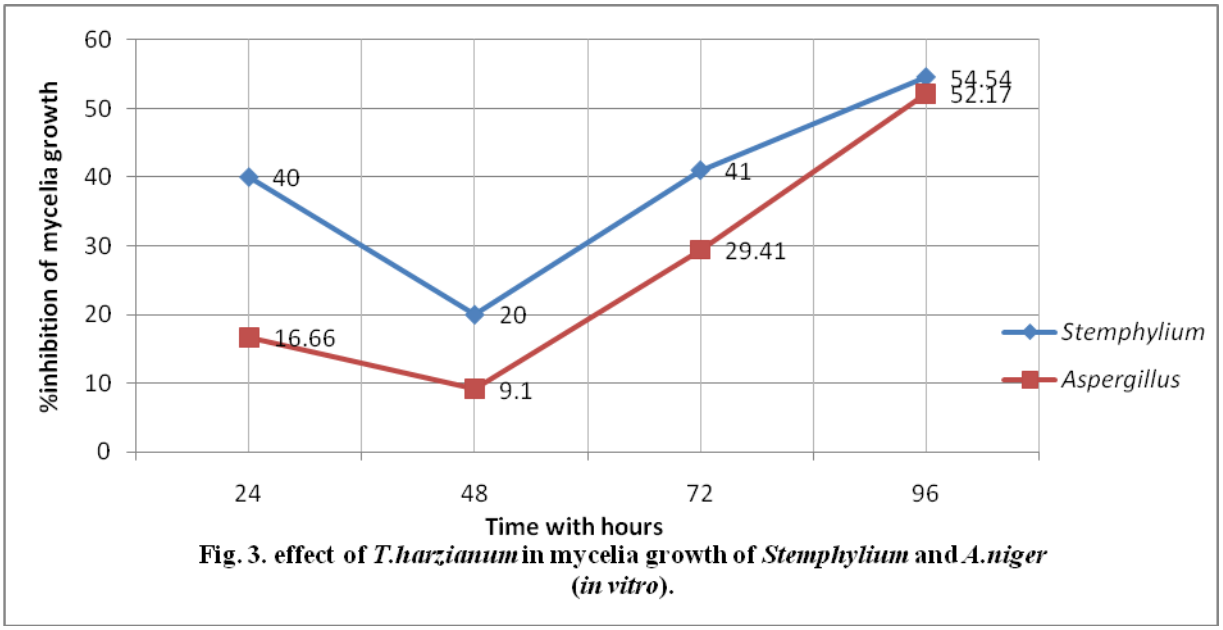

Fig. 3. effect of *T.harzianum* in mycelia growth of *Stemphylium* and *A.niger* (*in vitro*).

