An-Najah National University
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Biological Control of *Rhizopus* Soft Rot on Apple, Pear and Peach by *Trichoderma harzianum*

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Dedication

To my Parents and to my brothers with love

Acknowledgments

All praise to Allah for this accomplishment.

Thanks to Dr. Yacoub Batta for his guidance, encouragements and supervision during the study and dissertation preparation.

I would like to record my special thanks to my father, my mother for their efforts in all steps of my life and combine harvesting.

Thanks to my brothers.

At the end, my thanks to the many other people who helped in this work.

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List of Abbreviations

AACC: American Association of Cereal Chemist.

CFU: Colony - forming - units.

CWDE: Cell - wall – degrading enzymes.

ED: Effective dose.

EPA: Environmental Protection Agency.

IE: Invert emulsion.

OMA: Oat meal agar.

PDA: Potato dextrose agar.

RH: Relative humidity.

USDA: United States Department of Agriculture.

List of Appendices

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Abstract

This research aimed at evaluation of biological effectiveness of *Trichoderma harzianum* against the *Rhizopus* soft rot caused by *Rhizopus stolonifer*. Also, it aimed at determination of minimum protection period from infection with *Rhizopus* soft rot on three types of fruits (apple, pear, and peach). The fungus was mainly applied in form of invert emulsion (water - in - oil formulation) after being introduced into the emulsion in form of conidia in addition to using formulated and non – formulated forms of the fungus. The experiments (evaluation of efficacy) was carried out under laboratory conditions (20 $\pm$ 2°C and 30 $\pm$ 2°C). Results obtained have demonstrated that the fungus (*Trichoderma harzianum*) formulated in invert emulsion was effective in reducing *Rhizopus* soft rot lesion diameter compared to other treatments. Significant differences ($P \leq 0.05$) were obtained in reducing the lesion diameters of *Rhizopus* soft rot treated with *Trichoderma* in invert emulsion in copmarsion with the control treatment. Results have also indicated that *Trichoderma* formulated in invert emulsion on unwounded apple fruits gave the longest minimum protection period against *Rhizopus* soft rot disease, which demonstrated the biological effectivness of *Trichoderma harzianum*. More over, it is recommended to confirm the efficacy of the fungus against *R. stolonifer* especially in the formulated form under a wide range of temperatures and relative humidities, in addition to controlled atmosphere conditions and using other fungal strains of *T. harzianum* against *R. stolonifer* in the same formulation

and may be other formulations can be also tested. Using other kinds of fruits also may expand the knowledge and verify the concept of biological control.

Chapter One

Introduction

Introduction

Plant diseases caused by fungal pathogens, provoke severe losses of agricultural and horticultural crops every year. These losses can result in reduced food supplies while world population continues to increase, poorer quality agricultural products, economic hardship for growers and processors, and, ultimately, higher prices (Agrios, 1997; Monte, 2001). *Rhizopus* soft rot caused by the pathogenic fungus *Rhizopus stolonifer* is one of the most important postharvest diseases attacking wounded fruits and vegetables causing further rupture of softened skin during handling or under pressure. It causes severe economic losses for the following reasons: there are very few effective chemical fungicides which can control the disease and there is an increasing resistance to the effective fungicides; the public perception would prefer to have untreated fruits with chemical fungicides postharvest. Much of modern research in plant pathology aims at finding other environmentally friendly means of controlling plant diseases. This study try to use a biological means as using the antagonistic fungus *Trichoderma harzianum* to control *R. stolonifer* on three types of fruits (apple, pear, peach). Since biological control of postharvest diseases using antagonistic fungi is a relatively new approach, it has emerged as an effective alternative control means to chemical fungicides, and it can be targeted much more efficiently (Wilson and Pusey, 1985; Pusey, 1996). In the Palestinian territories, fruit trees constitute the largest percentage compared to the total planted area. It constitutes approximately 63.8%, and this equals to 1,158,000 dunums in west bank and Gaza strip. The total planted areas with peach, pear and apples were estimated at 2161, 485, and 1809 dunums, while the production of these fruits were 1124, 138, and 641 metric tons, respectively (Palestinian Central Bureau of Statistics, 2004).

This means that the three types of fruits contribute 0.72% from the total fruit production in 2002/2003, since the total fruit production in Palestinian territories was 263,612 metric tons and approximately (peach, pear, and apple) contribute 0.38% from the total planted fruit area, since the total planted fruit area was 1,158,050 dunums. The total revenues from these three fruit types in the Palestinian territories was 1,453,000 US $ in 2002/2003 which contributes 0.29% from the total fruit revenues (Palestinian Central Bureau of Statistics, 2004).

Objectives of the Study

1. To assess the biological effectiveness of *Trichoderma harzianum* against the *Rhizopus* soft rot caused by *Rhizopus stolonifer* on three types of fruits (apple, pear, peach) at two temperatures.

2. To determine the protection period from infection with *Rhizopus* soft rot on the same types of fruits following the *Trichoderma harzianum* treatment.

Chapter Two

Literature Review

1. *Rhizopus soft* rot

1.1 Description

1.1.1 Identification and Classification

Rhizopus stolonifer, causal organism of soft rot of fruits and vegetables, can be classified as a cosmopolitan filamentous lower fungus living in the soil, decaying fruit and vegetables, animal feces, and old bread. *R. stolonifer* belongs to Mucoraceae family, the order: Mucorales, and class zygomycetes which contains two other genera: *Choanephora* and *mucor*) known to cause diseases in plants (Agrios, 1997). The spores of zygomycetes are often floating around in the air, they are either saprophytes or weak parasites of plants and plant products on which they cause soft rots or molds (Agrios, 1997).

It is named as *Rhizopus stolonifer* because it produces a mycelium with long sporangiophores connected by an aerial stolon.The stolons connect sporangiophores along various points of host contact; a root-like structure called a "rhizoid" extends beneath the sporangiophores and fastens them with the host tissues (Agrios, 1997). The genus *Rhizopus* contains several other species, such as; *R. oligosporus, R. chinensis, R. oryzae, R. rhizopodiformis, R. arrhizus, R. azygosporus,R. microsporus* (Reinhardt et al., 1981). The most common one is *R. stolonifer*. Some morphological features, such as the length of rhizoids and sporangiophores, the diameter of sporangia, the shape of columellae, and the size, shape and surface texture of sporangiophores aid in differentiation of *Rhizopus* species from each other.

1.1.2 Macroscopic Features

Colonies of *Rhizopus* grow very rapidly at temperatures 25 ± 2ºC fill the Petri dish, and sporulate in 4 days. The colony texture is typically cotton-candy like. From the front, the color of the colony is initially white and then turns grey to yellowish brown. Pathogenic species of *Rhizopus* can grow well at 30ºC (Sutton et al. 1998).

1.1.3 Microscopic Features

Rhizopus has non septate or sparsely septate broad hyphae (6-15 μm in diameter), sporangiosphores, rhizoids (root-like hyphae), sporangia, and sporangiospores are visualized. The sporangiophores are brown in color and usually unbranched, they can be solitary or form of clusters. Rhizoids are located at the points where the stolons and sporangiophores are meeting. Sporangia (50 - 350 μm in diameter) are located at the tip of the sporangiophores, they are round with flattened bases.

Sporangiospores (4 – 11 μm in diameter) are unicellular, round to ovoid in shape, hyaline to brown in color, and smooth or striated in texture (St-German & Summerbell, 1996).

1.2 Distribution

Rhizopus soft rot of fruits and vegetables occurs throughout the world on harvested fleshy organs of vegetables, fruits and flower crops during storage, transit, and marketing of these products (Agrios, 1997). The disease, when occurs on wet or wounded fruits packed in card board boxes, can be an unsightly mess due to the watery leakage from fruits causing the boxes collapse (Alvarez & Nishijima, 1987).

