

## Contribution in isolation and identification of some pathogenic fungi from wheat seeds, and evaluation of antagonistic capability of *Trichoderma harzianum* against those isolated fungi in vitro.

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### ABSTRACT

The aim of this study was to isolate and to identify the pathogenic fungi accompanying interior in hard wheat seeds, and to evaluate the antagonism capability in vitro of (*Trichoderma harzianum*) against those pathogenic isolated fungi. Three samples of local hard wheat seeds (*Triticum durum* Desf) were brought from Oum-elbouaghi (Algeria) silos yields, follower for varieties: Vitron, Waha, and Cirta. ).They were stored for one year. The results of isolation on potato-dextrose-agar (PDA) medium and after identification of fungal accompanying interior in the seeds clarified about (83) fungus isolates following for species next : *Fusarium acuminatum* (1 isolate) in a ratio of (1.2%), and *Alternaria*(82 isolates) with a ratio of (98.795 %).The *Alternaria* isolates apportioned among two species: *Alternaria alternata* with 39 isolates(12 isolates for: *Alternaria alternata*1 and 27 isolates for : *Alternaria alternata*2) and *Alternaria infectoria* with 43 isolates).And, besides it was clearly seen that the Cirta variety was a lowest infected with fungi(19 isolates) in ratio of (22.89 %), and both varieties Waha and Vitron with(32 isolates)for each one in a ration equal (38.554 %) . The results of the antagonistic capability in vitro of (*Trichoderma harzianum*) against isolated fungi, showed that: 1- The direct confrontation in vitro of *Trichoderma harzianum* against the different fungus isolates on PDA medium, showed that: a- In the fourth day of the experiment, *Trichoderma harzianum* inhibited the mycelia growth of different pathogenic fungi in dual cultures, with different ratios, it was equal to: 6.66% for *Fusarium acuminatum*, and 23%, 26.66% and 40% for *Alternaria alternata*1, *Alternaria alternata*2, and *Alternaria infectoria*, respectively. b-The microscopic observations of the different interactions hyphal between *Trichoderma harzianum* and different fungus isolates, showed that the antagonistic fungus affected on *Alternaria* species with the decomposition phenomenon (Lyses), where it analyzed the mycelia and spores of *Alternaria altrenata*2 and *Alternaria infectoria*, while it analyzed the mycelia of *Alternaria alternata*1, compared with control. 2- The remote confrontation in vitro of *Trichoderma harzianum* against the different fungus isolates on PDA medium showed that : a- The volatile metabolic substances of *Trichoderma harzianum* affected the growth of the different pathogenic fungi, with different ratios over the seven days of treatment, compared with control.b- The microscopic observations Noted that the volatile metabolic substances of *Trichoderma harzianum* affected some isolated fungi with mycelia analysis prevent the spore's formation of the *Alternaria alternata*2, while stopped the spore formation of *Fusarium acuminatum*, compared with control.

**Key words:** wheat seeds, antagonistic, *Fusarium acuminatum*, *Trichoderma harzianum*, *Alternaria*.

### INTRODUCTION

Wheat seeds are a favorable medium for the pathogenic mycoflora and carrying them, those fungi cause a decrease in both seed vitality, and nutritional value, moreover the excretion of mycotoxins (Moreno and al., 1986). *Alternaria* genus contains a great

number of species plus of sixty, parasites or saprophytes, there effect announced on seeds; *Alternaria* parasites are the origin of the germination lacks, sowing dissolution and there are significant inoculum sources of the adult plants. (Rémi, 1997). Different taxa in the species-group of *Alternaria infectoria* (teleomorph *Lewia* spp.) are often isolated

