CATRINA (2007), 2 (2): 193 - 201
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PROCEEDINGS OF THE SECOND INTERNATIONAL CONFERENCE ON THE ROLE OF GENETICS AND BIOTECHNOLOGY IN CONSERVATION OF NATURAL RESOURCES, ISMAILIA, EGYPT, JULY 9-10, 2007

Evaluation of the Biocontrol Activity of Different *Trichoderma* Formulations

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ABSTRACT

Several natural organic products were tested for their capability to support the growth and sporulation of *Trichoderma*. These were pagass, rice straw, sawdust, peanut shell and whey. Rice straw gave the highest sporulation followed by sawdust. On the other hand, whey, which gave no spores when added to any of the aforementioned materials, increased the growth and sporulation. Spores viability of *Trichoderma* was measured at intervals of 10 days for two months. Field experiment revealed different responses of kidney bean plant to the treatment with these substrates. *Trichoderma* with rice straw were the most effective. It significantly reduced *Sclerotinia* rot disease and supported plant growth compared to control, and all other used materials. Pagass alone showed a great effect on plant vigor.
Key words: *Trichoderma,* formulation**,** *Phaseolus vulgaris,* viability.

INTRODUCTION

Diseases caused by phytopathogenic soil borne pose serious threats to the production of several crops in tropical, subtropical and temperate regions of the world (Willetts & Wong, 1980; Punja, 1988). Though chemical control of pathogens, especially of sclerotial pathogens viz. *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, etc., reduce the disease to some extent, it represents another hazardous issue to environment and man. An alternative and effective method to control these pathogens is the use of biological control agents (Harman *et al.*, 1994 and 2004).

White mold, also known as *sclerotinia* rot and *sclerotinia* wilt is caused by *Sclerotinia sclerotiorum* (Lib.) de Bary. This fungus attacks a wide range of susceptible hosts and has a worldwide distribution. It frequently causes serious and unpredictable yield losses in bean *(Phaseolus vulgaris* L.) Despite this, the disease has not yet been controlled effectively and economically. Control measures which are commonly used include the application of foliar bioprotectants, seed treatments, sclerotia germination inhibitors, soi1 disinfectants, crop rotation, sanitation, moisture and microclimate regulation (Tu, 1989). S*clerotinia sclerotiorum, Sclerotium rolfsii*, and *Sclerotinia minor*, represents three of the most destructive pathogens of many economically important crops (Purdy, 1979; Punja, 1985; Abawi & Grogan, 1979). These pathogens produce resting structures known as sclerotia, which have a strong resistance both to chemical and biological degradation (Punja, 1985) permitting the survival in the absence of the host (Abawi & Grogan, 1979). Sclerotia are composed of an outer rind layer of melanized cells that are thought to be responsible for resistance to microbial degradation in soil (Jones, 1970). The incidence of diseases incited by sclerotia-producing pathogens is frequently proportional to the inoculum density of these structures in soil (Benhamou & Chet, 1996; Tu, 1980). A number of fungal antagonists, including "*Trichoderma* spp." are able to parasitize the sclerotia of *Sclerotinia* spp., either in laboratory assays or in soil (Turner & Tribe, 1976; Phillips, 1989; Whipps & Budge, 1990; Jones *et al.*, 2003).

The mechanisms, whereby *Trichoderma* spp. control diseases caused by sclerotial fungi, may involve interference with sclerotial germination that may or may not be accompanied by sclerotial degradation, growth inhibition of the pathogen in soil and prevention of host penetration by the pathogen. Some species of *Trichoderma* can penetrate the rind and colonize the inner cell layers of sclerotia, often completely destroying them or rendering them unviable (Sarraco *et al*., 2006).

Development of the formulations and the delivery systems for biocontrol antagonistic microorganisms are of a great importance in the field of biocontrol. One of the important technologies for the formulation of biocontrol organisms is the immobilization of wet or dry biomass within cross linked polymers such as alginate or carrageean pellets (Walker & Connick, 1983; Papavizas *et al.*, 1987 and Cho & Lee, 1999). Alginate pellets were used in formulations of chemical and microbial herbicides (Walker & Connick, 1983). Alginate pellets containing spores of various biocontrol fungi have been used to control several diseases. In biotechnology industry, cell entrapment is often used to enhance production rates of bioproducts to reduce mortality of cells, and to facilitate their recovery (Gousen, 1987; Lewis & Papavizas, 1985 and Serp *et al.*, 2000). Such preparations offer many advantages comparing with conidial suspensions.

