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ORIGINAL ARTICLES

Long Activity of Stored Formulated Bio-Agents Against Some Soil-Borne Plant Pathogenic Fungi Causing Root Rot of Some Vegetables

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ABSTRACT

Viability and antagonistic ability of stored formulated bio-agents were tested periodically throughout ten months of storage. Long term activity of some antagonistic fungal and bacterial agents against vegetables root rot incidence was evaluated when applied as seed treatment in pot experiment under open greenhouse conditions. The carriers of (Sawdust) and (Sawdust + CMC) were the most suitable tested carries for keeping the viability of *B. subtilis, P. fluorescens* and *T. harzianum* with no significant reduction all over the storage period up to ten months. Meanwhile these antagonist started to lose their viability significantly when formulated on (Sawdust + Talc powder + Chitosan) carrier after the third month of storage followed by the carrier (Sawdust + Chitosan) and (Sawdust + Talc powder) after the fifth month and finally the carrier (Sawdust + CMC +Talc powder) after six months of storage. The inhibitory effect of stored antagonistic fungi and bacteria against the linear growth of root rot pathogenic fungi was evaluated *in vitro.* No significant differences were observed in the antagonistic ability of *B. subtilis, P. fluorescens* and *T. harzianum* either stored on (Sawdust) and (Sawdust + CMC) or fresh cultures against tested pathogens. Also, the antagonistic ability of *B. subtilis,* the highest tested antagonist, against tested pathogenic fungi was reduced with the range of 0.7-7.8% and 0.1-6.6% when formulated on (Sawdust), (sawdust + CMC) carriers and stored for ten months comparing with losing antagonistic ability by 6.2-21.3%, 4.9-31.5%, 2.0-28.1% and 15.5-36.2% when formulated on (Sawdust + Chitosan), (Sawdust + Talc powder), (Sawdust + CMC +Talc powder) and (Sawdust + Talc powder + Chitosan) carriers and stored for the same period. Similar trend was also observed with the other formulated antagonists *P. fluorescens* and *T. harzianum.* The obtained results could lead to conclude that the antagonistic ability not depended on the counts or population of the antagonist but mainly on its active viability. Under greenhouse conditions, all the tested fresh and ten months stored bio-agents showed interesting highly significant effect causing high reduction of root rot incidence at both pre-, and post-emergence stages of plant growth comparing with the check treatment. Promising applicable technique could be suggested on the light of the results obtained in the present study. The usage of stored formulated bio-agents might be considered as safe, cheep and easily applied biocontrol method against such soilborne plant pathogens.

***Key words*:** Biological control, long term antagonism, Root rot pathogens, Cucumber, Cantaloupe, Tomato, pepper.

Introduction

Fungal disease control is achieved through the use of fungicides which is hazardous and toxic to both people and domestic animals. This leads to environmental pollution. Therefore, a more balanced, cost effective and eco-friendly approach must be implemented and adopted by farmers. Biological control is an innovative, cost effective and eco-friendly approach. Use of natural enemies to control disease is termed biological control. Biological control is an alternative to the use of chemical pesticides. Biological fungicides may act to suppress the population of the pathogenic organisms through competition with pathogenic organisms. Stimulated plant growth, which may allow plants to quickly outgrow any pathogen effects, or damage the pathogen by means of toxins produced (Cook, 2000). Bio-control agents are derived from natural materials such as animals, plants, bacteria, fungi and certain minerals. With the knowledge of the adverse effects of synthetic fungicides worldwide, attention is rapidly, being shifted to non-synthetic, safer alternatives. The application of biological control using antagonistic microorganisms proved to be successful for controlling various plant diseases in many countries (Sivan, 1987). It is still not easy and costly in application, however it can serve as the best control measure under greenhouse conditions. In recent years, several attempts have been made to overcome this obstacle by applying antagonistic microorganisms. *Trichoderma* spp. is known for its mycoparasitic and antagonistic mechanism for the control of fungal disease. *Trichoderma harzianum* is a fungal biocontrol agent that attacks a range of pathogenic fungi and can be used in the biological control of several plant diseases

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(Papavizas, 1985; Chet, 1987; Samuels, 1996). *Trichoderma* spp. are well documented as effective biological control agents of plant diseases caused by soilborne fungi (Sivan and Chet, 1986; Whipps and Lumsden, 2001; McLean *et al*., 2004). Hadar *et al*. (1979 and 1984) and Elad *et al*. (1980) observed that the application of wheat bran colonized by *T. harzianum* to soil infested with *R. solani* and *Sclerotium rolfsii* reduced the incidence of root diseases caused by these pathogens in beans. As for antagonistic bacteria, Kim *et al.,* (1997) found that seed treatment with *Bacillus* spp. was actively controlled three fungal root diseases of wheat. Also, *Pseudomonas cepacia* or *Pseudomonas fluorescens* applied to pea seeds act as biological control agent against Pythium damping-off and Aphanomyces root rot and was able to reduce diseases incidence (Parke *et al*.,1991 and King and Parke, 1993). Sunick *et al.,* (1997) recorded that, *Bacillus* sp. gave a highly antagonistic effect against some pathogenic fungi including *Fusarium solani*. Moreover, Abdel-Kader (1997) reported that *Trichoderma harzianum* introduced to the soil was significantly able to reduce root rot incidence of bean plants more than the fungicide Rizolex-T.

The objective of the present study was to evaluate the long term viability and activity of some stored antagonistic fungal and bacterial agents against some root rot pathogens either in vitro or against disease incidence under open greenhouse conditions.