from various cereals including barley, maize and wheat grain, ornamental plants and skin lesions from animals and humans.(Andersen *and al.*,2009). *Alternaria alternata* present on all species of seeds, it met on the leaves, the fruits or seeds of many plants of various parasites or at the end of the vegetation (Rémi, 1997). In pathogenicity study of five *Fusarium spp* frequently isolated from wheat and barley roots in the southern Idaho during growth –chamber experiments and field studies showed that the presence of *Fusarium culmorum* from infected root tissue, followed by *F.acuminatum* and *F. reticulatum*, however *F.semitectum*, followed by *F.acuminatum* and *F.equiseti* had the greatest impact on total root length (Carl *and al.*, 2005). The addition of chemical fungicides may reduce or eliminate fungal growth on the seeds, but they can decrease the seed vitality at the same time. (Moreno *et al.*, 1986). chemical pesticides cause a significant damage to the public health, environment and groundwater pollution; also, it is uneconomical, so that, recently the scientists use the biological control. In bibliographical study of more than 200 research was found that the *Trichoderma sp* plays an important role in biological control, and it represents 60% of all other biofungicides include bacteria, nematode and virus, it used as a pesticide, and herbicide; also found that the use of this fungus was improved the plant growth (Mausam *and al.*, 2007). A treatment of *Crossandra infundibuliformis* var.Danica with *Trichoderma viride* and *T.harzianum* decreased the wilt diseases which caused by *Fusarium oxysporium*, and increased the plant growth, both in the field trials and in laboratory alike; that study strongly suggests that the *Trichoderma* isolates, especially, *T.viride* can be exploited for the biological control of wilt disease at field level (Jegathambigai *and al.*, 2009). *Trichoderma viride* and *T.harzianum* were used in the control against the associated fungi with seeds, including *Aspergillus flavus* and *Fusarium moniliforme*; also they were used as anti-fungal against *Lasiodiplodia theobroma*, *Diplodia natalensis*, *Botryodiplodia theobromae*, *Rhizoctonia sp*, *Aspergillus niger*, *A.tamarii*, *Penicillium oxalicum*, and *P.sclerotinum* (Calistru *and al.*, 1997; Thangavelu *and al.*, 2004; Okigbo and Okediugwu, 2000; Moreno and Paningbatan, 1995; Mortuza and Ilag, 1999). The *Gloicladium* and *Trichoderma* have been mostly were used as biofungicides agents; they showed a high inhibition against certain fungal diseases, their effects were equal or exceeded at, those which were of some chemical pesticides. (Illipronti *and al.*, 1993; Harman *and al.*, 1980).

The goal of this research was to isolate and to identify the interior associated fungi with three varieties of wheat seeds :( Vitron, Waha, and Cirta. ), and investigate the effect of one antagonistic local isolate of *Trichoderma harzianum* against the fungal isolates.

## MATERIALS AND METHODS

**Wheat seeds:** Three samples of local solid wheat seeds (*Triticum durum* Desf) were brought from Oum-elbouaghi (Algeria) silos yields, follower for varieties: Vitron, Waha, and Cirta. ). They were stored for one year. (Fig.1a).

**Isolation and identification of fungal accompanying interior on the seeds:** 200 seeds of each variety were sterilized with 10% sodium hypochlorite solution for 2 min and rinsed twice with sterilized distilled water, the seeds were dried with sterile filter paper and plated (15 seeds per plate), on fresh Potato Dextrose Agar(PDA) medium impregnated with (10% of Acetic Acid solution, 1ml /100ml of medium), each replicate has three plates, and incubated for 7-10 days at 27°C. The resulting of borne fungus colonies on seeds were sub-cultured by transferring a small mycelia plugs from the colony margins. Pure culture was obtained by sub-culturing three times (Booth, 1977). The fungi were identified on the basis of their morphological and reproductive characters (Booth, 1977; Booth *and al.*, 1990; Robert *and al.*, 1981).

**Fungus material:** Four isolates of pathogenic fungi, follower for species: *Alternaria alternata*1, *Alternaria alternata*2, (*Alternaria infectoria* /*Lewia infectoria*), and *Fusarium acuminatum*, were isolated from wheat seeds. Another sample of antagonist ( *Trichoderma harzianum* / *Hypocrea lixii*) was isolated from the soil of wheat plant. All samples were identified in Laboratory of Microbiology, University of Oum-elbouaghi (Algeria), and verified by Professor Thonart Philippe, Microbial biotechnology, Walloon Center of Biology Industrial, University of Liege, Belgium.

**In vitro. Evaluation of the antagonistic capability in vitro of *Trichoderma harzianum* against the pathogenic Fungi, on PDA medium (direct confrontation):**

**Dual culture technique:** For study the confrontation vis-à-vis between the antagonistic fungus and the other pathogenic fungi, two discs (8mm in the diameter) of one week old culture on (PDA), one carrying the stock of the antagonistic agent (*Trichoderma harzianum*) and the other of the pathogenic agent were then placed at the periphery of Petri plate (9cm in diameter) at the same distance

on PDA medium. One disc of each pathogenic agent was maintained as control (alone culture). Each replicates has three plates. Both the dual and alone cultures were incubated at 25°C for four days, and measurement of radial mycelia of the fungus was taken every 24 hours. The percentage inhibition of growth (I) was calculated using the formula given below:  $[I (\%) = (1 - T / C) \times 100]$ . Where: I=Percentage inhibition of pathogen growth by antagonists. C=Radial growth in control. T=Radial growth in the treatment.

The speed of the fungal colony growth (V) was measured using the formula given below (Rapilly, 1968):  $V = [(L2-L1) + (L3-L2) \dots (Ln-Ln-1)] / n-1$ , V = the speed of growth (mm / day), L =mycelia growth (mm), L1 = growth in the first day. Ln = growth in the last day.  $D = (D1 + D2) / 2$ .  $L = D-d / 2$ . L = the growth of the fungal mycelia (mm), D = diameter of the fungal colony (mm), d = diameter of the initial fungal disk (Azza and Allam, 2004; Camporota, 1985; Carl *and al.*, 2005; Rapilly, 1968).