1.3 Host Range

According to the USDA fungus – host distributions reports in 2003, *R. stolonifer* has a very broad host range (over 240 species in many countries around the world). Several fruits and vegetables are susceptible to infection and include the following genera: *Alium, Ananas, Brassica, Cucumis, Cucurbita, Fragaria, Lycopersica, Phaseolus, Pisum, Solanum* (Nishijima et al., 1990), in addition to sweet potatoes, strawberries, peaches, cherries, and peanuts. Corn and some other cereals are affected under fairly high conditions of moisture. Bulbs, corms, and rhizomes of flower crops, for example, gladiolus and tulips, are also susceptible to this disease (Agrios, 1997).

1.4 Symptoms of *Rhizopus* soft rot on Fruits

Symptoms of *R. stolonifer* on infected areas of fleshy fruits appear water soaked at first, and are very soft. If the skin of the infected organ remains intact, the tissue loses moisture gradually until it shrivels into a mummy; otherwise they break down and rupture softened skin during handling or under pressure. Fungal hyphae then grow outward through the wounds and cover the affected portions by producing tufts of whisker-like gray sporangiophores which carry sporangium. The bushy growth of the fungus often extends to the surface of the healthy portions of affected fruits and even to the surface of the containers within a few days when they become wet with the exuding whitish – yellow liquid, the infected fruit is often covered by coarse, gray, hairy mycelia that form a mass of black sporangia at there tips (Nishijima et al., 1990). Infected tissues at first give off mildly pleasant smell, but soon yeasts and bacteria move in and a sour odor develops (Agrios, 1997).

1.5 Factors Influencing the Growth of *R. stolonifer*

Since *R. stolonifer* is considered to cause a postharvest disease, there are many preharvest and postharvest factors that influence fruit decay.

1.5.1 Prehavest Factors Influence Postharvest Decay

It was found that conditions of producion at harvest stage determine how long the crop can be safely stored. For example, apples are picked slightly immature to ensure that they can be stored safely for several months, the on set of ripening in various fruits renders them more susceptible to infection by pathogens (Kader, 1985). On the other hand, fruit can be made less susceptible to decay by management of crop nutrition. For example, calcium has been more closely related to disease resistance than any other cations associated with the cell wall (Sams, 1994). This can be demonstrated in a study on effect of increased flesh calcium content of apples on storage decay fruit treated with solutions of $CaCl_2$ by dipping. Increased calcium contents in peaches have also been documented with reduced postharvest decay (Conway, 1989). Conversely, high nitrogen content in fruit predisposes them to decay (Conway, 1984). In pears, it has been found that management of trees for low nitrogen and high calcium content in the fruit reduced severity of postharvest fungal decay (Sugar et al., 1992). Also infections with *Rhizopus* soft rot depend on chosen cultivars. In a recent study, it was found that resistance of major apple cultivars to the fungi was dependent on cultivars (Spotts et al., 1990). According to Lisker et al (1996), mechanical wounding, or chloroform dips, and decline in acidity during growth and maturation, dramatically icreased the susceptipility of young grape berries to *R. stolonifier* inoculation.

1.5.2 Postharvest Factors Influence Decay

Rhizopus is a strictly wound – parasite, so it can penetrate host tissues only through fresh wounds and bruises made by harvesting, handling, insects, and rodents (Barnes, 1979; lisker et al., 1996). Poor storage conditions specially temperature and relative humidity (RH) play a role to cause infection. The optimum temperature for germination and growth ranges (5-52˚C) in storage rooms (Dennis and Cohen, 1976). Fungal spore germination is often enhanced at higher RH, but small differences in RH have significant effects in relation to the degree of postharvest decay (Spotts and Peters, 1981).

1.6 Biology and Life Cycle

Rhizopus exists everywhere, usually as a saprophyte and sometimes as a weak parasite on stored organs of plants. The mycelium of the fungus produces long, aerial sporangiophores at the tips of which black spherical sporangia develop (Agrios, 1997) (Figure 1).

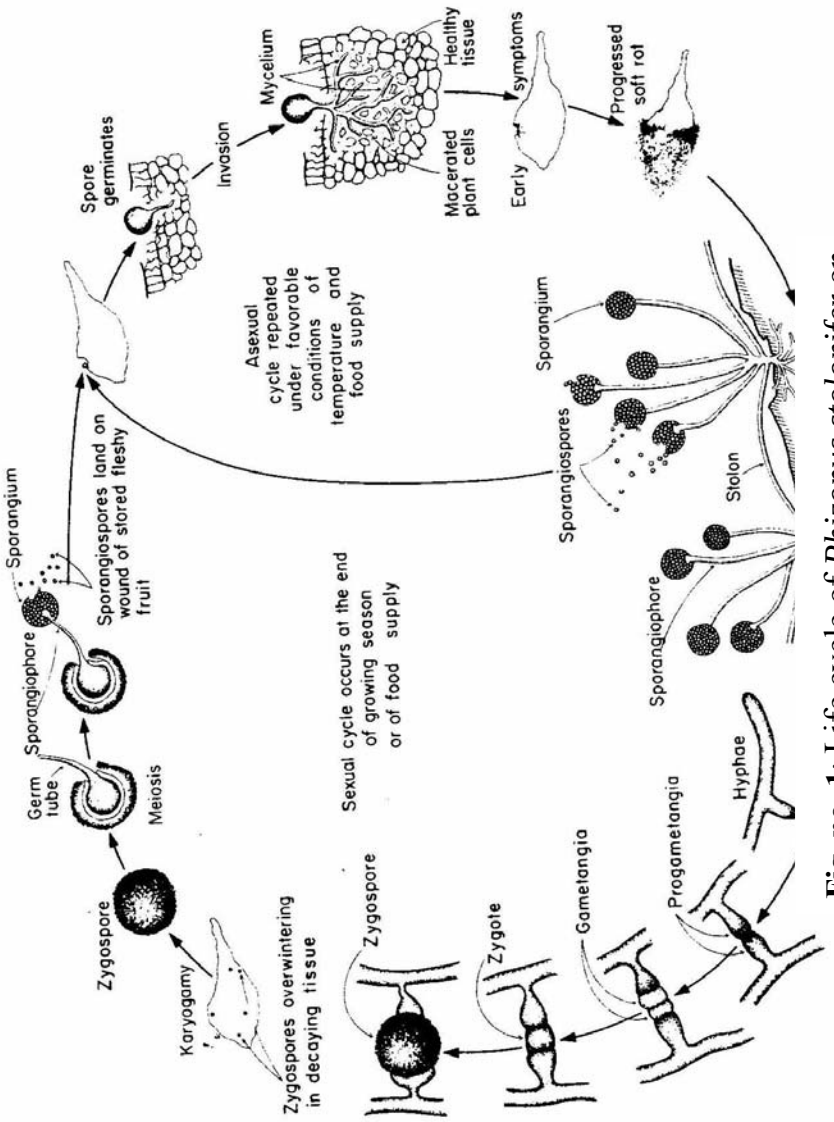

Fig. no. 1: Life cycle of *Rhizopus stolonifer* on fruits and vegetables (Agrios, 1997)

The sporangia contain thousands of spherical gray sporangiospores. When the mycelium grows on a surface, it produces stolons or superficial

hyphyae that arch over the surface and at the next point of contact with the surface produce both root-like hyphae or rhizoids which grow toward the surface piercing the softened epidermis and then go through the organic material, secreting the enzymes, absorbing water, and digesting sugars and starches (Agrios, 1997). The aerial sporangiophores bearing sporangia, and from each point of contact more stolons are produced in all directions. Adjacent hyphae produce short branches called progametangia, which grow toward one another. When they come in contact, the tip of each high face is separated from the progametangium by a cross wall. The terminal cells are the gametangia. These gametangia fuse together and their nuclei pair. The cell formed by fusion enlarges and develops a thick, black, and watery cell wall (Barness, 1979) (Figure 2).