Materials and Methods

Laboratory Experiments:

Different carrier materials for bio-agents propagules formulation, *i.e*. (1) Sawdust; (2) Sawdust + CMC; (3) Sawdust + Chitosan; (4) Sawdust + Talc powder; (5) Sawdust + CMC + Talc powder and (6) Sawdust + Talc powder + Chitosan were used in this test. The long-term antagonistic activity of stored formulated fungal and bacterial antagonists was evaluated using the dual culture technique (Ferreira *et al*. 1991). Bio-agents that formulated in different carrier materials were stored up to 10 months. Evaluation of both sufficient potential as antagonistic ability and determining viability as colony forming unit (cfu) of the stored formulated bio-agents were recorded monthly.

Preparation Of Fungal Spores And Bacterial Cells Suspensions:

One of antagonistic fungal inoculum (*Trichoderma harzianum*) was grown on PDA medium at 28±1^{o}C until an abundant heavy growth of conidia was evident. Conidia were harvested by scraping the surface of the colonies with a spatula, transferred to sterilized distilled water and filtered through nylon mesh. All spore solutions were adjusted with sterile water to give a spore concentration of 10^4-10^5 spores per milliliter. Meanwhile, antagonistic bacteria (*Bacillus subtilis*, *Pseudomonas fluorescens*) were grown on Nutrient broth medium and incubated in a rotary shaker at 200 rpm for 24 h at 28±1^{o}C. The bacterial cells were harvested by centrifugation at 6,000 rpm for 10 min, washed twice with 0.05 M phosphate buffer at pH 7.0, and re-suspended in distilled water. The concentrations of bacterial cells in the suspensions were adjusted to 10^5-10^6 cells per milliliter. Concentrations of both bacterial cells and fungal spores suspensions were adjusted with the aid of a haemacytometer slide. A few drops of the emulsifier Tween 20 (Sigma Co.) were added to the prepared bio-agent to obtain a distributed separated spores/cells suspensions.

Formulation Of Fungal And Bacterial Bio-Agents:

Different carrier materials for bio-agents propagules formulation were used as follows:

(1) Sawdust.
(2) Sawdust + CMC (1g:0.1g, w:w).
(3) Sawdust + Chitosan (1g:0.5 mL, w:v)
(4) Sawdust + Talc powder
(5) Sawdust + CMC + Talc powder (1g:0.1g:0.5g, w:w:w).
(6) Sawdust + Talc powder + Chitosan (1g: 0.5g: 0.5 mL, w:v).

Sawdust and Talc powder were autoclaved (120 ^{o}C/1.5lb for 60 min.) before use then mixed to obtain the proposed stated above different formula. Fungal and bacterial spores/cells suspensions were added individually to sterilized different prepared different formula at the rate of 2:1 (carrier: suspension, W:V), then mixed thoroughly to even ensure equal distribution of microorganism suspension through the mixed carriers. The prepared mixture then placed onto paper sheet and left for air dry 2-3 hrs at room temp. (22-25 ^{o}C). Then after the mixtures were passed through sieve (0.25 mm-diameter) to get the formulated bio-agent in granules shape. The obtained granules were packed into dark bottles (250 mL) sealed and stored in incubators at three different temperatures 10, 15 and 20 ±2^{o}C until use.

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In Vitro Tests:

Evaluation of sufficient potential as antagonistic ability as well as determining viability of stored formulated bio-agents were recorded monthly.

For determining the antagonistic activity, 25g of each formulated bio-agent were added to 75 mL of sterilized water agar medium (20g agar/1000 mL distilled water) before solidifying and rotated gently to obtain equal distribution of the added material, then poured into 9-cm-diameter Petri dishes and left for 2 hrs to reach the solid state. Disks (5 mm-diameter) of solid formulated bio-agent were obtained using cork borer. The prepared disks of each formulated bio-agent were used for testing the antagonistic ability of stored bio-agents against different soil-borne pathogenic fungi followed the method described by Ferreira *et al*. (1991). Disks obtained from fresh bio-agents cultures were used as control check treatment.

As for determining viability of stored formulated bio-agents, the method developed by Louw and Weblely (1959) for studying the microflora of the root region was used. The plate count technique according to Allen (1961) was followed for both total fungal and bacterial counts. One gram of each stored formulated bio-agent was added to reagent bottle 99 mL of sterilized distilled water, shacked for 15 min. using bench shaker (150 rpm/min.). Serial dilutions (10^{-2} to 10^{-5}), of each stored formulated bio-agent were made up. A volume of 1.0 ml of 10^{-2} and 10^{-3} dilution of stored formulated fungi was poured into Perti dishes with 20 ml of Martin's medium. Meanwhile, a volume of 1.0 ml of 10^{-4} and 10^{-5} dilution of stored formulated bacteria was poured into Perti dishes with 20 ml of nutrient agar medium. Petri dishes were then swirled gently to ensure even distribution of fungi and bacteria in the media. Five Petri dishes were used as replicates for each particular treatment. All plates were incubated at 28 ± 1°C for 5 days for fungi and 48 hr for bacteria, then examined. Formed fungal and bacterial colonies were counted and the number of colony forming units (cfu) per 1.0g of stored formulated bio-agent was calculated.