Effect of the volatile substances of *Trichoderma harzianum* on the growth of the pathogenic fungi on the PDA medium (remote confrontation): This method consists in mending the antagonist fungus and the pathogenic one, in two plates separated

thereafter, an assembly is carried out by the superposition of two plates, antagonist in bottom and the pathogenic one in top, the junction between the two plates is ensured by layers of parafilm in order to avoid all loss of volatile substances. for study the effect of the volatile substances of *Trichoderma harzianum* on the growth of the pathogenic fungi on PDA medium, two discs (8mm in the diameter) of one week old culture on (PDA), one carrying the stock of the antagonistic agent (*Trichoderma harzianum*) and the other the pathogenic agent, were then placed at the center of Petri plates (9cm in diameter) containing PDA medium. The lids are removed aseptiquement, then the bottom of each plate containing the antagonist tested is placed below that containing the pathogenic fungus, the two juxtaposed bottoms are closed by layers of Parafilm. For the control, a bottom of plate containing the medium alone is invested below a bottom plate containing the pathogenic fungus. Each replicates has three plates, figure (2). Both the dual and the alone cultures were incubated at 25°C, in the darkness for four days, and measurement of radial mycelia growth were taken every 24 hours. The percentage growth inhibition (I) was calculated like previously (Camporota, 1985; Fadwa *and al.*, 2009). (fig.1c).

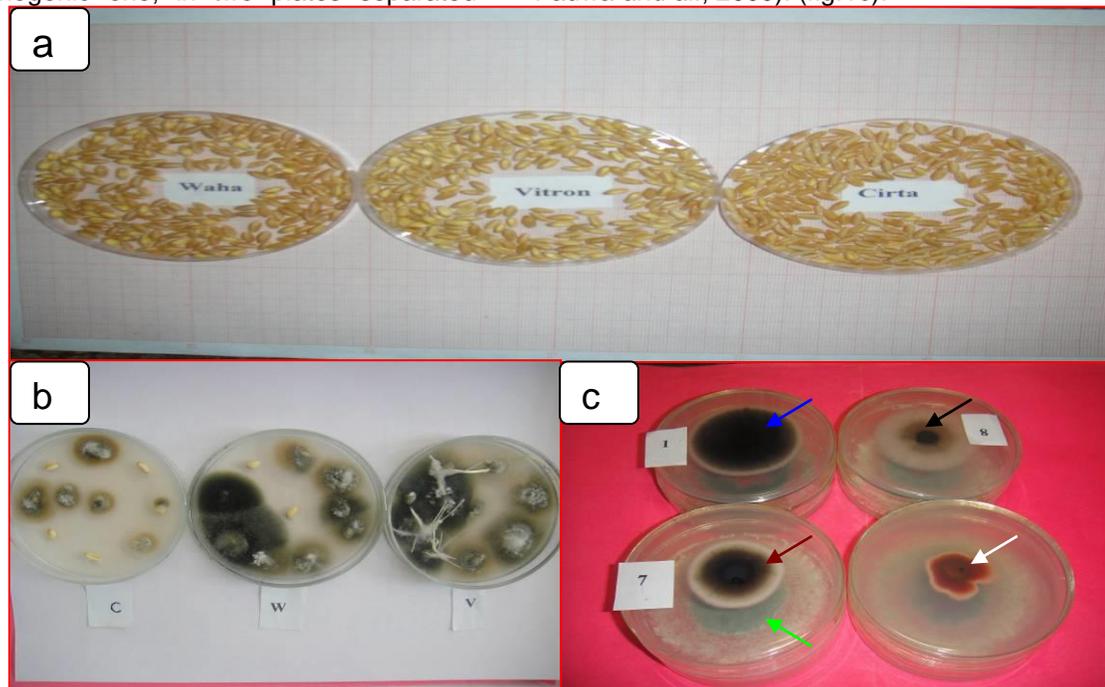


Figure 1: Wheat seeds and isolated fungus used in present study: a: Varieties of wheat seeds used in this study. b: Some Petri plates with borne fungus on wheat seeds C= Cirta, W=Waha, V=Vitron. c: Method used in the impact study of the volatile substances of *Trichoderma harzianum* (green arrows), against the isolated fungus. 1 = *Alternaria alternata*1 (blue arrow). (7)= *Alternaria alternata*2 (brown arrow). (8)=*Alternaria infectoria* (black arrow). *Fusarium acuminatum* (white arrow).