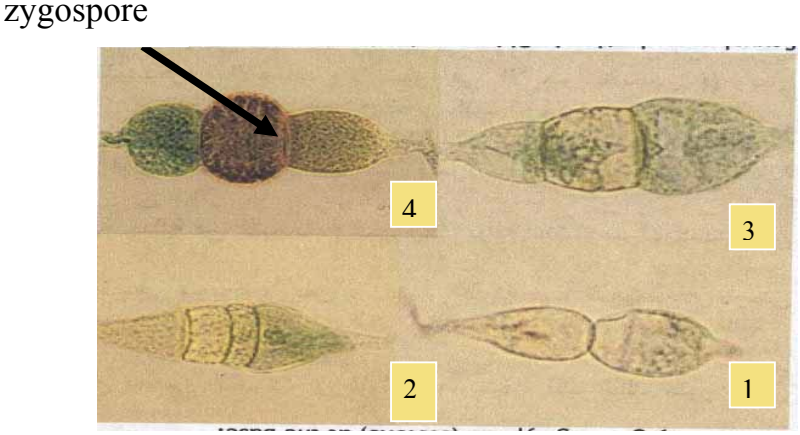

Fig. no. 2: Sexual reproduction in *Rhizpous stolonifer*: Hyphae meeting (1+2) and making a zygospore (3 + 4) (Barness, 1979).

This sexually produced spore is called zygospore, it is used by the fungus in the overwintering or as a resting stage. When it germinates, it produces a sporangiophore bearing sporangium full of sporangiospores. Throughout

the year, sporangiospores float about and if they land on wounds of fleshy fruits, roots, corms, or pulps, they germinate. Wounds made by harvesting, handling, insects, rodents enhance the infection (Barnes, 1979). The produced hyphae secrete pectinolytic enzymes, which break down and dissolve the pectic substances of the middle lamella that hold the plant cells in place in the tissues. This results in loss of cohesion among the cells and development of "soft rot". The pectinolytic enzymes secreted by the fungus advance ahead of mycelium and separate the plant cells, which are then attacked by the cellulolytic enzymes of the fungus. The cellulases break down the cellulose of the cell wall, and the cells disintegrate. Mycelium does not seem to invade cells but it is surrounded by dead cells and non living organic substances, and it is living more likely as a saprophyte than a parasite. The fungus continues to grow inside the tissues. When the epidermis breaks, the fungus emerges through the wounds and produces aerial sporangiosphores, sporangia, stolons, and rhizoids. In extremely fleshy fruits, the mycelium can penetrate even healthy fruit. Unfavorable temperature and humidity or insufficient maturity of the fruit slow down the growth and activity of the fungus, so it reproduces sexually (Moniz de Sà, 2003).

1.7 Effect of Infected Fruits by *R. stolonifer* on Their Nutrient Content

In the case of storage rot of fruits caused by *R. stolonifer*, nutrient content may be greatly reduced. Freshly harvested bread fruit, associated with *R. stolonifer* and other fungi, was shown to decline from about 70% carbohydrate to about 60%, the total fat, protein, and energy of the bread fruit also declined at room temperature storage (Amusa et al., 2002). In 2003, the same investigators studied biodeterioration of the African star

apple (*Chrysophylum albidum*) in storage occurred by many fungi including *R. stolonifer* and the effect on its food value. Mineral analysis was also carried out according to the standard AACC (1983) method that revealed the uninfected freshly harvested African Start apple fruit had crude protein contents (CP) of 8.75%, carbohydrate content (CHO) of 29.6%, crude fat (CF) of 16.2%, and moisture content (MC) of 42.1%. However, 9 days after harvesting, the CP, CHO and CF contents decreased to about 5.01%, 20.2% and 13.2%, respectively due to infection with *R. stolonifer* according to (Amusa et al., 2003). Also, they deduced from this study that deterioration of the fruit by the pathogen might have led to an increase in the mineral contents such as K, Ca, Na and decrease in metabolic synthetates of the African Star apple fruits. Changes in nutrient composition caused by infection of the fruit will adversely affect the uses for jam and other food products.

1.8 Control of *R. stolonifer*

1.8.1 Chemical Control

Fungicides used for postharvest decay control should only be used after the following critical points are considered: type of pathogen involved in the decay; location of the pathogen in the product; best time for application of the treatment; maturity of the host; and environmental conditions during storage, transportation and marketing of product (Ogawa and Manji, 1984). Preventive field fungicide sprays control *Rhizopus* soft rot reducing field inoculum levels, fungicide sprays also reduce the incidence of fruit lesions, caused by other fungi since *Rhizopus* can act as courts of entry into the papaya fruit (Alvares and Nishijima, 1987). Iprodione has been used for several years as a preharvest spray in combination with wax and / or oil. Its

decay control spectrum is increased and will also control postharvest fungi such as *Rhizopus*, and *Alternania* (Ogawa et al., 1992). Many of the former products that were used postharvest are no longer permitted to be used or discontinued because of concerns with residues and possible toxic effects. The most notable fungicides that contained Benomyl, Thiabendazole, Dichloron, and Imazalil are examples of postharvest chemical treatments that are presently used. However, resistance to Thiabendazole and Imazalil is widespread (Holmes and Eckert, 1999; Conway et al., 1999) and their use as effective materials is declining. Preservative or antimicrobial food additives are not generally thought of as postharvest treatments but they do control decay, these products include sodium benzoate, sorbic acid, propionic acid, SO_2, acetic acid, Nitrites and Nitrates, and some antibiotics such as Nisin (Chichester and Tanner, 1972). The demand for new postharvest fungicide treatments is strong, especially since the discontinuation of Iprodione in 1996. Fludioxinil was granted an emergency registration in 1998 to curb potential losses in nectarines, peaches, and plums that would have resulted (Foster and Adaskaveg, 1999). Sanitation is the cornerstone of any effective postharvest decay reduction program. It must be a partnership between grown and packer and it must begin in the orchard. Storage containers and warehouses must be disinfected with a copper sulfat solution, formaldehyde, sulfur fumes, Chloropicrin (Agrios, 1997). Recently, several botanical essential oils have shown potential as a natural fungicide against *R. stolonifer*, including *Ocimum amerecanum L*. (Tajo and Thoppil, 1999), peppermint and sweet basil vapor (Edris and Farrag, 2003), and Kava root extract (Xuan et al., 2003).

1.8.2 Cultural Control

As *Rhizopus* soft rot acts as a saprophyte which exists everywhere, it can affect the fleshy organs when it reaches the maturity through wounds and bruises made by harvesting and handling (Agrios, 1997). At this point, disease may begin at the field if the previous conditions are available. Host eradication (roguing) is one of the cultural control methods carried out routinely in many nurseries, greenhouses, and fields to prevent the spread of numerous diseases by elimination of infected plants that provide a ready source of inoculum within the crop. This elimination prevents greater losses from the spread of the pathogen to additional plants. Crop rotation can reduce population of the pathogen in the soil, and appreciable yields from the susceptible crop can be obtained every third or fourth year of the rotation. Plowing under infected plants after harvest, such as left over infected fruit, stems, tubers, or leaves, helps cover the inoculum with soil and speeds up it's disintegration (rotting) and concurrent destruction of most pathogens carried in or on them. Pruning infected or dead branches, and removing infected fruit and any other plant debris that may harbor the pathogen to grow into still healthy parts of the tree. Spacing plants properly in the field or greenhouse prevents the creation of high humidity conditions on plant surfaces and inhibits infection (Agrios, 1997). Also, appropriate choice of fertilizer such as low nitrogen and high calcium in the fruit reduced severity of postharvest decay (Sugar et al., 1992). Handling fruit properly at harvest, not including fruit for storage that has fallen on the ground or has been in contact with grass or soil as fungi often enter through wounds, and using wood chips where bins are held to minimize contact with soil (Kupferman, 1990).