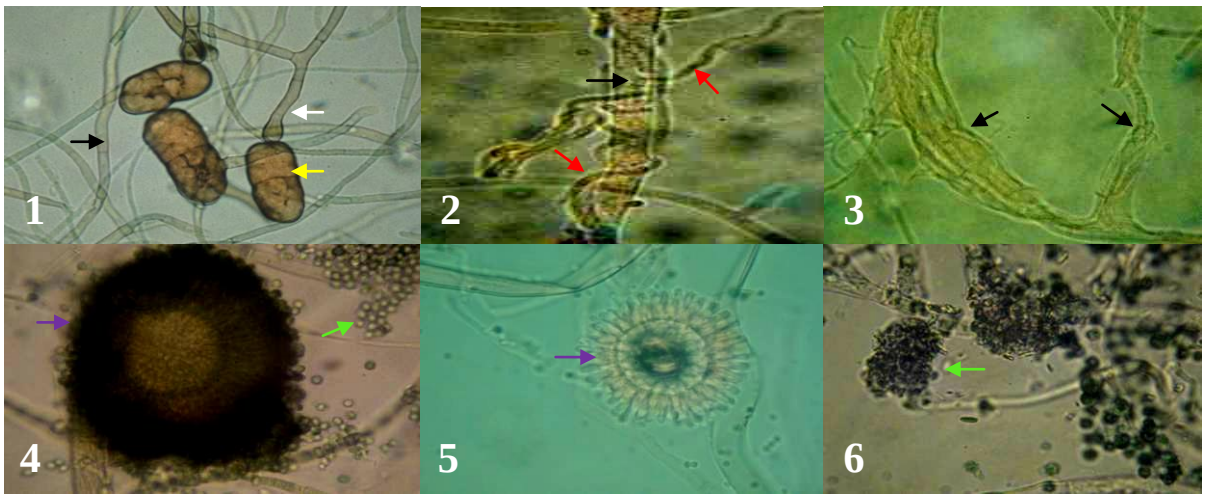

Fig.4 Microscopic observations of the *in vitro* effect of *T.harzianm* against *Stemphylium sp.* and *A.niger*. Decomposition phenomenon(3), (5) and(6); Mycoparasitism phenomenon (*Trichoderma* hyphal coiling around of *Stemphylium* hyphal),2. Controls,1 and 4. *Stemphylium*(hyphe=black arrow, sporophore = white arrow, dictyospore= yellow arrow)., *A.niger* (conidia= green arrow, vesicle=purple arrow., *Trichoderma* hyphe = red arrow.

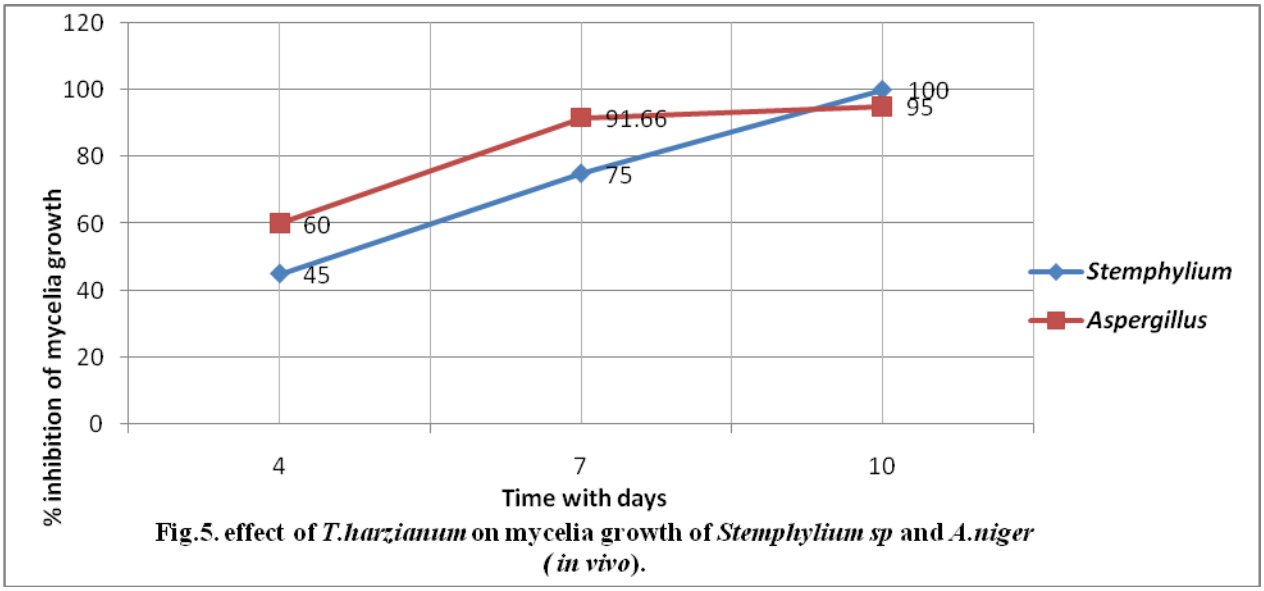

Fig.5. effect of *T.harzianum* on mycelia growth of *Stemphylium sp* and *A.niger* (*in vivo*).