**RESULTS:**

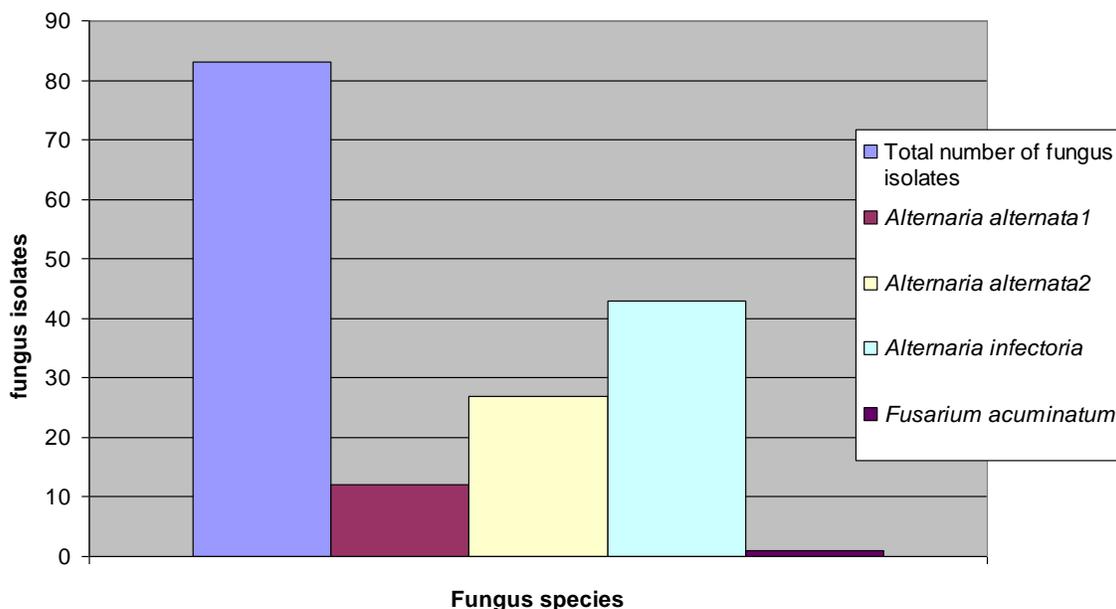
**Isolation and identification of fungi accompanying interior in the seeds:** The results of isolation on potato-dextrose-agar (PDA) medium and identification of fungal accompanying interior in the seeds clarified about (83) fungus isolates following for species next : *Fusarium acuminatum* (1 isolate) in a ratio of (1.2%), and *Alternaria*(82 isolates) with a ratio of (98.795 %).The *Alternaria* isolates

apportioned among two species: *Alternaria alternata* with 39 isolates(12 isolates for: *Alternaria alternata*1 and 27 isolates for : *Alternaria alternata*2) and *Alternaria infectoria* with 43 isolates).And, besides it was clearly seen that the Cirta variety was a lowest infected with fungi(19 isolates) in ratio of (22.89 %), and both varieties Waha and Vitron with(32 isolates) in a ration equal (38.554 %) for each one [Figures (1b)and(2)],(Table 1).

**Table 1: Isolated fungus from wheat seeds.**

Seeds varieties	Fungus species				Total number of isolates
	<i>Alternaria alternata</i> 1	<i>Alternaria alternata</i> 2	<i>Alternaria infectoria</i>	<i>Fusarium acuminatum</i>	
Cirta	6	7	6	0	19
Waha	0	9	22	1	32
Vitron	6	11	15	0	32
Total number of isolates	12	27	43	1	83

**figure 2: Isolated fungus from Wheat seeds,**



**The antagonistic capability in vitro of *Trichoderma harzianum* against the pathogenic fungus:**

The results showed that: 1- The direct confrontation of *Trichoderma harzianum* against the different fungal isolates in vitro on PDA medium, showed that when the mycelium of both the cultures came in contact with each other the hyphal growth of the pathogenic fungus were found to be inhibited by the hyphae of *Trichoderma harzianum*. This inhibition with different ratios, it was equal in the fourth day of the experiment to 23% , 26.66% and 40% for *Alternaria alternata*1, *Alternaria alternata*2 and

*Alternaria infectoria*, respectively, but amounted a lowest ratio in the fourth day to 6.66% for *Fusarium acuminatum* .[Table (2) and figures (3), (4-1A),(4-2A),(4-3A)and (4-4A)]. The present results did not showed any growth of the different *Alternaria* species when re-planting a disk from the interaction hyphal areas between *Trichoderma harzianum* and the different *Alternaria* species from different dual cultures, while *Trichoderma harzianum* grew [ Figures (4-2B),(4-3B),(4-4B)], except *Fusarium acuminatum* when grew alone in the plate Figure (4-1B).