1.8.3 Physical Control

Soil can be sterilized in greenhouses, and some times in seed beds and cold frames, by the heat carried in live or aerated steam or hot water. The soil could be steam sterilized either in special containers (soil sterilizers), into which steam is supplied under pressure, steam is piped into and is allowed to diffuse through the soil. Soil sterilization is completed when the temperature in the coldest part of the soil has remained for at least 30 minutes at 82ºC or above, which almost kills all soil borne plant pathogens (Agrios, 1997). Also hot-water treatment of certain seeds, bulbs, and nursery stock is used to kill any pathogen with which they are infected or which may present inside seed coats, bulb scales, etc., or which may be present in external surfaces or wounds (Agrios, 1997). High temperature may be used to control postharvest decay on crops that are injured by low temperatures, such as mango, pepper, and tomato (spotts, 1984). Heating of pears at temperatures from 21 to 38ºC for 1 to 7 days reduced postharvest decay (Spotts and Chen, 1987). Decay in "Golden Delicious" apples was reduced by exposure to 38ºC for 4 days (Sams et al., 1993). Many fruits can be stored dry for a long time and can be kept free of disease if they are dried sufficiently before storage and if moisture is kept below a certain level (about 12 percent) during storage, even slices of fleshy fruits as apples, peaches, and apricots can be protected from infection and decay by fungi if they are sufficiently dried by exposure to the sun or to warm air (Agrios, 1997). The most widely and effective method of controlling postharvest diseases of fleshy plant products is refrigeration. Although low temperatures at or slightly above the freezing point do not kill any of the pathogens that may be on or in the plant tissues, they do inhibit or greatly retard the growth and activities of all such pathogens and thereby reduce

the spread of existing infections and the initiation of new ones (Agrios, 1997; Sommer, 1989). Various types of electromagnetic radiation, such as ultraviolet (UV) light, and particulate radiation, such as X particles and B particles have been studied their ability to control postharvest diseases of fruits and vegetables like peaches, strawberries, and tomatoes.

Unfortunately, with many of these diseases the dosage of radiation required to kill the pathogen may also injure the plant tissues on which the pathogens exist. Although found safe and properly licensed by the USDA, it is vigorously opposed by certain segments of the population. So far, no plant diseases are commercially controlled by radiation (Agrios, 1997). Modified atmosphere is also used when there is little possibility of adjusting gas composition during storage or transportation (Sommer, 1989). Because the pathogen respires as does produce, lowering the O_2 content above 5% can suppress pathogenic growth in the host. In crops such as stone fruits, a direct suppression occurs when fungal respiration and growth are reduced by the high CO_2 of the modified atmosphere. Low O_2 does not appreciably suppress fungal growth until the concentration is below 2%. Important growth reductions result if the O_2 is lowered to 1% or lower although there is a danger that the crop will start respiring and develop off – flavor. Other technologies that have been anaerobically tested for lowering postharvest decay with limited success are the storage and transport under low O_2 and the use of carbon monoxide (Spotts, 1984; Sommer, 1989).

1.8.4 Biological Control Using Bacteria

So far, only three strains of bacteria have been registered and are commercially available for use as antagonistic microorganisms for

biological control of plant diseases, they are: *Agrobacterium radiobacter* K – 84, sold as Gallex® or Galltrol® used against crown gall disease caused by *Agrobacterium. tumefaciens*. *Pseudomonas fluorescens*, sold as Dagger G® used against *Rhizoctonia* and *Pythium* damping – off of cotton; and *Baccillus subtilis,* sold as Kodiak® used as a seed treatment and postharvest biological control agent of stone fruit brown rot caused by *Monilinia. fructicola* (Pusey and Wilson, 1984; Agrios, 1997). Then other studies have been finally appeared that increased the information on antagonistic microorganisms such as *Enterobactor cloacae* partially controlled postharvest diseases as *Rhizopus* rot of peach fruits (Wilson et al., 1987; Qing and Shiping, 2000). Also, *Pseudomonas* species had a biological effect against postharvest rot of nectariens and peaches (Smilanick et al., 1993).

1.8.4.1 *Pantoea agglomerans* EPS 125:

Treatment of stone fruits (apricot, peach and nectarine) with *Pantoea agglomerans* strain EPS 125 decreased the incidence and diameter of lesions of brown rot caused by *Monilinia laxa* and soft rot caused by *Rhizopus stolonifer*. Rot control was achieved on fruits either wounded and subsequently inoculated with the pathogens or non – wounded and naturally infected from orchards. The efficacy of biocontrol was dependent on the concentration of the biocontrol agent and pathogen. At medium to low pathogen dose, optimal concentrations of *P. aggolmerans* EPS 125 were above 10^7 CFU / ml. The medium effective dose of EPS 125 was 2.2 $\times$ 10^5 CFU / ml in case of controlling *R. stolonifer*. Significant inhibition of conidial germination and hyphal growth of *R. stolonifer* and *M. laxa* was achieved when the fungal and EPS cells were cocultivated on peel leachate

on nectarine juice. However, no effect was observed when the antagonist and the pathogen cells were physically separated by a membrane filter which permits nutrient and metabolite interchange. Therefore, wound colonization and direct interaction between the strain and the pathogen cells is necessary for antagonism, which proposed as the mechanism of biocontrol, without a significant contribution of the production of antibiotic substances or nutrient competition (Bonaterra et al., 2003).

1.8.4.2 *Pantoea agglomerans* CPA – 2:

Two hundreds and forty seven epiphytic microorganisms isolated from the fruits and leaf surfaces of apples and pears were tested for antagonistic properties against *Penicilluim expansum*, *Botrytis cinerera* and *Rhizopus stolonifer*. A bacterium strain identified as *Pantoea agglomerans* (CPA - 2) was selected (Nunes et al., 2001). Complete control at the three tested concentrations (2×10^7, 8×10^7 and 1×10^8 CFU / ml) was obtained on wounded pears inoculated with 10^3, 10^4 and 10^5 conidia / ml of each *P. expansum* and *R. stolonifer*, respectively. In over 3 years of experiments in semicommerical trials, *Pan. agglomerans* CPA-2 provided excellent control against the previous pathogens. It grew well inside wounds of pears at both room and cold temperatures, and under modified atmospheres. In contrast, it grew poorly on the surface of intact fruit (Nunes et al., 2001).

1.8.4.3 *Pseudomonas syringae:*

This strain of bacteria acts as an active ingredient in Bio – Save 11 LP, a biological – based decay control product. It was recently registered by the U.S Environmental Protection Agency (EPA) for aiding in control of *Rhizopus* soft rot on sweet potatoes. Bio – Save 11 LP is marketed as a frozen powdered formulation (Holmes, 2005). Efficacy data against *Rhizopus* soft rot is limited but very encouraging. In 2004, two small trials on sweet potato roots (CV: Hernandez) were impact – wounded and inoculated with spores of *R. stolonifer*. Inoculated roots were submerged for thirty seconds in a Bio – save 11 LP solution (799 grams of Bio – Save 11 LP per 40 gallons of water). This treatment resulted in an average of 95 percent control of *Rhizopus* soft rot compared to no control in the untreated check, and average 58 percent control by Botran® (dicloran) treatment (0.25 pound or 113 grams per 40 gallons). Bio – Save 11 LP should not be added directly to waxes, soaps, sanitizers or chlorinated water. The product should be applied to freshly washed sweet potatoes and recycled suspension need to be recharged periodically throughout the day. It is a natural product that provides an alternative control method for decay control for packers shipping to markets which do not accept Botran® – treated sweet potatoes (Holmes, 2005).