Greenhouse Experiment:

Pot experiment was carried out in the open greenhouse of Plant Pathology Dept., National Research Centre, Egypt for evaluating bio-control activity of stored bio-agents comparing with fresh ones against root rot disease incidence. Cucumber and pepper plants were chosen in this experiment as a model of widespread vegetable crops mainly grown under protected cultivation system in Egypt and showed susceptibility to attack with root rot pathogens (El-Mougy *et al*., 2011). The selected aggressive isolates of *Fusarium solani, Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum* and *Pythium* sp. which isolated from vegetables throughout Egypt (El-Mougy *et al*., 2011) were used. Loamy soil was artificially infested individually (at the rate of 5% w:w) with a mixture inoculum of pathogenic fungi which was previously grown individually for two weeks on sand barley medium (1:1, w:w and 40% water) at 25 ± 2ºC, then mixed thoroughly with the soil, then filled in plastic pots (20 cm in diameter). Another set of varied infested soils only with pathogens mixture was filled in plastic pots (20 cm in diameter), and used for comparison check treatment. Seeds of either Cucumber or Pepper were treated with inocula of stored bio-agents, fungi (*Trichoderma harzianum*) or bacteria (*Bacillus subtilis, Pseudomonas fluorescens*) at the rate of 3g/Kg of stored formulated bio-agents, meanwhile fresh bio-agents were applied as seed immersing for one hour in sticky (1g Arabic gum/L) suspension inocula ($3x10^6$ cfu/mL), then air dried before sowing. Five seeds of each of Cucumber, and Pepper were planted in each pot and six replicated pots were used for each particular treatment. Percentage of root rot incidence at pre-emergence growth stage was recorded after two weeks from sowing date. Meanwhile, percentage of root rot incidence at post-emergence stage was calculated 45 days after emergence. This experiment was repeated twice in order to confirm the obtained results.

Statistical Analysis:

All experiments were set up in a complete randomized design. One-way ANOVA was used to analyze differences between antagonistic inhibitor effect and linear growth of pathogenic fungi *in vitro.* A general linear model option of the analysis system SAS (SAS Institute Inc. 1996) was used to perform the ANOVA. Duncan's multiple range test at $P < 0.05$ level was used for means separation (Winer 1971).

Results And Discussion

Laboratory Experiments:

The obtained results of the determination the viability and antagonistic ability of formulated different antagonists on different carriers and stored for ten months on three different incubation temperature were found

to be the similar. Therefore the average results of the viability and antagonistic ability at the three incubation temperatures were calculated and presented in the following Tables and Figures.

Data in Table (1) and Figure (1) showed the effect of storage period on the viability of antagonistic microorganisms stored for ten months on different carrier materials. Presented data revealed that the carriers of (Sawdust) and (Sawdust + CMC) were the most suitable tested carries for keeping the viability of *B. subtilis, P. fluorescence* and *T. harzianum* with no significant reduction all over the storage period up to ten months. Meanwhile these antagonist started to lose their viability significantly when formulated on (Sawdust + Talc powder + Chitosan) carrier after the third month of storage followed by the carrier (Sawdust + Chitosan) and (Sawdust + Talc powder) after the fifth month and finally the carrier (Sawdust + CMC +Talc powder) after six months of storage.

On the other hand, the inhibitory effect of antagonistic fungi and bacteria against the linear growth of root rot pathogenic fungi was evaluated *in vitro.* The tested inhibitor factor in this study was the antagonistic agents applied as discs of either fresh growth culture or stored bio-agents formulated on different carriers as stated before.

Table 1: Effect of storage period on the viability of antagonistic microorganisms stored on different carriers.

Carriers	Antagonistic microorganism	Microorganism count unit per gram of dry carrier material										
		Storage Period (month)										
		0	1	2	3	4	5	6	7	8	9	10
Sawdust	*B. subtilis*	34.4 a*	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	33.8 a	33.6 a	33.2 a	23.8 b
	P. fluorescens	34.4 a*	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	32.8 a	32.4 a	32.1 a	23.8 b
	T. harzianum	28.4 a**	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.3 a	28.2 a	28.0 a	23.6 b
Sawdust + CMC	*B. subtilis*	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.1 a	34.0 a
	P. fluorescens	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.2 a	34.0 a
	T. harzianum	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.3 a	28.1 a
Sawdust + Chitosan	*B. subtilis*	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	28.8 b	22.7 c	17.9 d	16.4 d	14.2 de
	P. fluorescens	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	22.7 b	18.7 c	14.7 d	12.2 de	10.3 ef
	T. harzianum	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	21.4 b	18.4 c	16.4 d	15.1 d	12.4 e
Sawdust +Talc powder	*B. subtilis*	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	23.4 b	18.3 c	16.2 cd	14.2 cd	12.2 e
	P. fluorescens	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	23.4 b	17.5 c	14.4 cd	12.8 cd	10.4 ef
	T. harzianum	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	24.4 b	15.7 c	12.1 cd	11.3 cd	9.8 f
Sawdust + CMC +Talc powder	*B. subtilis*	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	31.3 cd	24.0 d	23.2 d	21.6 de
	P. fluorescens	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	34.4 a	30.4 c	23.1 d	22.3 d	20.2 de
	T. harzianum	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	28.4 a	22.2 c	17.0 d	16.2 d	13.4 e
Sawdust +Talc powder + Chitosan	*B. subtilis*	34.4 a	34.4 a	34.4 a	34.4 a	33.8 a	32.2 ab	22.2 d	18.1 c	12.0 d	10.4 e	8.8 f
	P. fluorescens	34.4 a	34.4 a	34.4 a	34.4 a	33.6 ab	31.0 b	23.1 c	17.0 d	11.8 e	10.2 e	8.4 f
	T. harzianum	28.4 a	28.4 a	28.4 a	28.4 a	26.2 ab	24.6 b	19.4 c	16.8 d	14.2 de	12.8 d	10.6 ef

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).
* Each figure represent bacterial count estimated as $1x10^6$ cfu/g dry carrier material
** Each figure represent fungal count estimated as $1x10^4$ cfu/g dry carrier material

Presented data in Table (2) and Figure (2) showed the antagonistic ability of bio-agent microorganisms stored for ten months on different carrier materials against soil-borne pathogens comparing with the same fresh cultures bio-agents.