The most critical impediment to biological control, in the field, was the lack of knowledge for mass production and proper delivery system of biocontrol agents (Papavizas, 1985). Regardless of the organism used, an important criterion for a successful implementation of bioagent is the preparation of microbial biomass of high population counts with a high level of viability and vigor. Formulation of biological control agents depends upon biomass production and maintaining viability at the end of the process

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(Adekunle *et al.*, 2001). For mass production of two of the most commonly used fungal antagonists viz. *Trichoderma* and *Gliocladium*, solid media have been frequently used.

The present study was conducted to seek an environmentally, economically and a suitable natural substrate for the biomass production of *Trichoderma*, to study the effect of formulations on *Sclerotinia* rot disease of kidney bean *(Phaseolus vulgaris* L.) under laboratory and field conditions.

MATERIALS AND METHODS

Screening of antagonistic potential against *Sclerotinia sclerotiorum*

Fifty *Trichoderma* isolates were tested in a laboratory experiment against phytopathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary. Plugs of the biocontrol agent and the pathogen were paired in a dual culture. Each plug (0.5 cm diameter) was placed 2 cm away of Petri dish edge and 4 cm a part of the other plug. Petridish containing either fungus alone was served as control. Plates were incubated at 25°C in dark. They were watched daily for radial growth. Inhibition potential was calculated using the following equation:

$$\% \text{ of inhibition} = A - a / A \times 100$$

Where: A= diameter of the pathogen colony grown alone, and a = diameter of the pathogen colony in dual culture.

Growth and sporulation on different agricultural and industrial wastes

Five natural agrowastes were tested for their capability to support the growth and sporulation of *Trichoderma*, viz. (pagass, rice straw, sawdust, peanut shell and whey). On the other hand, whey was mixed with each one of the aforementioned materials. All substrates, were sterilized and inoculated with 1ml of *T. viride* spore suspensions (6×10^6 spores ml^{-1}), after 7 days of inoculation 2 g of each formula were suspended in 20 ml distilled water contain drops of tween 80 and filtered through mesh cloth. Strength of the resulted spore suspension was measured by turbiditimeter and by haemocytometer. This was expressed as National Turbidity Units (NTU).

Effect of formulations on shelf life of *Trichoderma* spores.

Different preparations were stored at two temperatures (25°C and 5°C). They were screened at 10 days interval for the viability of the spores. One gram of dried preparation was serially diluted and plated onto malt extract agar (MEA) medium. Plates were incubated at 25°C; number of developed colony was counted and taken as a measure of viability. This was expressed as number of Colony Forming Units (CFU). Three replicates were carried out for each formulation.

Effect of formulation on antagonistic potential of *Trichoderma* against *Sclerotinia*

This experiment was carried out according to a design that could be called "Sandwich technique" (Fig.1). Briefly a layer of sterilized or non sterilized soil was spread out onto the bottom of sterilized Petri dishes. Above this layer of soil sclerotia of *S. sclerotiorum* were scattered and covered with a layer of any one of the aforementioned substrates colonized by *T. viride*. Finally, the *T. viride* layer was topped with another layer of sterilized soil, the dish was then sealed with parafilm and incubated at ambient temperature. Percentage of external and internal colonization of sclerotia was calculated after 15 days. This experiment was repeated in presence of the fungicide "benomyl" as a soil contaminant.

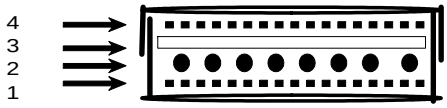

Figure (1): Illustration of sandwich technique; **1**: Layer of soil, **2**: layer of sclerotia, **3**: layer of carrier colonized by *T. viride*, and **4**: layer of soil.

Field exploration of different *Trichoderma* formula on disease incidence

The *T. viride* was grown in sterilized polyethylene bags containing any of the previously mentioned substrates mixed with whey at % 0.5- 1 ratio. The bags were incubated at dark for about 10 days at 25°C, some bags were subjected to spore harvesting to be used as coat for seeds of kidney bean plant, while the contents of the other bags were used as a layer directly in the field. This layer of substrate colonized by *T. viride* was covered with another layer of soil. Kidney bean seeds were then planted on the soil layer and covered with another layer of soil (layering technique). Field experiment was a separate plot design it comprised of four separate plots, each plot composed of six rows (Fig. 2). Each row represents a different treatment. After 65 days of *Trichoderma* applications different parameters were measured, such as shoot length, root length, fresh

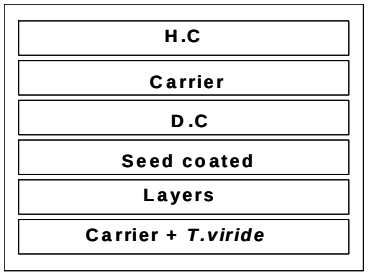

Figure (2): Illustration of the field experiment. **H.C**: healthy control, **Carrier**: different agrowastes, and **D.C**: diseased control.

and dry weight, No. of leaves, legumes count and weight as well as percentage of disease incidents.