1.8.5 Biological Control Using Fungi and Yeasts

So far, only three strains of fungi have been registered and are commercially available for use as antagonistic fungi, they are: *Gliocladuim virens,* Sold as Glio G® for control of seedling diseases of ornamental and bedding plants; *Trichoderma harzianum*, sold as F- stop® and others, for control of several soil borne plant pathogenic fungi; and *T. harzianum* / *T.*

polysporum, sold as Binab T® for control of wood decays (Agrios, 1997). Most postharvest rots of several fruits could be reduced considerably by spraying with spores of antagonistic fungi and saprophytic yeasts at different stages of fruit development, or by dipping the harvested fruit in their suspensions. Several antagonistic yeasts (as a unicellular fungi) protected grapes and tomatoes from *Botrytis cinerea*, *Penicillium expansum, Monilinia fructicola, and Rhizoctonia* rots (Agrios, 1997; Karabulut and Baykal, 2003). The yeast *Candida oleophila* was approved for postharvest decay control in citrus and apples under the trade name Aspire® (Agrios, 1997). DR52 was significantly superior to all the other yeasts in effectiveness against all the previous pathogens. DR52 was identified by Central bureau voor Schimmeel cultures (Baarn, The Netherlands) as *Kloeckera apiculata*. *K. apiculata* controlled *B. cinerea* during 30 days of storage. It's efficacy was 83.4% reduction in *B. cinerea* incidence and 87.5% reduction in *P. expansum* incidence during 45 days of storage (Karabulut and Baykal, 2003). Also, *K. apiculata* partially controlled postharvest *Rhizopus* rot of peaches (Mc Laughlin et al., 1992; Qing and Shiping, 2000). Roberts (1990) discovered that *Cryptococcus laurentii* has antagonistic activity against many postharvest pathogens. *Rhodotorula glutinis* also limited *Rhizopus* rot in apple, table grapes, and strawberries (Lima et al., 1998; Qing and Shiping, 2000). Lima et al. (1997) reported that treated strawberries with *Aureobasidium pullulans* yeast before storage reduced 70% of decay caused by *Rhizopus spp.*

1.8.5.1 Biofumigant Fungus *Muscodor albus*:

The potential of the volatile – producing fungus *Muscodor albus* for controlling postharvest diseases of fresh fruit (apples and peaches) by biological fumigation was investigated. *In vitro* tests showed that *M. albus* volatiles inhibited and killed a wide range of storage pathogens belonging to species of *Botrytis, Colletotrichum, Geotrichum, Monilinia, Penicillium* and *Rhizopus.* Since *M. albus* has a sterile mycelium and does not require direct contact with the crops to being treated, it could be an attractive biological fumigant for controlling postharvest diseases. In wound – inoculated peaches, 24-72h fumigation with *M. albus* provided complete control of brown rot (*Monilinia fructicola).* The volatile profile of *M. albus* colonized grain was measured by gas chromatograph connected to a flame ionization detector (GC-FID) and showed that 2-methyl-1-guatanol and isobutyric acids were the major volatile compounds found (Mercier and Jimóenez, 2004).

1.8.5.2 *Candida guilliermondii*:

postharvest rot of peach fruits was studied *in vitro* and *in vivo* under different storage temperatures using *Candida guilliermondii*, to show if the presence of *C. guilliermondii* had any antagonistic effect against *R. stolonifer,* and what is the mode of action that *C. guilliermondii* may use it's biocontrol efficacy against *R. stolonifer. C. guilliermondii* at 5.0×10^8 CFU /ml of washed cells provided complete control of 5×10^4 spores /ml of *R. stolonifer* during storage at 25ºC for 4 days, at 15ºC for 7 days and at 3ºC for 30 days. Temperature had no significant effect on the biocontrol efficacy. Cell free culture filtrate of *C. guilliermondii* was not effective in preventing decay and resulted in even greater lesion diameter than those of

sterile distilled water at 3ºC. These results showed that competition for nutrient, but not antibiotic production plays a major role in the biocontrol capability of *C. guilliermondii* against *Rhizopus* rot of peach fruits. As the interval between wounding and inoculation with the pathogen increased from 0 to 72h, susceptibility of wounds to decay by *R. stolonifer* decreased from 100% of 0h to 5% of 4h and 0% of 24h, then increased to 10% of 48h and 40% of 72h (Fan et al., 2000).

1.8.5.3 *Pichia membranefaciens*:

A new yeast antagonist, *Pichia membranefaciens,* isolated from wounds of peach fruit, was evaluated for it's biocontrol capability against *R. stolonifer* on nectarine fruits at different temperatures and with other treatments. *P. membranefaciens* at 5×10^8 CFU/ml of washed cell suspension completely inhibited *Rhizopus* rot in nectarine wounds artificially inoculated with 5×10^4 spores per ml at 25, 15, and 3ºC. A culture filtrate of the yeast antagonist failed to provide any protection against *Rhizopus* rot in nectarine fruits compared with the washed cells, which supported the premise that competition for nutrients may play a major role in the biocontrol capability of *P. membranefaciens* against *R. stolonifer*. The importance of nutrient competition has been previously demonstrated with other antagonistic yeasts (Droby and Chalutz, 1994; Janisiewicz and Roitman, 1988). The yeast mixed with iprodione at 100 μg a.i. / ml gave better control of *R. stolonifer* than either yeast or iprodione alone. A solution of 20g $CaCl_2$ per liter enhanced the efficacy of *P. membranefaciens* (10^7 to 10^8 CFU/ ml) as an aqueous suspension. This is due mainly to the role of calcium in ameliorating physiological disorders and thus indirectly reducing pathogen activity (Conway et al., 1992). The role of calcium in resistance may be in

interfering with the activity of pectinolytic enzymes (Conway, 1984). Rapid colonization of the yeast in wounds was observed during the first 48h at 25ºC and 15ºC and then stabilized for the remaining time, as previously observed for other antagonistic yeasts (Piano et al., 1997; Mercier and Wilson, 1995). *P. membranefaciens* at 5×10^8 CFU/ml was effective when applied O to 72h before the pathogen, while at 1×10^8 CFU/ml, its efficacy was best when applied 24 to 48h prior to inoculation with *R. stolonifer*. However, it's efficacy was significantly reduced when the yeast was applied simultaneously with the pathogen, with disease incidence of 60% and lesion diameter of 37mm (Qing & Shiping, 2000). Some reports have demonstrated that a direct relationship exists between the population density of an antagonist and the efficacy of postharvest biological control treatment (Hong et al., 1998; Janisiewicz, 1988).

2. Trichoderma harzianum Rifai

2.1 Description

Trichoderma is among the most common saprophytic fungi. They all within the subdivision Deuteromycotina. Most *Trichoderma* strains have no sexual stage, but instead produce only asexual spores. For a few strains, the sexual stage is known; however, these do not include strains that have usually been considered for biocontrol purposes. The sexual stage, when found, is within the Ascomycetes in the genus *Hypocrea* (Monte, 2001). Colonies of *Trichoderma* grow rapidly and mature in 5 days. At 25ºC and on potato dextrose agar, the colonies are wooly and become compact in time. The color is white, yellow, or green cushions of sporuating filaments (De Hoog et al., 2000; St – Germain and Sumerbell, 1996). Colonies have either floccose or elliptical conidia, or tufted non – floccose globose.

Conidia are single – celled, usually green (typically 3 μ m in diameter) while typical fungal hyphae are 5 to 10 μm diameter. Conidia are smooth – or rough – walled and grouped in sticky heads at the tips of the phialides (hyaline, flask-shaped and inflated at the base). These clusters frequently get disrupted during routine slide preparation procedure for microscopic examination (Sutton et al., 1998; Kubicek and Harman, 1998). Taxonomy recently have gone from consisting of nine to at least 33 species. As an example, the best biocontrol species *T. harzianum* which is tolerant to stress imposed by nutrient scarcity, has been separated into an array of species *T. harzianum*, *T. inhamatum, T. longibrachiatum*, *T. atroviride* and T. *asperellum* (Hermosa et al., 2000; Monte, 2001; Hagedorn, 2004; Kuhls et al., 1999).

Morphological features of the conidia and phialides help in differentiation of these species from each other, the most secure way for most investigators to identify a species of *Trichoderma* is through DNA sequences. DNA sequences provided the much – needed independently derived data that would enable a better understanding of species of *Trichoderma* (Gams and Bissett, 1998; Kinderman et al., 1998; Kulhls et al., 1997).

2.2 Distribution

Trichoderma is widely distributed in plant material, decaying vegetation, wood, and in almost all soils. *Trichoderma* is able to grow in soils having a pH range from 2.5 – 9.5, although most prefer a slight to moderately acidic environment (Hagedorn, 2004). They have been considered to be at

least partially responsible for the control of 'suppressive soils', soils on which crops or trees are unaffected by a given pathogen (Agrios, 1997; Gams and Bissett, 1998). *T. harzianum* or *T. hamatum* identified as two of the usual soil species exert its effect by competing for nutrients and producing toxins against phytopathogenic species (Bora et al., 2000). Several new species of *Trichoderma* from eastern and Southeast Asian soils have been recently described by John Bissett and his collaborators (Bissett et al., 2003).