Data revealed that no significant differences were observed in the antagonistic ability of *B. subtilis, P. fluorescens* and *T. harzianum* either stored on (Sawdust) and (Sawdust + CMC) or fresh cultures against tested pathogens. In this regards, data also showed that (Fig. 2) the antagonistic ability of *B. subtilis,* the highest tested antagonist, against tested pathogenic fungi was reduced with the range of 0.7-7.8% and 0.1-6.6% when formulated on (Sawdust), (sawdust + CMC) carriers and stored for ten months comparing with losing antagonistic ability by 6.2-21.3%, 4.9-31.5%, 2.0-28.1% and 15.5-36.2% when formulated on (Sawdust + Chitosan), (Sawdust + Talc powder), (Sawdust + CMC +Talc powder) and (Sawdust + Talc powder + Chitosan) carriers and stored for the same period. Similar trend was also observed with the other formulated antagonists *P. fluorescence* and *T. harzianum.* On the other hand, it is interested to note that these bio-agents significantly lost their antagonistic ability when formulated and stored up to ten months on (Sawdust + Chitosan), (Sawdust + Talc powder), (Sawdust + CMC +Talc powder) and (Sawdust + Talc powder + Chitosan) carriers. The obtained results could lead to conclude that the antagonistic ability not depended on the counts or population of the antagonist but mainly on its active viability.

These findings were confirmed with other reports. Various carriers and polymers have been used to increase the survival rate of the organism with mixed success. Some of the biological control agents are adversely affected by the combination with some traditional chemical seed protection. These products come in dry formulations as dusts, dry spores, and gum/talc powders. Many liquid formulations are also available for sprays, dips, fluid drilling gels and solid matrix priming. These may be designed for large-scale application or planter box treatments (Sallam *et al.*, 2008).

The market for biological control products is not only determined by agricultural aspects such as the number of diseases controlled by one biocontrol product in different crops but also by economic aspects as cost-effective mass production, easy registration and the availability of competing means of control including fungicides. Shelf life is a very important parameter to be considered in the development of a formulation, because most products will have to be stored for long periods of time before they can be marketed and later applied. More recently commercial formulations of biological controls have been developed which have consistently given good control of some plant diseases (Stewart *et al.*, 2001).

Table 2: Effect of storage period on the antagonistic ability of bio-agent microorganisms stored for ten months on different carrier materials against soil-borne pathogenic fungi.

Carriers	Stored Antagonistic microorganism (after 10 month of storage)	Growth reduction of tested soil-borne pathogenic fungi (%)								
		S. rolfsii	*R. solani*	*F. solani*	*F. oxysporum*	*S. sclerotiorum*	*S. minor*	*M. phaseolina*	*Pythium* sp.	*A. solani*
Sawdust	*B. subtilis*	72.4 b	75.6 b	80.0 a	80.0 a	55.6 d	57.6 d	62.3 c	76.4 b	76.6 b
	P. fluorescens	73.2 b	78.6 b	74.7 b	74.4 b	50.0 d	50.0 d	56.6 d	73.8 b	73.6 b
	T. harzianum	82.4 a	84.7 a	80.2 a	80.3 a	60.6 c	70.4 b	82.6 a	82.4 a	80.0 a
Sawdust + CMC	*B. subtilis*	73.4 b	76.6 b	80.0 a	80.0 a	54.6 d	56.6 d	60.3 d	76.4 b	74.6 b
	P. fluorescens	74.2 b	77.6 b	72.7 b	72.4 b	51.0 d	52.0 d	55.6 c	74.8 b	74.6 b
	T. harzianum	80.0 a	84.2 a	80.0 a	82.3 a	60.6 c	70.4 b	80.6 a	84.4 a	80.0 a
Sawdust + Chitosan	*B. subtilis*	66.6 c	65.6 c	63.3 c	70.4 b	48.8 e	45.0 d	60.4 c	66.6 c	62.6 c
	P. fluorescens	60.0 c	62.4 c	62.0 c	60.1 c	42.2 e	44.4 e	62.2 c	58.8 d	60.0 c
	T. harzianum	58.8 d	54.6 d	55.5 d	69.2 c	55.5 d	50.0 d	55.5 d	55.5 d	56.8 d
Sawdust + Talc powder	*B. subtilis*	62.2 c	60.0 c	61.4 c	67.4 c	46.4 e	44.4 e	61.2 c	54.4 d	60.2 c
	P. fluorescens	58.6 d	53.6 d	57.7 d	58.8 d	40.0 e	40.2 e	56.4 d	55.5 d	54.6 d
	T. harzianum	60.0 c	64.2 c	63.3 c	70.0 b	50.0 d	55.5 d	62.2 c	50.0 d	61.0 c
Sawdust + CMC+ Talc powder	*B. subtilis*	64.4 c	60.0 c	62.7 c	66.6 c	45.0 e	42.2 e	63.1 c	60.0 c	64.4 c
	P. fluorescens	60.0 c	63.6 c	61.4 c	48.4 e	45.0 e	40.0 e	60.0 c	57.3 d	62.0 c
	T. harzianum	72.4 b	71.6 b	70.4 b	62.2 c	52.2 d	45.0 e	71. 4b	62.2 c	70.4 b
Sawdust +Talc Powder + Chitosan	*B. subtilis*	58.8 d	44.4 e	56.6 d	55.5 d	42.2 e	38.8 f	54.4 d	50.0 d	57.8 d
	P. fluorescens	56.4 d	48.8 e	54.4 d	44.4 e	43.3 e	40.0 e	48.6 e	52.2 d	55.4 d
	T. harzianum	64.4 c	43.6 e	62.2 c	60.0 c	45.0 e	41.6 e	60.0 c	58.8 d	62.4 c
Fresh cultures	*B. subtilis*	78.6 b	76.7 b	80.8 a	80.6 a	57.6 d	58.7 d	64.4 c	78.4 b	79.6 b
	P. fluorescens	76.2 b	79.6 b	76.7 b	75.4 b	52.6 d	50.4 d	58.6 d	78.8 b	76.6 b
	T. harzianum	84.4 a	86.7 a	82.2 a	83.3 a	62.6 c	72.4 b	86.6 a	87.4 a	81.4 a

Mean values within columns for each organism followed by the same letter are not significantly different ($P \leq 0.05$).