RESULTS

Screening of antagonistic potential against *Sclerotinia sclerotiorum*

According to the results of the *in vitro* screening of the fifty isolates of *Trichoderma* against *Sclerotinia sclerotiorum* three patterns of inhibition were recorded. Group (A) comprises *Trichoderma* isolates that were able to parasitize and inhibit sclerotia formation. This group contains 6 isolates, of which *T. viride 6* showed the highest percentage of inhibition (75%) (Fig. 3). Group (B) contains isolates that had the ability to inhibit only sclerotia formation (Fig. 4). This group accommodated *T. viride* 1, *T. harzianum* 5, *T. pseudokoningii* 4 and *T. atroviride* 24. The third group (C) includes isolates that suppressed radial growth without affecting sclerotia and contain 39 isolates. *T. auroviride* 18 showed the highest percentage of inhibition in this group (45%), among this group were the isolate *T. harzianum* 34, and *T. viride* 15 (Fig. 5).

Growth and sporulation on different agricultural and industrial wastes

Sawdust amended with whey supported the highest production of spores (273.5 NTU) followed by rice straw amended with whey (252.2 NTU). The lowest crop of spores was recorded with sawdust alone (51.6 NTU).It was noticed that addition of whey to any one of the used substrates, greatly increased spores production (Fig. 6). On the other hand, whey alone did not enhance sporulation of *Trichoderma*. Data of Table (1) showed the different spores count produced by *T. viride 6* when grown in various substrates alone or in combination with whey. *Trichoderma* grown on sawdust plus whey produced the high amount of spores (5×10^8 CFU ml^{-1}) followed by mixture of rice straw and whey (8×10^7 CFU ml^{-1}).

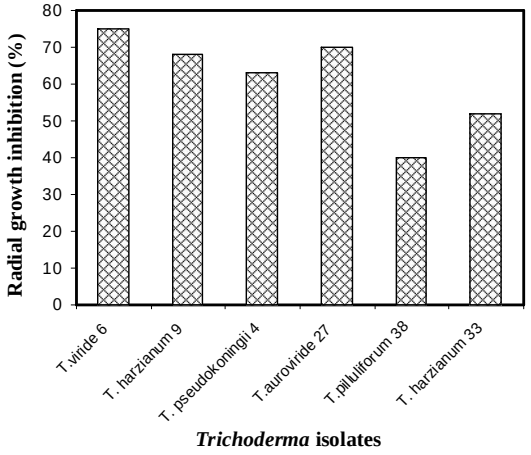

Figure (3): Effect of *Trichoderma* "group A" on radial growth of *Sclerotinia sclerotiorum*.

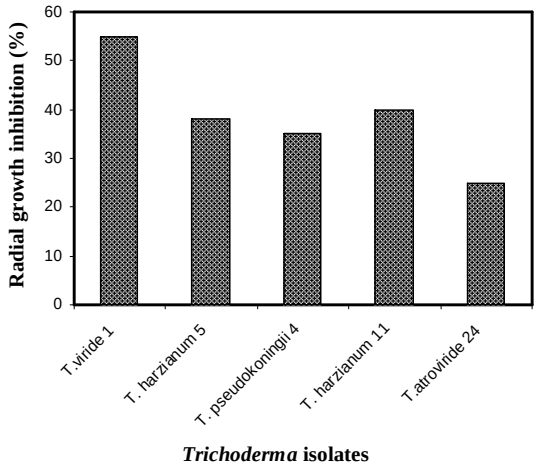

Figure (4): Effect of *Trichoderma* "group B" on radial growth of *Sclerotinia sclerotiorum*.