2.3 Host Plant

Trichoderma has a very wide host range, since *Trichoderma* species are found in almost all soils (Hagedorn, 2004). Once established in a host plant, vegetables, fruits, ornamentals, *Trichoderma* has been shown to co – exist for up to five years. It has been found that plant benefits correlate with increased population of *Trichoderma*. In other words, the more the better, whether it's larger doses or more frequent application – or both (Winter, 2000).

2.4 Pathogenicity

The most commonly reported biocontrol agent of *Trichoderma* is *T. harzianum*. However, this species was implicated as the cause of the green mould epidemic of commercially grown mushrooms in North America and Europe. The consequences of *T. harzianum* being a pathogen of such an economically important crop as mushrooms would have been disastrous to biological control (Seaby, 1996; Samuels and Doder, 2002; Savoie and Mata, 2003).

2.5 Role of *Trichoderma* in Controlling Fungi

2.5.1 Fungal Diseases Controlled by *T. harzianum*

Many *Trichoderma* strains have been identified as having potential applications in biological control, they are effective against a wide range of plant pathogenic fungi including: *Armillaria, Botrytis, Colletotrichum, Dematophora, Endothia, Fulvia, Fusarium , Chondrostereum, Fusicladium, Macrophomina, Monilia, Nectria, Phoma, Phytophthora, Plasmopara, Pseudoperospora, Pythium, Rhizoctonia, Sclerotinia, Sclerotium, Venturia, Verticillium*, and wood-rot fungi (Monte, 2001;Harman, 2000,Agrios, 1997;Batta, 2004;Sawant et al., 1995). Many recent studies have been demonstrated the effect of *T. harzianum* on postharvest diseases which cause fruit rot, for example, significant curative and preventive effect was provided by the antagonistic strain *Trichoderma* –Th1of *T. harzianum* against *Alternaria alternata* causing black fruit spot on persimmon fruits (Batta, 2001). This disease infects fruits in the field near the harvesting time, but develops during the postharvest period causing fruit rot (Batta, 2001). Another significant effect was obtained in controlling *Penicilluim expansum*, the causative fungus of blue mold on apples, through studying the effect of treatment with *T. harzianum Rifai* formulated in invert emulsion on postharvest decay of apple blue mold (Batta, 2004). Significant differences were obtained between means of percent reduction in decay –lesion diameter relative to sterile distilled water control in the treatments with formulated and non formulated conidia in invert emulsion (48.8%, 24.8% and 0.6%, respectively). Also, a significant long period of protection from *P. expansum* infection (up to 2 months) was also obtained when unwounded apple fruits were dipped for

30 second period in formulated *T. harzianum* conidia before being inoculated by *P. expansum* compared to the wounded fruits. This indicate the importance of this type of treatment in protecting apple fruits from blue mold infection for long time at postharvest stage without refrigeration (Batta, 2004). *T. harzianum* are also used in biological control of damping – off diseases caused by *Pythium* species (Figure 3) and *Rhizoctonia* (Figure 4). (Omarjee et al., 2001; Agrios, 1997; Harman, 1998; Biswas, 1999; Dutta and Das, 1999).

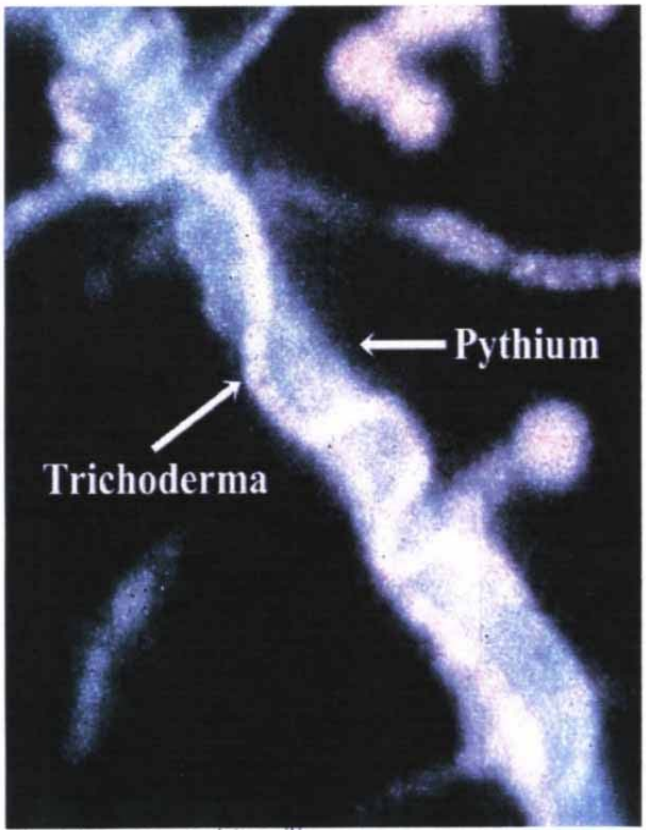

Fig. no. 3: Mycoparasitism by a *Trichoderma* strain on the plant pathogen (*Pythium*) on the surface of pea seed. Used with permission of American Phytopathological Society (Hubbard et al., 1983. Phytopathology 73: 655 – 659).

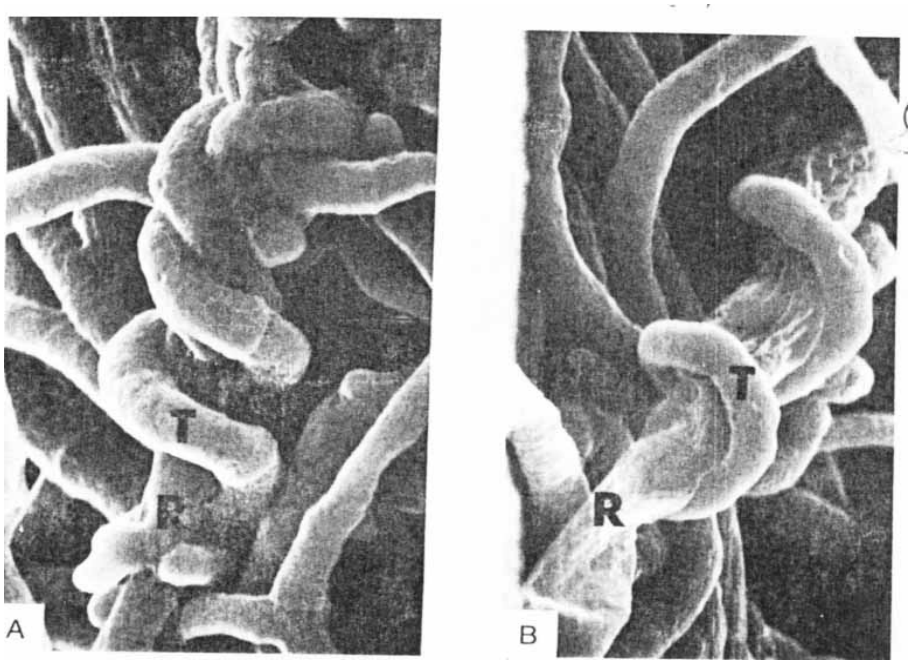

Fig. no. 4: Effect of the biological control agent T*richoderma harzianum* on the plant pathogenic fungus *Rhizoctonia solani*. (A) Hyphae of *Trichoderma* (T) forming dense coils and tightly encircled hyphae of *Rhizoctonia* (R) within 2 days after inoculation (Magnification: 6000X.) (B) by 6 days after inoculation, *Rhizoctonia* hyphae show loss of turgor and marked cell collapse, whereas *Trichoderma* hyphae continue to look normal (Magnification: 5000X.) [From Benhamou and Chet (1993), Phytopathology 83, 1062 – 1071.].