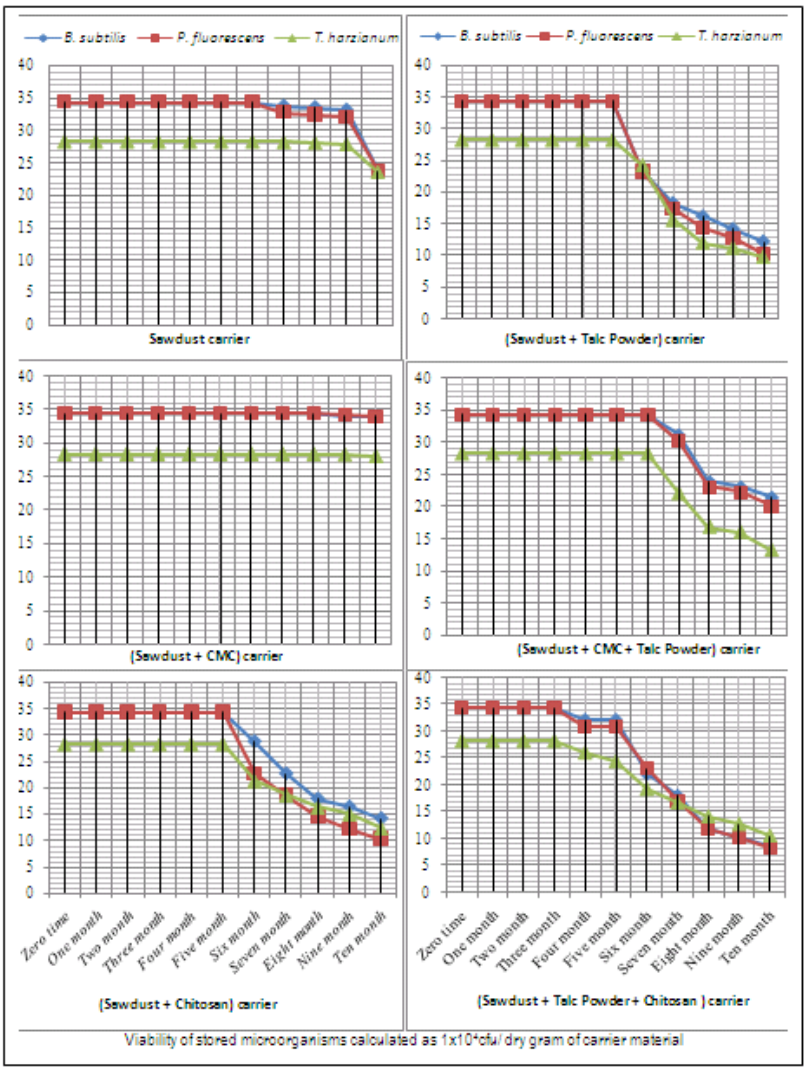

Fig. 1: Average reduction (%) in viability of formulated bio-agents microorganisms stored for ten months on different carrier materials.

Sallam *et al*., (2008) reported that the effect of storage time at room temperature on the viability of *Trichoderma* spp. in the prepared formulations showed that more than 40% viability of the colonies was recorded at room temperature storage after 4 months. The lowest viability was observed in all fungal formulates after 4 months. Similar results were obtained by Walker and Connick (1983) who declared that formulations of *Trichoderma* spp. after stored at ambient conditions for 6 to 8 months.