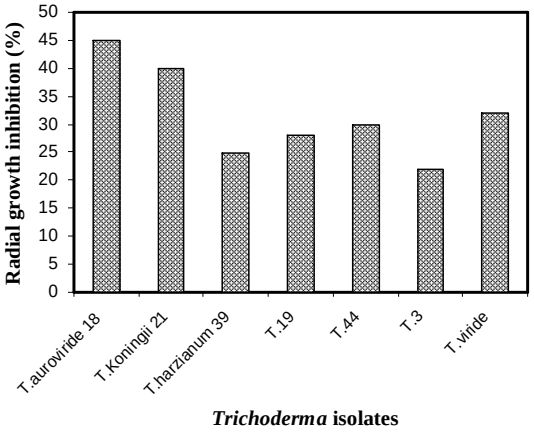

Figure (5): Effect of *Trichoderma* "group C" on radial growth of *Sclerotinia sclerotiorum*.

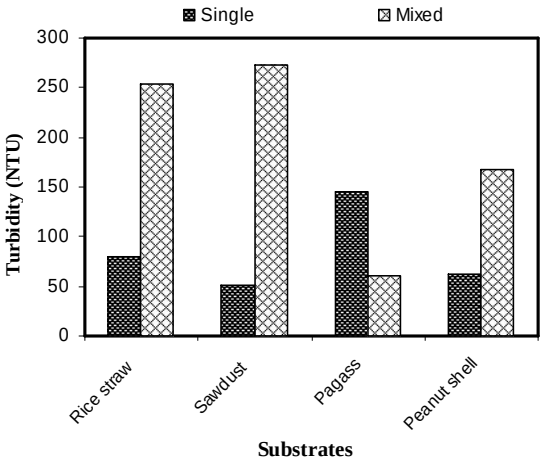

Figure (6): Sporulation of *T.viride* on different substrates.

Table (1): Amount of spores produced by *T. viride* grown on different substrates alone (single) or in combination with whey (mixed).

Substrates	spores count ml^{-1}	
	single	mixed
rice straw	7×10^6	8×10^7
Sawdust	6.5×10^6	5×10^8
Pagass	5×10^6	3.5×10^6
peanut shell	4×10^6	5.3×10^6

Effect of formulations on shelf life of *Trichoderma* spores

Viability of spores was decreasing gradually with increasing storage period. Temperature of storage and substrate on which spores were originally produced had a great effect on viability of spores. Spores produced on sawdust amended with whey showed the highest number of survive spores after 60 days of storage at 5°C (8×10^5 C.F.U). The lowest survival was noticed with spores produced on peanut shell under the same storage condition (Table 2).

Effect of formulation on antagonistic potential of *Trichoderma* against *Sclerotinia*

Percentage of visible colonization of sclerotia was taken as a measure of antagonistic potential in sterilized and non sterilized soils (Fig. 7). It was noticed that percentage of colonization in sterilized soil was more than that of non sterilized soil. *Trichoderma viride* grown on rice straw was more potent than all other formulated *Trichoderma*. On the other hand peanut shell gave great support to *T. viride*. The parasitic potential of *T. viride* grown on peanut shell was steady in both sterilized and non sterilized soil. It was clearly evident that external parasitism was greater than the internal parasitism either in case of sterilized or non sterilized soils (Fig. 8) and (Fig. 9). Pollution of agricultural soil with fungicides greatly reduces the biocontrol potential of *T. viride* to about 50% of its original capability (Fig. 10). It was also noticed that number of internally parasitized sclerotia was reduced to zero in some cases (Fig. 11).

Field exploration of different *Trichoderma* formula on disease incidence

It was clearly evident that *T.viride* had a great potential to reduce disease incidence (DI) to at least 50%. *T. viride* grown on rice straw or peanut shell was more effective than *T. viride* grown on Pagass and sawdust (Fig. 12). On comparison of methods of *Trichoderma* formula delivery, layering technique was highly efficient than seed coating (Fig. 13). Pagass alone support the growth of kidney bean (*Phaseolus vulgaris*). The two other substrates increased the growth of plant but to less extent while peanut shell reduces the plant growth (Fig. 14).

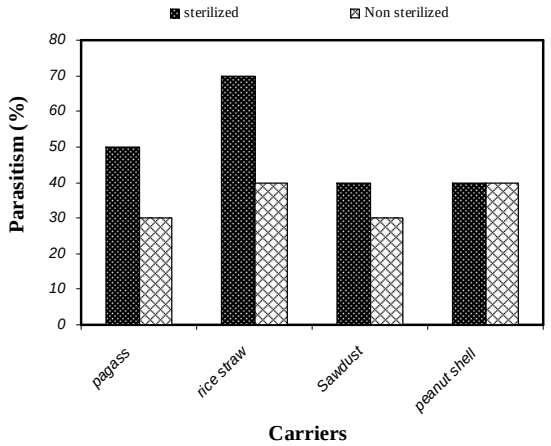

Figure (7): Percentage of sclerotia parasitized by *T. viride* in soil.