**Table 2: In vitro. Effect of *Trichoderma harzianum* on the mycelia growth of the pathogenic fungi, and speed mycelia growth in dual cultures, on PDA medium.**

Test number	Fungus species	Percentage inhibition of mycelia growth after:				Speed of mycelia growth in dual Cultures(cm/day)
		1 day	2 days	3 days	4 days	
01	<i>Alternaria alternata</i> 1	/	00	00	23	1.2
	<i>Trichoderma harzianum</i>	/	/	/	/	3.6
02	<i>Alternaria alternata</i> 2	/	00	00	26.66	1
	<i>Trichoderma harzianum</i>	/	/	/	/	4
03	<i>Alternaria infectoria</i>	/	00	14.28	40	0.8
	<i>Trichoderma harzianum</i>	/	/	/	/	4
04	<i>Fusarium acuminatum</i>	/	00	00	6.66	1.2
	<i>Trichoderma harzianum</i>	/	/	/	/	3.4

Figure 3 :In vitro, effect of *Trichoderma harzianum* on the mycelia growth of the pathogenic fungi (dual cultures),on PDA medium.

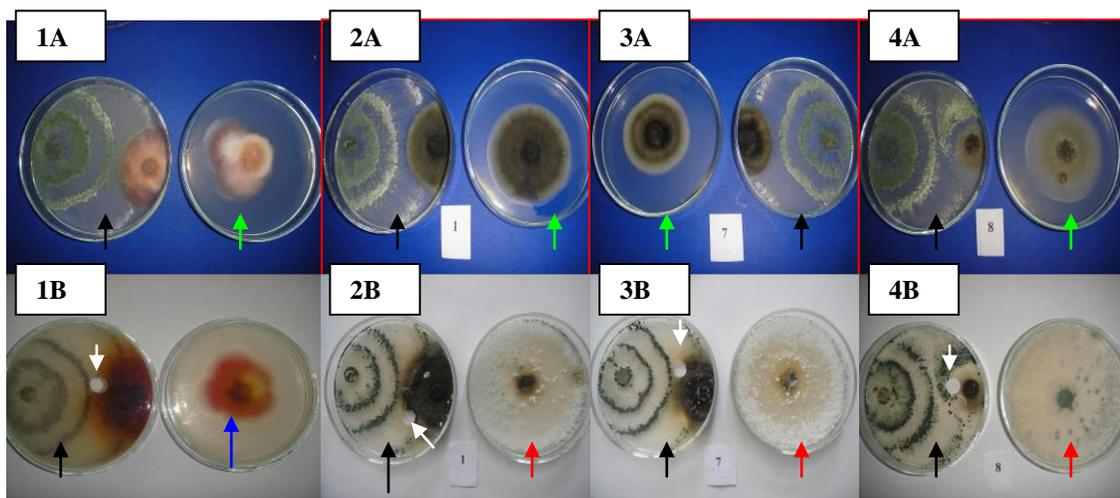
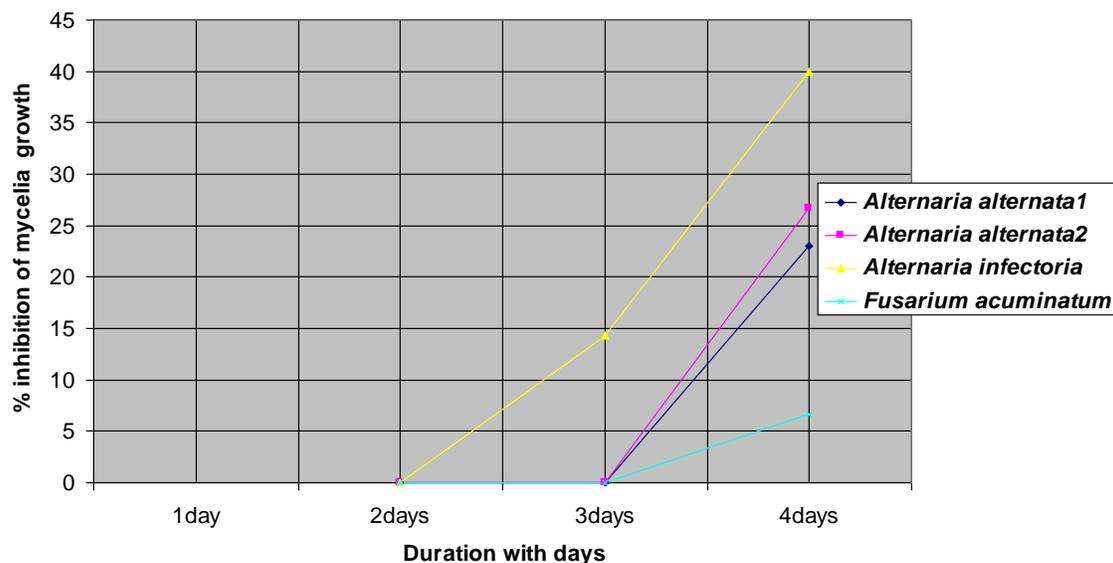


Figure 4: Direct confrontation on PDA medium, between *Trichoderma harzianum* (green colony), and the different pathogenic fungus, on (dual cultures) (black arrows). Compared with the alone cultures (controls) (green arrows). (1)= *Fusarium acuminatum*. (2)= *Alternaria alternata1*. (3)= *Alternaria alternata2*. (04)= *Alternaria infectoria*. (A)= First test. (B)=After replanting a disc from the interaction areas (white arrows), where *Trichoderma* growth only appear (red arrows),or *Fusarium acuminatum* growth only appear (bleu arrow).