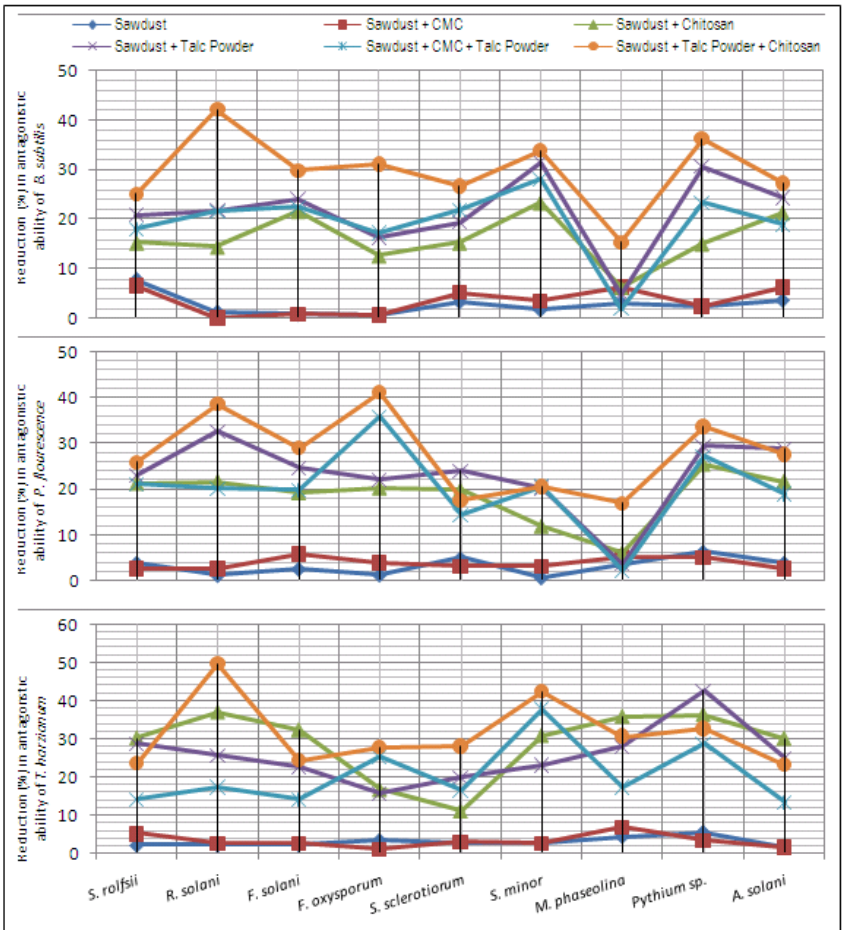

Fig. 2: Average reduction (%) in the antagonistic ability of formulated bio-agent microorganisms stored for ten months on different carrier materials against soil-borne pathogens.

However, Küçük and Kivanç (2005) mentioned that no viability was observed in different soils at 30°C after 9 weeks, whereas there was viability in all soils at 4°C even after 24 weeks. In nature wide range of organic substrates could be used for the solid-state fermentation for mass multiplication. Solid fermentation media consisting of inert carriers with food bases was used for mass production of biocontrol agents (Lewis, 1991).The media with relatively low microbial content would be suited for solid-state fermentation and for the amendment of biocontrol agents. Solid substrates include straws, wheat bran, sawdust, moistened bagasse, sorghum grains, paddy chaff, and decomposed coir pith, farmyard manure and other substrates rich in cellulose for inoculums production. In the present study formulated bio-agents on base of sawdust and sawdust + CMC was found to be the most suitable carriers tested for keeping both viability and antagonistic ability of stored both fungi and bacteria bioagents for up to ten months (300 day). Also, Vidhyasekaran and Muthamilan, 1995 recorded that *Pseudomonas fluorescens* strains showed inhibitory action against the chickpea (*Cicer arietinum*) wilt pathogen *Fusarium oxysporum* f. sp. *ciceris* under *in* vitro studies. They assessed the efficacy of various carriers in sustaining the population of these strains during storage and found that in talc-based and peat-based formulations the bacteria survived even up to 240 days of storage although the population declined from 30 days, while chickpea seeds treated with talc-based formulations, *P. fluorescens* survived on the seeds for at least 180 days. Furthermore, growth population and viability of antagonists–primed seeds during storage were reported also by several workers. Harman *et al.* (1989) found that *T. harzianum* strain T-22 increased 10-fold during matrix priming of tomato and cucumber seeds. Viability of the encapsulated *T. harzianum* remained high

for at least six months when stored at 5°C. The suppressiveness of Zeolite- and peat-based of *Paenibacillus* sp. and *Streptomyces* sp. formulation stored at room temperature or at 4° C was retained for over six months (Sing *et al.,* 1999).

On the other hand several carriers for formulating bio-agents had been reported. Formulations of fluorescent Pseudomonas were developed through liquid fermentation technology. The fermented biomass was mixed with different carrier materials (Talc/ Peat/ Kaolinite/ Lignite/ Vermiculite) and stickers (Vidhyasekaran and Muthamilan, 1995). Krishnamurthy and Gnanamanickam (1998) developed talc based formulation of *P. fluorescens* for the management of rice blast caused by *Pyricularia grisea*, in which methyl cellulose and talc was mixed at 1: 4 ratio and blended with equal volume of bacterial suspension at a concentration of 10^{10}cfu/ml. Nandakumar *et al*. (2001) developed talc based strain mixture formulation of fluorescent pseudomonades. It was prepared by mixing equal volume of individual strains and blended with talc as per Vidhyasekaran and Muthamilan (1995). Talc based strain mixtures were effective against rice sheath blight and increased plant yield under field conditions than the application of individual strains. Talc and peat based formulations of *P. chlororaphis* and *B. subtilis* were prepared and used for the management of turmeric rhizome rot (Nakkeeran *et al*., 2004). One school of thought explains that CMC is added as a sticker at 1:4 ratio to talc. Though it is effective in disease management, it would lead to the increase in the production cost, which would prevent the growers to adopt the technology. More over another school of thought explain that CMC and talc should be used at 1:100 ratios. Hence feasibility of the technique and shelf life of the product has to be evaluated to make the technology as a viable component in disease management so as to promote organic farming.

Greenhouse Experiment:

So far, few biological control agents have achieved success under field conditions. Among the hundreds of organisms identified as potential biological disease control agent, only few have resulted in proving commercially acceptable control of these diseases (Warrior *et al*., 2002). A fungal biocontrol preparation for control or prevention of plant fungal diseases comprises sporulated fungal biomass and a carrier preferably is vermiculite. Different formulations have been used in control soil borne pathogens, these are, fungal spores (Harman *et al.*, 1980), and powdery preparations of fungal mycelium (Latunde-Dada, 1993). A biocontrol formulation with agricultural potential should possess several desirable characteristics such as: easy preparation and application, stability, adequate shelf life, abundant viable propagules, and low cost (Churchill, 1982). The formulation should be amenable for application to both phylloplane and rhizosphere, depending on the pathogens and plants to be controlled. Formulation of the bio-agents to reduce incidence of the diseases caused by soilborne pathogens in the field is of great importance in biocontrol of such diseases. Therefore the present work was aimed also to determine the efficacy of application of formulation contained the *B. subtilis, P. fluorescens* and *T. harzianum* as seed treatment against root rot diseases of Cucumber and Pepper under greenhouse conditions.