Botrytis cinerea is another postharvest disease that causes grey mold on apple, it was biologically controlled by *T. harzianum Rifai* formulated in invert emulsion (Batta, 2003; Batta, 1999). Formulated *T. harzianum* conidia in invert emulsion had a significant preventive effect against *B. cinarea* on wounded apple fruits compared to non – formulated *T. harzianum* conidia and control treatments. The diameter of typical *Botrytis* lesions on treated apple fruit was significantly reduced. In addition, the application of formulated *T. harzianum* conidia inhibited *Botrytis* sporulation (no production of conidia) on the surface of typical Botrytis lesions. Dipping healthy apple fruit in formulated conidia of *T. harzianum*, followed by inoculation with *B. cinerea* by spraying a conidial suspension

of the pathogen on the treated fruits, protected treated fruits from infection with *B. cinerea* for 16 days, when using micro – wounded fruits. According to Batta (2003), formulation of invert emulsion had low viscosity and contained both coconut and soybean oil with two emulsifiers (oil – soluble emulsifier Tween 20 and water-soluble emulsifier Dehymuls K). The invert emulsion produced was stable and compatible with the Th2 strain of *T. harzianum*. Conidia in this formulation remained viable much longer than non – formulated conidia of the same strain held at 20 $\pm$ 1 °C and 30% ambient RH. The ingredients of the invert emulsion especially oils and emulsifiers are safe and not toxic to apple fruit. These ingredients are also likely to be non – toxic to humans as they are also used as food additives and in the manufacture of cosmetics (Batta, 2003).

2.5.2 The Commercial Products of *T. harzianum*

2.5.2.1 Types, formulation and methods of application of commercial strains products: These versatile fungi are used commercially in a variety of types, including the following:

A) Foods and textiles: *Trichoderma spps*. Are highly efficient producers of many extracellular enzymes. They are used commercially for production of cellulases and other enzymes that degrade complex polysaccharides. They are frequently used in the food and textile industries for these purposes. The enzymes are also used in poultry feed to increase the digestibility of hemicelluloses from barley or other crops.

B) Plant growth promotion: for many years, the ability of *Trichoderma* spps to increase the rate of plant growth and development, including,

their ability to cause the production more robust roots has been known. It was found that one strain increases the number of even deep roots (at as much as a meter below the soil surface). These deep roots cause crops, such as corn, and ornamental plants such as turfgrass, to become more resistant to drought. Perhaps even more importantly, recent research indicates that corn whose roots are colonized by *Trichoderma* strain T- 22 require about 40% less nitrogen fertilizer than corn whose roots lack the fungus.

C) Biocontrol agents: *Trichoderma* spps are used, with or without legal registration, for control of plant diseases (Harman, 1998). It has been investigated as biological control agent for over 70 years (Samuels, 1996), but only relatively recently have strains become commercially available on the open market. Some of their commercial products are listed in Table 1 (Monte, 2001; Fravel, 2002; Harman, 2000).

Table no. 1: Commercial products of *Trichoderma* spp. used as a biocontrol agents.

Commerical name	Biocontrol agent / strain	Pathogen / Disease and treated crops	Formulation	Application method
Binab - T	Various *Trichoderma* products	With diseases: root rot, decay in tree wounds. Crops, flowers, fruits, ornamental, and vegetables	Wettable powder and pellets	Spray, mixing with water and painting on tree wounds.
Bio – Fungus	*Trichoderma spp.*	*Sclerotinia, Phytophthora, Rhizoctonia solani, pythium spp, Fusaruim, Verticillium*. Crops: flowers, trees, vegetables.	Granular, wettable, powder, sticks and crumbles	Applied after fumigation, incorporated in soil; sprayed or injected
Root Pro, Root Protato	*T. harzianum*	*Rhizoctonia solani, Pythuim spp, Fusarium spp, and Sclerotuim rolfsii.* Crops: flower.	Fungal spores mixed with peat and other organic material	Agents mixed with growing media at time of seeding.
Root Shield (bio –Trek, T-22G)	*T. harzianum Rifai* strain KRL – AG2 (T-22)	*Pythium spp., Rhizoctonia solani, Fusaruim spp*. Crops: trees, shrubs, transplants, all ornamentals, tomato, cabbage, cucumber.	Granules, wettable powder	Granules mixed with soil. Powder mixed with water and added as a soil drench.
Triaco	*T. viride*	*Rhizoctonia spp., Pythium spp., Fuasruim spp*., root rot, seedling rot, collar rot, damping off, *Fusarium* with crop: oil seeds, soybean, cotton, chickpeas, tobacco, coffee,and vegetables	Powder	Dry or wet seed, tuber, or set dressing or soil drench, spread / broadcast over field
Trichopel, trichoject.	*T. harzianum* and *T. viride*.	*Armillaria, Fusarium, Botryosphaeria, Chondrosternum.*	Powder	Soil drench

Other commercial products of *Trichoderma* which is under registration or on the open market are: Trichodex (Israel) against *Botrytis* of vegetables and grapevines. Soil Gard (USA), Supresivit (Denmark), Tusal (Spain), and *Trichoderma* 2000 (Israel) are used against damping – off diseases caused by *Pythium*, *Rhizoctonia spp.* (Monte, 2001), and *Macrophomia phaseolina* (Adekunle et al., 2001) as a seed treatment.

2.5.2.2 Tolerance assessment of using *T. harzianum* commercial strains products:

An exemption from the requirement of a tolerance for residues of *T. harzianum Rifai* strain T-39 on all food commodities when used as ground and certain foliar applications. This regulation eliminates the need to establish one maximum permissible level for residues of *T. harzianum Rifai* strain T-39. An exemption had been granted since testing of the biofungicide showed no toxic effects. Another exemption from the requirement of a tolerance for residues of the microbial pesticide active ingredient T. hKRL - AG2, known as strain T- 22 when used as seed treatment, on cuttings and transplants, or as soil application. In a study of the biological efficiency by *Trichoderma* on the germination of winter wheat grain, the isolates *Trichoderma* also not toxic for germinating plants and in some cases they stimulated the growth of above ground and underground wheat organs (Michalikova and Kohacik, 1992).

2.5.3 Biological Activity and Mode of Action

Trichoderma spp. have evolved numerous mechanisms for attacking other fungi and for enhancing plant and root growth. Several new general methods for biocontrol and for enhancement of plant growth have recently

been demonstrated, and it is now clear that there must be hundreds of separate genes and gene products involved in the following processes (Agrios, 1997; Viñas, 2004; Monte, 2001), known as modes of action:

1) Mycoparasitism: relies on the recognition, binding and enzymatic disruption of the host - fungus cell wall and death of the pathogen by direct parasitism (Goldman and Goldman, 1998; Monte, 2001).

2) Nutrient or site competition: for example; sugars such as maltose, sucrose and glucose, have been suggested to play a role in the bicontrol of moulds by yeasts against diseases (Filonow, 1998).

3) Antibiosis: direct toxic effects on the pathogen by antibiotic substances released by the antagonist. The concentrations of the antibiotic (S) in solution (crude filtrates and crude antibiotic solutions) will be estimated from the probit regression line of inhibition of germination of spores – log concentration of antibiotic as described by Madrigal et al. (1991). This probit of response – log concentration curve will be calculated from the result of the relative toxicity of different concentration levels of the pure antibiotic on the germination of spores of every pathogen by following the probit analysis method (Finney, 1971). From these curves the effective doses (ED) of 50% inhibition for both the germination and the germ tube growth will be calculated.

4) Production of volatile compounds: volatile compounds from the biological control agents can be an important factor of the inhibitory mechanism, especially under closed storage condition, such as ethylene, released by the metabolic activities of the antagonist. Effects

will be recorded as changes in radial growth, spore formation and CFU's of the target fungi such as, *Penicillium expansum, Botrytis cinerea, Rhizopus stolonifer* (Viñas, 2004). If inhibition by volatile compounds is indicated, this will be confirmed by investigating whether the effects can be removed by continuous ventilation. For biological control agents showing a high degree of inhibition through the gas phase a tentative identification of volatile agents will be done through gas – chromatography, using known controls.