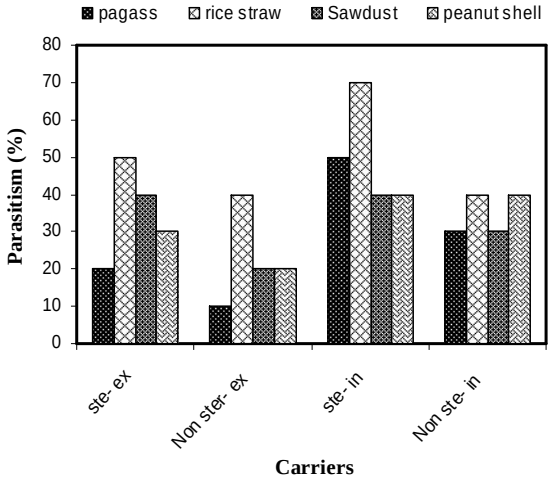

Figure (8): Effect of different carriers on parasitic potentiality of *T.viride* against sclerotia.

Table (2): Survival of *Trichoderma* spores produced on different substrates alone or in combination with whey and stored at two temperatures for 60 days.

Substrates	Colony forming units (C.F.U) of *Trichoderma* g^{-1}											
	10		20		30		4		50		60	
	25°C	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C	5°C
pagass	7×10^6	7×10^6	5×10^6	5.7×10^6	4×10^5	4.8×10^6	1×10^5	5×10^5	4×10^4	2×10^5	5×10^4	8×10^4
rice straw	8.5×10^6	8.5×10^6	6×10^6	7×10^6	4×10^6	4.2×10^6	6×10^5	3×10^6	3.5×10^5	7×10^5	2×10^5	4×10^5
sawdust	6×10^6	6×10^6	3.4×10^6	7×10^5	5×10^5	2×10^5	2×10^5	1.5×10^5	6×10^4	8×10^4	3×10^4	8×10^5
peanut shell	8×10^6	8×10^6	4×10^6	5.6×10^6	4×10^5	3×10^6	3×10^5	5×10^5	7×10^4	3×10^5	4×10^4	2×10^4

DISCUSSION

One of the major constrains of biological control is its cost. Using of valueless materials for the production of biocontrol agents could minimize the finance of production. Sawdust, rice straw, peanut shell, pagass and whey are agrowastes and industrial wastes. In addition to be of no value, they represent a great environmental problem through polluting the water, soil and air. Recycling of these substances for production of

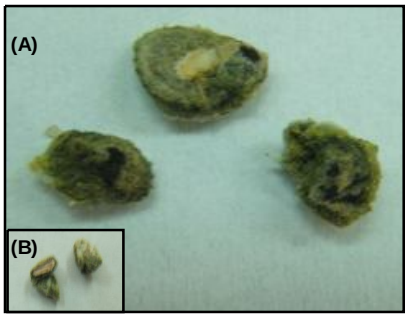

Figure (9): External parasitism (A) and internal parasitism (B) on sclerotia.

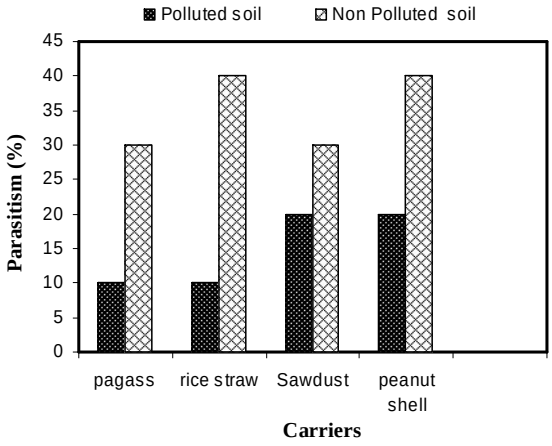

Figure (10): Percentage of sclerotia parasitized by *T. viride* in benomyl polluted soil.