Table 3: Effect of stored antagonistic bio-agents against root disease incidence of cucumber and pepper under open greenhouse conditions.

Carriers	Stored Antagonistic microorganism (after 10 month of storage)	Root rot incidence %			
		Pre-emergence growth stage		Post-emergence growth stage	
		Cucumber	Pepper	Cucumber	Pepper
Sawdust	*B. subtilis*	13.3 g	13.3 g	11.5 f	13.0 g
	P. fluorescens	16.0 f	16.0 f	13.0 e	14.2 f
	T. harzianum	20.0 e	20.0 e	14.2 de	14.2 f
Sawdust + CMC	*B. subtilis*	16.0 f	16.0 f	12.5 e	13.6 g
	P. fluorescens	20.0 e	20.0 e	15.7 d	15.0 ef
	T. harzianum	20.0 e	20.0 e	15.0 d	15.0 ef
Sawdust + Chitosan	*B. subtilis*	23.3 d	23.3 d	12.5 e	16.6 e
	P. fluorescens	26.6 cd	23.3 d	15.7 d	16.6 e
	T. harzianum	26.6 cd	26.6 cd	13.6 e	18.1 c
Sawdust +Talc powder	*B. subtilis*	26.6 cd	30.0 c	16.0 c	16.6 e
	P. fluorescens	30.0 c	30.0 c	18.1 bc	18.1 c
	T. harzianum	30.0 c	33.3 bc	18.1 bc	19.0 bc
Sawdust + CMC +Talc powder	*B. subtilis*	33.3 bc	33.3 bc	16.6 c	16.0 e
	P. fluorescens	36.6 b	36.6 b	18.1 bc	16.6 e
	T. harzianum	36.6 b	36.6 b	18.1 bc	17.3 cd
Sawdust +Talc powder + Chitosan	*B. subtilis*	33.3 bc	33.3 bc	17.3 bc	17.3 cd
	P. fluorescens	36.6 b	36.6 b	18.1 bc	19.0 bc
	T. harzianum	36.6 b	36.6 b	20.0 b	20.0 b
Fresh cultures	*B. subtilis*	13.3 g	13.3 g	12.5 e	13.6 g
	P. fluorescens	16.0 f	16.0 f	13.0 e	15.0 ef
	T. harzianum	20.0 e	20.0 e	14.2 de	16.6 e
Control		54.6 a	51.9 a	24.3 a	27.4 a

Mean values within columns followed by the same letter for each growth stage are not significantly different ($P \leq 0.05$).

In the present study, the efficacy of using stored formulated various antagonistic fungi and bacteria as seed treatment against root rot incidence of cucumber and pepper was evaluated in pots experiment using soil artificially infested with the disease incidents under greenhouse conditions. Results of pre-, and post-emergence root rot incidence were presented in (Table 3 and Fig. 3). Presented data reveal that all the tested fresh and stored bio-agents showed interesting highly significant effect causing high reduction of root rot incidence at both pre-, and post-emergence stages of plant growth comparing with the check treatment. No significant differences were observed between fresh applied bio-agents cultures and stored ones formulated on (Sawdust) and (Sawdust + CMC) carriers.

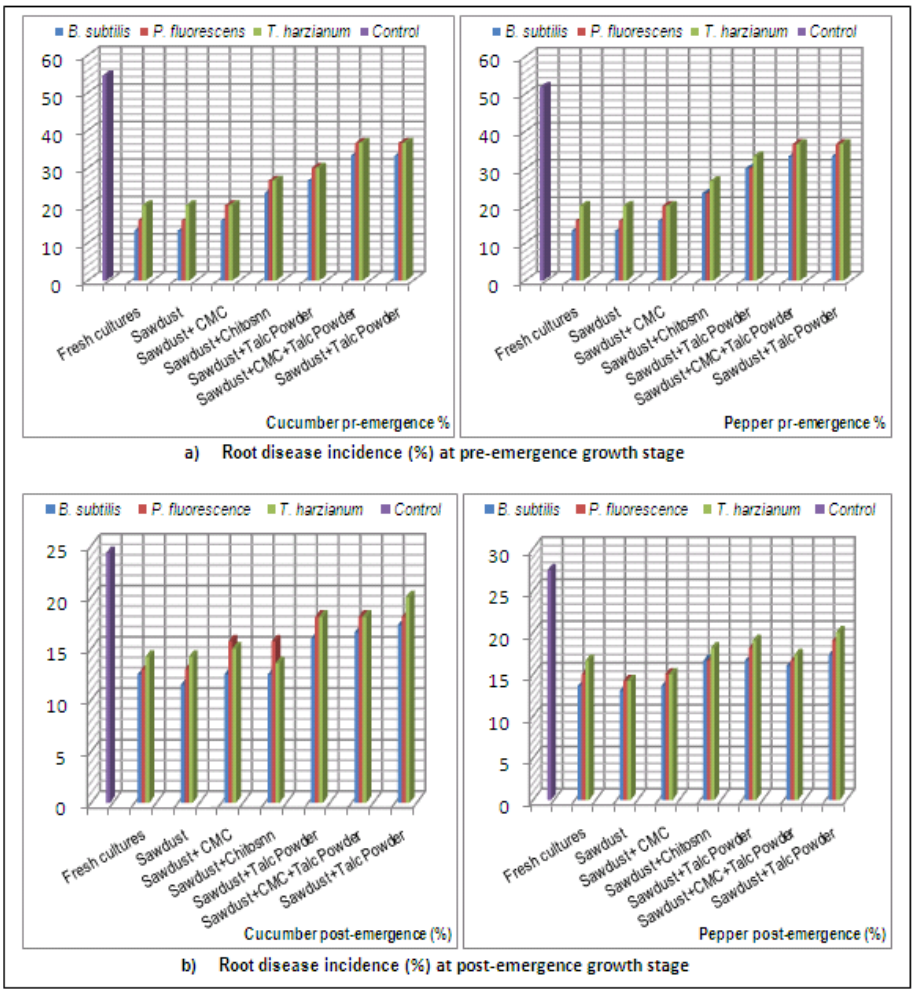

Fig. 3: Effect of stored antagonistic bio-agents against root disease incidence (a & b) of cucumber and pepper under open greenhouse conditions.