2-The microscopic observations of the different interactions hyphal displayed that the antagonistic fungus affected the pathogenic fungi with decomposition phenomenon (Lyses): the antagonistic fungus was analyzed the mycelia and spores of

*Alternaria alternata2* and *Alternaria infectoria*, figure (5 -3B, 4B), while was analyzed the mycelia of *Alternaria alternata1*, compared with control. Figure (5-2B).

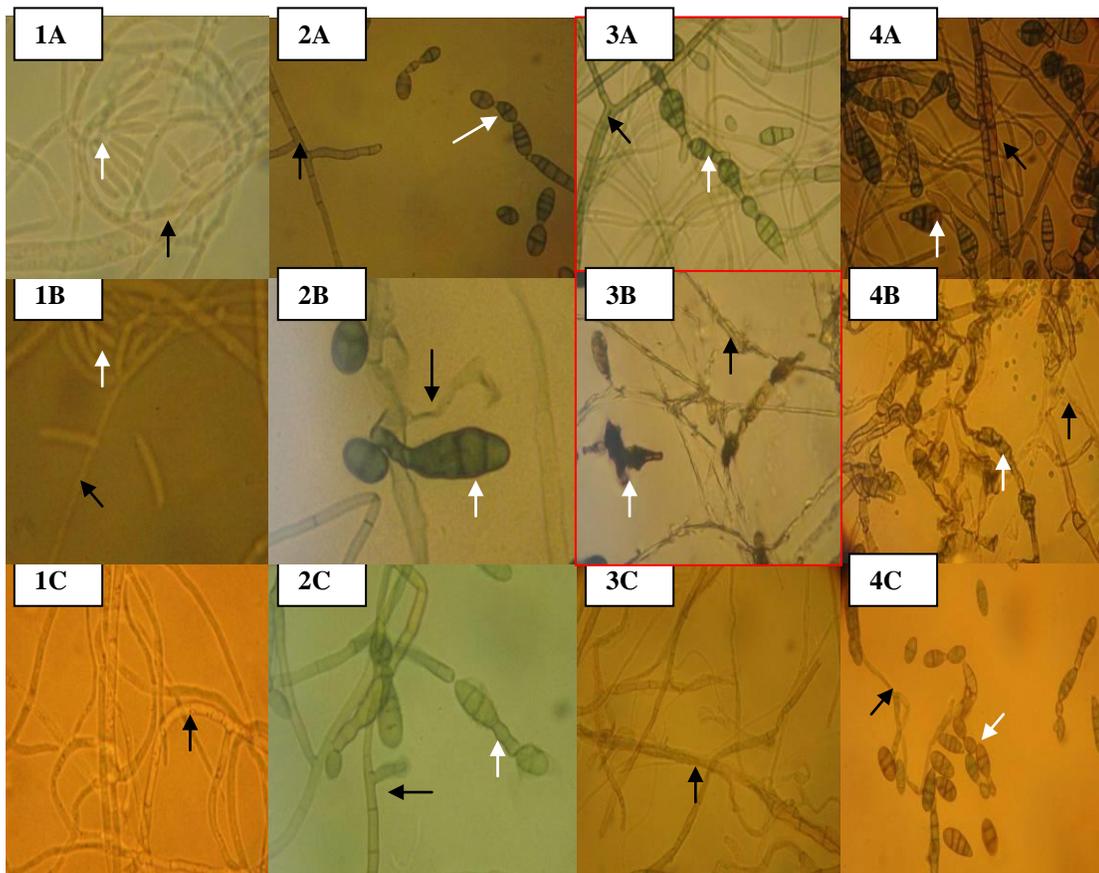


Fig 5: Microscopic observations showed the affect of the *Trichoderma harzianum* and its volatile metabolite substances on mycelia (black arrows) and on spore formation (white arrows) of the pathogenic fungi compared with controls. (1) = *Fusarium acuminatum* . (2)= *Alternaria alternata1*. (3)=*Alternaria alternata2*. (4)= *Alternaria infectoria*. (A)=Controls. (B)= Direct confrontation affect. (C)=Volatile substances affect. 40 x.