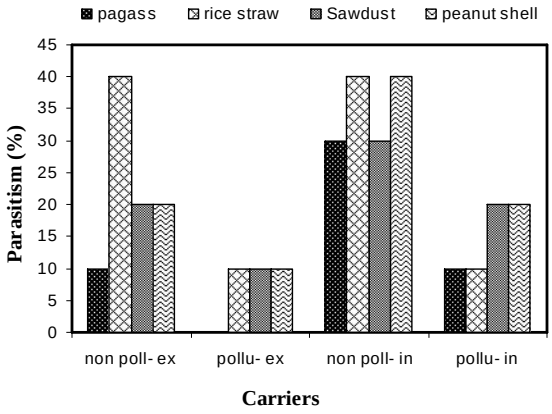

Figure (11): Effect of carriers on parasitic potentiality of *T. viride* against sclerotia.

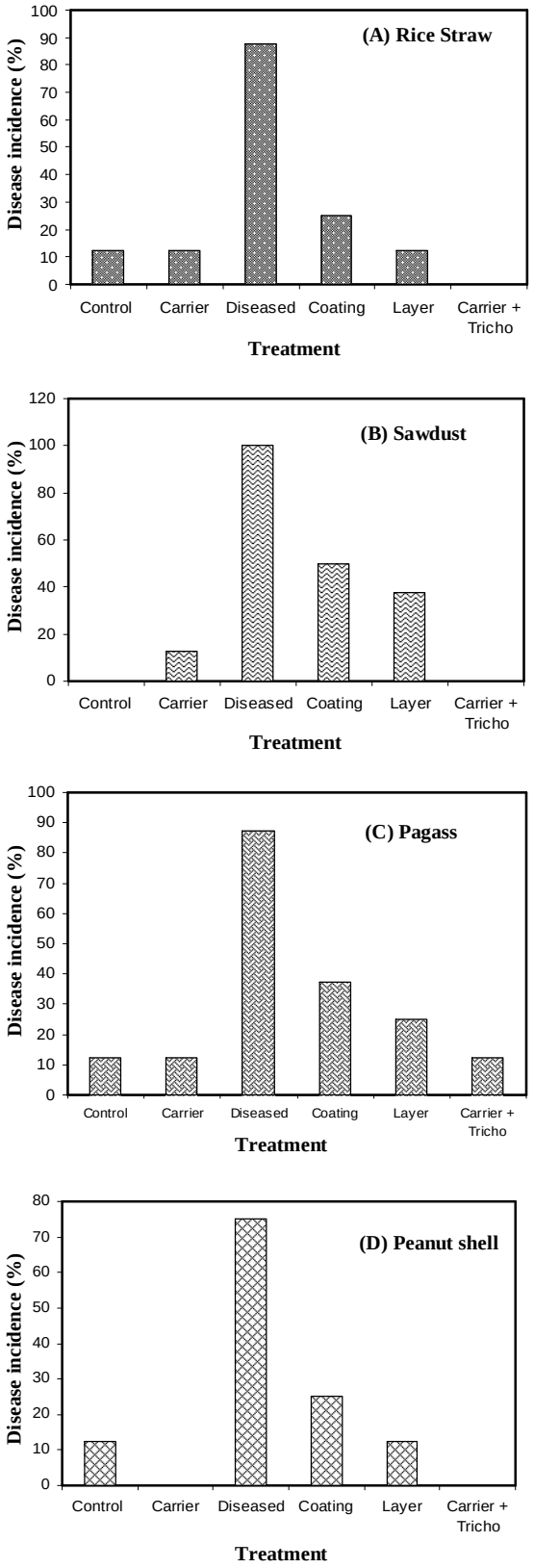

Figure (12): Effect of *T.viride* on disease incidence of white mold rot caused by *Sclerotinia sclerotiorum* by different carriers and two delivery methods.