Meanwhile stored bio-agents on (Sawdust + Chitosan); (Sawdust +Talc powder); (Sawdust + CMC +Talc powder) and (Sawdust +Talc powder + Chitosan) carriers showed less significant protective effect against root rot disease incidence. They showed low significant pre-emergence root rot incidence ranged between 23.3-36.6% for applied fungal and bacterial, respectively comparing with untreated and check treatments which recorded as 54.6 and 51.9% for cucumber and pepper, in respective order. Similar trend concerning post-emergence root rot incidence (Table 3) was observed. All treatments varied in their effect on disease incidence. Treated seeds showed higher significant reduction on disease incidence than untreated ones. Moreover, bacterial treatment showed superior effect on disease incidence (11.5-14.2%) followed by fungal treatment (14.2-20.0%).

It is also interesting to note that, more reduction in disease incidence was observed at post-emergence stage of plant growth than at pre-emergence. This observation could be attributed to their sensitivity to the fluctuations in environmental conditions and are inconsistent in their performance. Similar observation was also reported by several investigators. Backman *et al*. (1977) stated that 60-75% of the cotton crop in US is treated

with *B. subtilis* for the management of soilborne pathogens encountered in cotton ecosystem. Among several PGPR strains *Bacillus* based products gains momentum for commercialization. Because, *Bacillus* spp., produce endospores tolerant to extremes of abiotic environments such as temperature, pH, pesticides and fertilizers (Backman *et al*., 1997). Furthermore, seed treatment of pigeonpea with talc based formulation of fluorescent pseudomonads at the rate of 4g/kg of seed followed by soil application at the rate of 2.5 kg/ha at 0, 30, and 60 days after sowing controlled pigeonpea wilt incidence under field conditions. The additional soil application of talc based formulation improved disease control and increased yield compared to seed treatment alone (Vidhyasekaran *et al*., 1997). Delivering of *P. fluorescens* as seed treatment followed by three foliar applications suppressed rice blast under field conditions (Krishnamurthy and Gnanamanickam, 1998). Combined application of talc based formulation of fluorescent pseudomonads comprising of Pf1 and FP7 through seed treatment, seedling dip, soil application and foliar spray suppressed rice sheath blight and increased plant growth better than application of the same strain mixture either through seed, seedling dip or soil (Nandakumar *et al*., 2001). Biological control of plant pathogens is becoming an important component of plant disease management practices. In the present study the used fungal and bacterial antagonists proved their highly inhibitor effect against root rot pathogens under *in vitro* and *in vivo* conditions. These results are also confirmed by several researchers (Bell *et al.,* 1982; Abdel-Kader, 1997 and El-Mougy, 2001). Biological control of seedling diseases using antagonistic fungi and bacteria has received increasing attention. Antagonists applied to seeds prior to planting colonize the rhizosphere of seedlings and thus are present at or near the pathogen' infection court, where they act by producing antifungal or antibiotic compounds, through hyperparasitism, or by competitively colonizing spermosphere and rhizosphere substrates (Taylor and Harman, 1990). Seed treatment is an attractive delivery system either fungal or bacterial bioprotectants (Wright *et al.* 2003). Bio-protectants applied to seeds may not only protect seeds (Sivan and Chet, 1986) but also may colonize and protect roots (Chao, *et al*., 1986). The term bio-priming has been used by at least research groups, and each group has used a different technique to achieve bio-priming. Harman and Taylor (1988) added their biocontrol agents directly to the Solid Matrix Priming (SMP), which allowed the *T. harzianum* to colonize the seeds during the priming process. On the other hand, Callan *et al* (1990) added a suspension of the bacterium *Pseudomonas fluorescens* to 1.5% methyl cellulose coated, surface sterilized sweet corn seed prior to hydrating the seeds between moistened paper towels. The two methods were used to protect tomato and sweet corn against Pythium damping-off. In the present study bio-priming cucumber and pepper seeds bio-treatment was applied through mixing with stored formulated bio-agents or imbibing seeds into solution of fresh bio-agents cultures for 1h as a period time for allowing seeds' colonization before drying. A successful antagonist should colonize rhizosphere during seed germination (Weller, 1983). It is evident that antagonistic biagent can affect plant resistance to a pathogen either by inducing the basal level of defense reactions immediately after treatment or by enhancing a capacity for rapid and effective activation of cellular defense responses, which are induced only after contact with a challenging pathogen, a process known as "sensitization" or "priming" (Conrath *et al*., 2002). Also, Jensen *et al.*, (2004) reported that based on microscopic evaluation of the growth a distribution of the antagonist during priming, *Clonostachys rosea* colonized the whole surface of the pericarp, including the apex of carrot seed where the primary root emerges. These reports are in agreements with the obtained results in the present study.

Promising applicable technique could be suggested on the light of the results obtained in the present study. The usage stored formulated bio-agents might be considered as safe, cheep and easily applied biocontrol method as seed or soil application against such soilborne plant pathogens particularly in organic farms taken in consideration avoidance of environmental pollution.

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