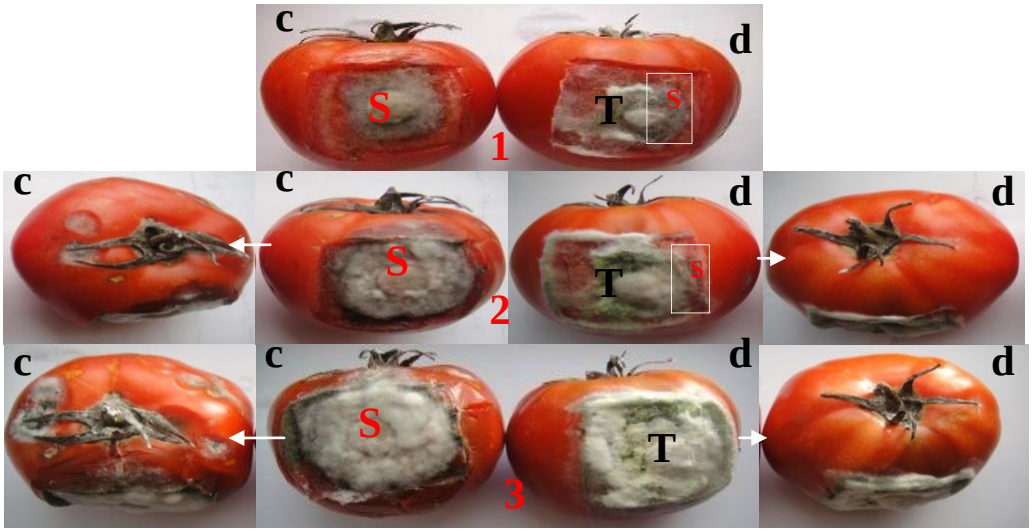

Fig. 6 *In vivo* effect of *T.harzianum* against *Stemphylium sp*. Test(after 4 days, 1. ; after 7 days,2. ; after ten days,3.) ; c= control., d= dual culture ; *S=Stemphylium*, T= *Trichoderma*.

Fig.7 *In vivo* effect of *T.harzianum* against *A.niger.* test(after 4 days,1.; after 7 days,2.); c= control., d= dual culure. A= *A.niger,* T=*Trichoderma*.

In this investigation, this local strain of *T. harzianum* showed a high efficiency both *in vitro* and *in vivo* against *Stemphylium sp.* and *A.niger* . This results was confirmed by many paper studies, where found that the *T.harzianum* could restrict growth of *A.niger in vitro* (dual culture) with 75% and amounted to reach 78.77% (Agrwal *et al.*, 2011; Lone *et al.,* 2012). The *T.harzianum* can inhibited the growth of *Bipolaris sp., F.oxysporium*, *Fusarium sp.* and *R.solani* with a different ratios, and inhibited there spore's formation, with recording a different degrees of parasitism (Azza and Allam, 2004; Berber *et al.,* 2009; Comporota, 1985; Hibar *et al.,* 2005). *T.harzianum* strains produced an inhibitor metabolites as 1, 3- b-glucanase and chitinase which were inhibited the growth of *G.graminis* var. *tritici*, *F.culmorum* and *F.moniliforme* on PDA medium (Cigdem and Merih, 2004). *T.harzianum* can produced nonanoic acid into a liquid culture medium. The latter has a strongly affected both mycelial growth and spore germination of the cacao pathogen (*Crinipellis perniciosa* and *Moniliophthora roreri*) (Anejaa *et al.,* 2005). *T.harzianum* reduced disease incidence significantly against *P.ultimum* and *R.solani* on both cucumber and tomato on greenhouse (Johanne *et al.,* 2002). In the similar study, Yacoub, (1999), found that the *Trichoderma sp* reduced the lesion development and number of conidiophores of *Botrytis sp.* in foliar discs of strawberry test, compared with the non–treated (control). This local strain of *T.harzianum* may offer potential for biological control of tomato *Stemphylium* and *A.niger* soft rot.

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