**In vitro. Effect of the volatile substances of *Trichoderma harzianum* on the growth of the pathogenic fungus on PDA medium:** The remote confrontation showed that the volatile metabolic substances of the antagonistic fungus inhibited the mycelia growth of the different pathogenic fungus, with different rates over the seven days of treatment, the percentage inhibition of mycelia growth peaked after two days of treatment to reached 12.5% and 20% for *Alternaria alternata1* and *Alternaria alternata2*, respectively, and decreased to 3.22% in the sixth day in *Alternaria alternata1* and scored in the sixth day a ratio equal to 28% for *Alternaria*

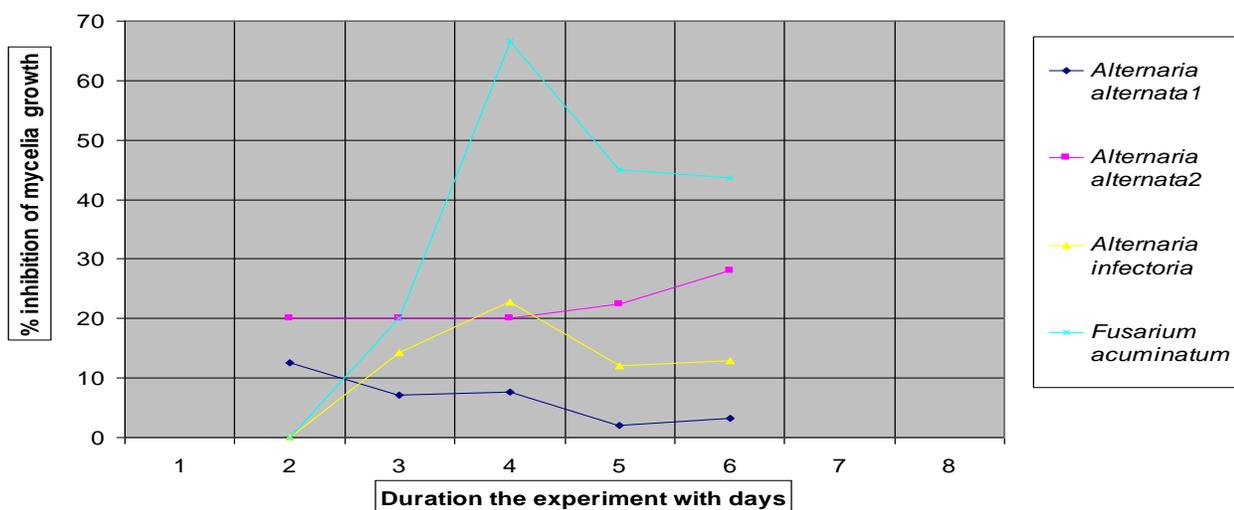
*alternata2*, and scored the maximum ratio in the fourth day in *Alternaria infectoria* to 22.72%, and lowered to 12.9% in the sixth day, but in the *Fusarium acuminatum* has recorded the lowest inhibition percentage 20% in the third day and a highest inhibition percentage 66.66% in the fourth day. [Table (3) and figure (6)].

The microscopic observations noted that the volatile metabolic substances of *Trichoderma harzianum* affected on the isolated fungus with the mycelia analysis and prevent the spore's formation of the *Alternaria alternata2*, (figure, 7-3C), while stopped only the spore formation of *Fusarium acuminatum*, compared with control. (Figure, 7-1C).

**Table 3: Percentage inhibition of the mycelia growth of isolated fungi by the volatile substances of *Trichoderma harzianum*, in the dual cultures, on PDA medium.**

Fungus species	Percentage inhibition of mycelia growth after:							
	1day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
<i>Alternaria alternata1</i>	/	12.5	7.14	7.69	1.96	3.22	/	/
<i>Alternaria alternata2</i>	/	20	20	20	22.5	28	/	/
<i>Alternaria infectoria</i>	/	0	14.28	22.72	12	12.9	/	/
<i>Fusarium acuminatum</i>	/	0	20	66.66	45	43.63	/	/

**Figure 6 :The volatile substances effect of *Trichoderma harzianum* on mycelia growth of isolated fungus.**



**DISCUSSION**

The results of this study revealed that the antagonistic fungus (*Trichoderma harzianum*) has a high inhibitory effect against the different isolated fungi, with a several biological modes: 1- Competition with his faster growth in the dual cultures compared with those which were of the different

isolated fungus (Table 2). 2-lyses (figure 5(2B, 3B, 4B). 3- Volatile substances effect (Table 3) and [Figures 5(1C, 3C)]. This results has been reported and confirmed by Fadwa *and al.*(2009) when studying the effect of the antagonism in vitro between six isolates of the antagonistic fungus *Trichoderma harzianum* and *T. viride* against four pathogenic isolates of *Bipolaris* and found that the *Trichoderma harzianum* inhibited the pathogenic