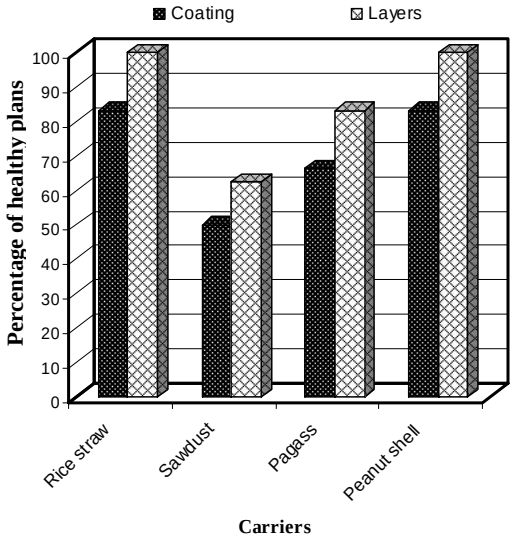

Figure (13): Comparison between the two delivery methods of *T. viride.*

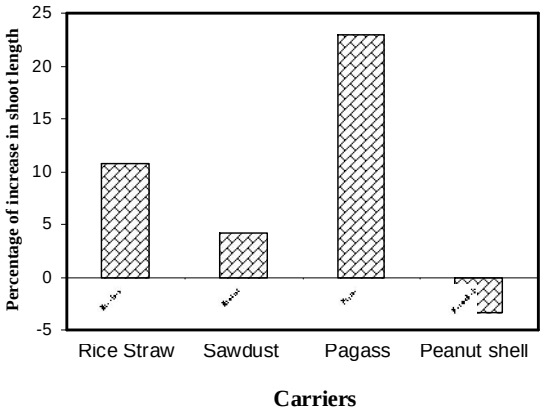

Figure (14): Effect of carrier alone on growth of *Phaseolus vulgaris.*

another valuable product will solve the environmental problem, add a value to these wastes as well as it will diminish the cost of biocontrol agent (BCA) production, leading to low price commodity.

Different formulations of *Trichoderma* were proposed aiming at increasing activity, longevity and immobilizing of BCA (Knudsen *et al*., 1990; Papavizas, 1992; Lewis *et al*., 1998; Cho and Lee, 1999; Lewis and Lumsden, 2001; Batta, 2007). Complex carbon sources such as rice straw and sawdust amended with whey provide a food base that aids the "BCA" to establish a new community at the site of application. Verma *et al.* (2007) stated that complex carbon source supplemented with nitrogen source increases conidia concentration in the media. *Trichoderma viride* grew well on all of the used substrates with various percentage of CFU, but it failed to sporulate on whey.

Many agrowastes were used for growing *Trichoderma* species. Illuyemi *et al*. (2006) used palm kernel cake, while Singh *et al*. (2007) used several other agrowastes including rice husk, and sawdust .They got out with good growth, but population density varied considerably with different substrates and this supports our results of the present study. Where number of CFU, were varied greatly with used agrowastes (Rice straw 252.2 N.T.U, sawdust 272.6 N.T.U, peanut shell 167 N.T.U and pagass 60.2 N.T.U). The variation in percentage of CFU could be attributed to the variation in chemical composition of the used materials. Gutierrez-Correa *et al*. (1999) and Singh *et al.* (2007) demonstrated that population density of *Trichoderma* is greatly affected by nutritional status of the substrates they grown on.

When whey was added to other substrates the growth and conidiation increased tremendously. This could be attributed to the composition of these different substrates. Sawdust, rice straw, peanut shell and pagass are very rich in carbon source but very poor (if any) in amino acid content. Whey, on the other hand, has a great amount of amino acids (Abd Elsalam, 2001). Combination of whey and any one of the aforementioned substances represents a very rich medium that supports the growth of *T. viride.* Addition of whey alters the C: N ratio. Variation in C: N ratio has a strong influence on conidiation (Papagianni, 2004) and activity of (Celar, 2003 and Olsson *et al.*, 2003). Addition of nitrogen to complex carbon source increases the growth and conidiation of (Verma *et al.*, 2007). This is in coincidence with the data of this investigation. It was noticed from the present data that high activity of *T. viride* is correlated with high production of CFU. This finding is in agreement with results of some other investigators (Harmen & Bjorkman, 1998; Ganassi *et al*., 2000 and Verma *et al.*, 2007).

Viability of conidia produced on different agrowastes varied with substrates used. It almost remains constant for about 30 days at 5°C, but declined slowly at 25°C. Decreasing of fungal preparation viability with time was reported by some other scientists (e.g. Kücük and Kivanc, 2005).

Trichoderma viride showed different degree of parasitism on sclerotia of phytopathogenic fungus *Sclerotinia sclerotiorum.* The percentage of internal parasitism was lower than that of external parasitism. *T. viride* grew on sclerotia and under the rind, but it was not certain if it grew deeper into cortex and medullary regions. However the destruction of sclerotia suggested that the mycelium of *T. viride* seemed to grow through these regions. Sarracco *et al.* (2006) proved that *T. harzianum* grew well under rind and through cortex and medullary regions. Biocontrol activity of *T. viride* was affected significantly by the fungicide "Benomyl" either in sterilized or non sterilized soil (70%, 40% respectively) comparing to non polluted soil (40% and 10%, respectively). Khattabi, *et al.* (2001) showed that

T. harzianum responded differently to the effect of three different fungicides. While the fungicide "Hymexaxol" enhanced the antagonistic activity of *T. harzianum*, "Benomyl" strongly inhibited its activity.