fungus growth with a different ratios, including the following: 68.55-72% and 69.52-73.32% for each of *B.maydis* and *B.sorghicola* respectively, and 67.02-70.02% for each of *B.sorokiniana* and *B.tetramra*, and *Trichoderma viride* inhibited the mycelia growth of (*B.maydis* and *B.sorghicola* and *B.sorokiniana* and *B.tetramera*) with ratios as follows: 67.55-74.48% and 69.52-82.85% and 68.12-73.61% and 71.22-76.66%, respectively, and they inhibited the spore's formation, and found that the volatile metabolic substances of different antagonistic isolates affected the mycelia growth and spores formation of the pathogenic fungus with a different rates, with recording a different degrees of parasitism of various antagonistic fungi at the pathogenic isolates. Hibar *and al.* (2005) found that the antagonism in vitro of *Trichoderma harzianum* against *Fusarium oxysporium* showed an inhibition on the pathogenic fungus growth with a ratio more than 65%, moreover the volatile metabolism substances of the antagonism reduced the pathogenic fungus growth by 63% compared with controls. Comporota, (1985) studied the antagonism in vitro, between 28 biological isolation of *Trichoderma*, follower for species: 14 isolates of *T.harzianum*, 5 isolates of *T.hamatum*, 3 isolates of *T.viride*, one of *T.koningii* and 5 non-specific type, on 3 isolates of the pathogenic fungus *Rhizoctonia solani* Kuhn, and the different antagonistic isolates showed a different effect on the pathogenic fungus which affected the mycelia growth with a different degrees, and their volatile metabolism substances inhibited the mycelia growth and spores formation of the pathogenic fungi. And also including by Larralde *et al.* (2008) when they chose 9 fungal isolates of *Trichoderma*: 2 of *T.atroviride*, 2 of *T.longibrachiatum* and 1 of each *T.reesi* and *T.koningiopsis* and *T.citrinoviride* and 02 did non-specific type from 30 isolates of *Trichoderma*, where they inhibited the growth of pathogenic fungus (*Macrophomina phaseolina*) with proportions higher than 50% during the antagonism study, and the microscopic observations in the hyphal interaction showed that the fungal antagonistic fungus has an ability to analyze the hyphae and sclerotes of the pathogenic fungus, the analysis of the metabolic substances of these antagonistic fungi in laboratory revealed that there is a positive correlation between

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the strength of inhibition of these fungi with the high quantity of enzymatic production of B-1, 3glucanase and N-acetylhexosaminade. Azza and Allam (2004) discovered that the *Trichoderma sp* isolates have a strong antagonism against wilt diseases caused by *Fusarium sp*, in vitro, on potato dextrose agar medium, when decreased his growth with the following proportions: (88%), (86%) and (80%) for *Trichoderma harzianum*, *T.hamatum* and *T. viride* respectively. Ramsy (1991) Found that the *Rhizopus stolonifer* and *Trichoderma harzianum* and *T. viride*, inhibited the mycelia growth, and lowered the proportion of spore germination and spore tube lengths of *Bipolaris oryzae* and *Pyricularia oryzae*. The treatment of cowpea seeds with spore suspension of *Trichoderma viride* has protected them against the brown blotch disease which was caused by *Colletotrichum truncatum* and found that the *T.viride* produced a volatile and non-volatile organic compounds, Viridin and antibiotics, biofungicides (Bankole and Adebanjo, 1996). Also was found that the *Trichoderma viride* isolate (T60) when used as a commercial biopesticide against *Coniophora puteana* and *Postia placenta* and *Serpula lacrymans* has a multiple effects with a volatile and non-volatile organic compounds, Lytic enzyme and soluble antibiotics in the water, and nutrient competition (Brown and Bruce, 1999; Brown *and al.*, 1999). Interactions between *Trichoderma harzianum* strains and some soil borne plant pathogens (*Gaeumannomyces graminis* var. *tritici*, *Fusarium culmorum* and *F. moniliforme*) were studied on PDA medium. All *T. harzianum* strains tested produced a metabolite that inhibited growth of plant pathogenic fungi on PDA medium. When grown in liquid cultures containing laminarin, chitin or fungal cell walls as sole carbon sources, 2 strains of *T. harzianum* produced 1, 3-b-glucanase and chitinase in the medium, higher levels of these enzymes were induced by *T. harzianum* T15 (Cigdem and Merih, 2004). These results showed that a high efficacy of this local isolate of *Trichoderma harzianum*, against a few dangerous pathogenic fungi, this study strongly suggests that this *Trichoderma harzianum* isolate can be exploited for the biological control of seed diseases at the field level.

will be presented as part of the doctoral dissertation of the first author, Hamitou, M. We are grateful to Professor Thonart Philippe, Microbial biotechnology, Walloon Center of Biology Industrial, University of Liege, Belgium, for confirmation the identification of the fungus isolates.

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