Application of *T. viride* to soil reduced dramatically the incidence of white rot disease caused by *Sclerotinia sclerotiorum*. The variation of reduction rate showed by different formulation could be due to the effect of substrate on the activity and count of *Trichoderma* conidia. Singh *et al*. (2007) illustrated that initial count of *Trichoderma* conidia was essential for effectiveness of used formulation for controlling soil borne plant pathogens. Fluctuation of reduction rate of disease incidence in the field due to treatment with different *Trichoderma* formulations was documented by many other workers (Harman *et al*., 2004; Singh and Singh, 2004 and Singh *et al*., 2007).

Another serious problem that facing the field application of BCA is the method of delivery of BCA. The most implemented ways of application for soil borne pathogens are the soil treatment and seed coating. In the present study, two techniques were tested. The first was layering technique where a layer of *Trichoderma*- colonized substrates was laid down into the soil. The second technique was seed coating, where seeds of kidney bean were coated with conidia of *T. viride* produced on different substrates (agrowastes). During this study layering technique gave a higher percentage of disease reduction comparing to seed coating (100% and 83%, respectively). These results are in contradiction with the findings of Strashnov *et al*. (1985) who found that seed coating was more effective than soil treatment. Adekunle *et al*. (2006) with seed coating using *T. harzianum* comparable to the fungicide Benlate. The high reduction obtained by layering during this study could be attributed to the continuous supplement of soil with *T. viride* through the colonized substrates, which help *T. viride* to establish a great community and aid it to overcome the inimical behavior of the resident microorganisms.

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Received August 20, 2007
Accepted December 10, 200

تقييم أثر الصيغ التركيبية المختلفة على نشاط المقاومة الحيوية للتريكوديرما

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الملخص العربـــى

استخدمت خمسة أنواع من المخلفات الزراعية والصناعية لقياس قدرتها على تدعيم نمو وإنتاج الجراثيم لفطر *Trichoderma viride* . تم إختيارهذا الفطر معمليا من بين 50 عزلة لقدرته العالية على التطفل وكذا الحد من تكوين الاجسام الحجرية لفطر *Sclerotinia sclerotiorum* . تمثلت هذه المخلفات الزراعية والصناعية فى قش الارز - نشارة الخشب- قشر الفول السودانى - بقايا القصب و شرش اللبن. تم زراعة الفطر على هذه المصادر كلا على حده أو مخلوطا مع شرش اللبن.

وقد سجلت أعلى نسبة نمو فى حالة إنمائه على قش الارز ويليه نشارة الخشب. على الجانب الاخر عند تنمية الفطر على شرش اللبن لم ينتج جراثيم ولكنه أعطى زيادة ملحوظة فى النمو عند خلطه بأى من المخلفات السابقة. كما تم قياس مدى حيوية الجراثيم المنتجة على هذه المخلفات تحت ظروف التخزين على مدى 60 يوما فى درجات حرارة مختلفة. بالإضافة إلى قياس القدرة التطفلية للفطريات المنتجة على هذه المخلفات بإستخدام الأجسام الحجرية فى تربة معقمة وأخرى غير معقمة وتم حساب نسبة التطفل الخارجى والداخلى لهذه الأجسام. كان للمبيد الفطرى Benomyl تاثيراً فى الحد من نسبة التطفل. ولإستكشاف قدرة الفطر المنتج على هذه المخلفات فى المقاومة الحيوية لمرض العفن الأبيض فى نبات الفاصوليا تم تصميم تجربة حقلية ووجد أن الفطر النامى على قش الارز وقشر الفول السودانى كان أكثر تأثيرا فى الحد من المرض. وعند إختبار تأثير المخلفات فقط على نبات الفاصوليا أظهرت بقايا القصب زيادة فى طول المجموع الخضرى لنبات الفاصوليا.

من النتائج السابقه يتضح لنا أهميه إستخدام مواد ناتجه من مخلفات زراعيه مثل قش الارز وبقايا القصب كأحد الصيغ التى يمكن للفطريات النمو عليها ويعتبر ذلك حل صديق للبيئه ورخيص الثمن للمزارع حتى يتمكن من القضاء على أمراض تعفن الجذور.