5) Induced host resistance: a state of enhanced defensive capacity developed by a plant or plant part when appropriately stimulated and

can occur naturally as a result of limited infection by a pathogen. Resistance that has been occurred from genes of *T*. *harzianum* inserted

into plants was demonstrated in (Figure 5) (Harman, 2000).

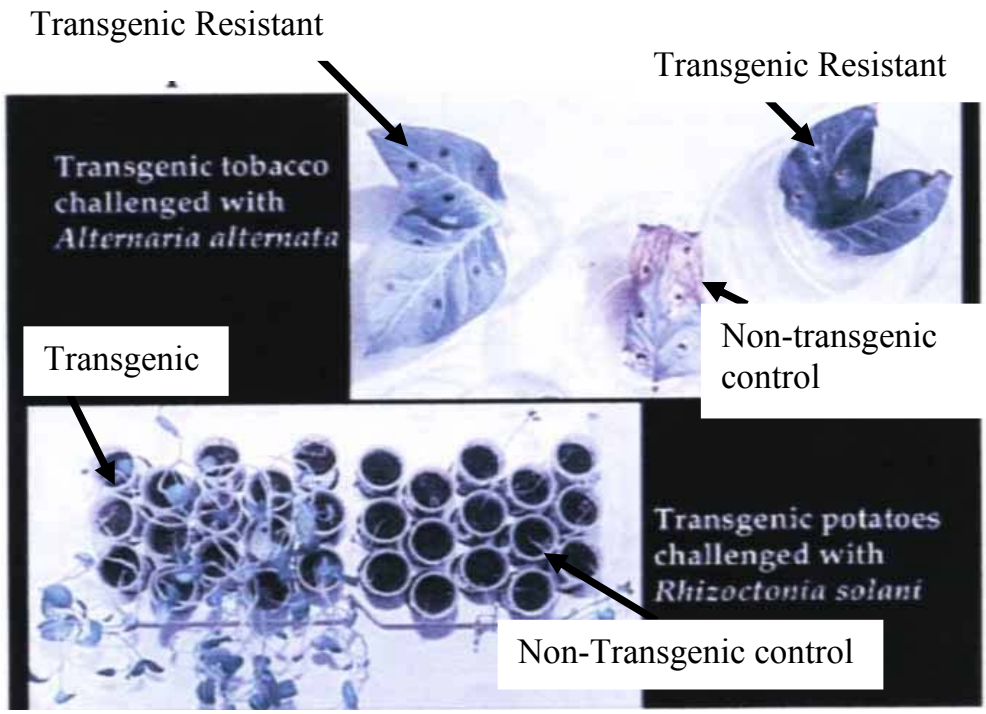

Fig. no. 5: Some biocontrol genes from *T. harzianum* have been inserted into plants, where they provide resistance to several diseases. Tobacco and potatoes, shown in this figure, were transformed to express the fungal endochitinase gene, which resulted in high levels of resistance to *Alternaria alternat*a (tobacco) and *Rhizoctonia solani* (potato). Data are from Lorito et al., 1998. Proc. Am. Sci. USA 95: 7860 – 7865.

6) Solubilization and sequestration inorganic nutrients: production of hydrolytic enzymes through direct interactions between the biocontrol agent and the pathogen (Viñas, 2004; Altomare et al., 1999).

A major part of *Trichoderma* antifungal system consists of a number of genes encoding for an astonishing variety of secreted lytic enzymes, including endochitinases, N-acetyl- ß -glucoseminidases, chitin 1,4- ß -chitobiosidases, proteases, endo- and exoglucan ß-1,3- glucosidases (Haran et al.,1996a) endoglucan ß -1, 6- glucosidases, lipases, xylanases, mannanases, pectinases, pectin lyases, amylases, phospholipases, RNases,

and DNases (Haran et al., 1996b; De La Cruz et al., 1992; Lorito et al., 1994). Particularly useful for biocontrol applications are chitinolytic and glucanolytic enzymes because of their ability to efficiently degrade the cell wall of plant pathogenic fungi by hydrolyzing biopolymers not present in plant tissues. A substantial amount of work performed mainly during the past 7 years has indicated that cell-wall- degrading enzymes (CWDEs) from *Trichoderma* strains have great potential in agriculture as active components in new fungicidal formulations (Benitez et al., 1998). This is because purified CWDEs from different strains of *T. harzianum* are highly effective in inhibiting spore germination and mycelial growth in a broad range of pathogens. In contrast to plant enzymes, chitinases and glucanases form *Trichoderma* can degrade not only the immature wall at hyphal apices but also the strong chitin-glucan complexes of mature cell walls, as well as survival structures such as sclerotia and chlamydospores, which reduces not only disease symptoms but also pathogen spread. In particular, enzymes absent from plants such as ß -1, 6- glucanses can degrade important fungal cell-wall structures such as ß -1, 6- glucans by linking chitin or ß -1, 3- glucans to cell – wall proteins. The antifungal activity of *Trichoderma* CWDEs can be enhanced synergistically by combining enzymes with different lytic activities (such as exo – and endochitinases and / or glucanases). For instance, a combination of an endochitinase, an exochitinase ands ß -1, 3- glueanase purified from *T. harzianum* has an effective dose (ED50) on *Botrytis* of about 1ppm, which is comparable to the effective dose of most chemical fungicides. Fungicides synergistic with the *Trichoderma* CWDEs include several compounds used for chemical control of plant diseases, such as azoles, benzimidazoles and pyrimidines. Tests show that *Trichoderma* chitinases and glucanases have no effect on

the plant even when relatively large quantities are injected into plant tissues. CWDEs are not harmful to humans or animals, as indicated by EPA tests for registration of strains of *Trichoderma* for use as biocontrol agents in the United States, and they degrade into environmentally friendly residues. CWDEs are particularly suited to postharvest control. Low – temperature controlled storage conditions will favor these applications as the level of enzyme activities will be more easily predicted than in the greenhouse or the field. Purified CWDEs or mixtures of CWDEs with high antifungal activity obtained from *Trichoderma* culture filtrates can be included in commercial formulations since they are easily characterized, stable, resistant to drying, freezing, temperatures up to 60ºC (Monte, 2001).

Chapter Three

Materials and Methods

1. Materials

1.1 Plant Materials

Three types of fruits were picked at harvesting stage to be used in the experiments. They were: apple (*Malus pumila*) variety: "Golden Delicious", pear (*Pyrus communis*) variety: "Spadona", peach (*Prunus persica*) variety: "Fayette". Firstly, all fruits were washed with tap water and disinfected superficially with sodium hypochlorite (0.025%) before rinsing them three times with sterile distilled water and then putting in closed plastic cans to be protected from contamination during the experiments and to obtain humid chamber conditions.

1.2 Fungal Materials

Pure fungal cultures of *Trichoderma harziarum Rifai* (strain: Th2) were used in the experiments. They were obtained from laboratory of plant protection (An – Najah National University), *Rhizopus stolonifer* (strain: RS1) isolated by the same laboratory from naturally infected peach fruits. The first strain was subcultured on oat meal agar (OMA) medium plates and the second one was subcultured on potato dextrose agar (PDA) medium plates.

1.3 Chemical Materials

Water – soluble wax (Dehymuls K®), Glycerine, plant oils (coconut and soybean oils), oil – soluble emulsifier (Tween 20), sterile distilled water, oat meal agar and potato dextrose agar culture media, sodium hypochlorite for disinfection.

2. Methods

2.1 Technique of Culturing the Fungi and Preparation of Spore Suspension

The strains of *Trichoderma harzianum* and *Rhizopus stolonifer* were subcultured on (OMA) and (PDA) culture media, respectively, under aseptic conditions. The plates were incubated at 20 $\pm$ 2ºC and 16 hours of illumination per day (growth chamber conditions) for 10-14 days in order to obtain enough quantities of fungal conidia or sporangiospores for inoculation. Fungal growths on plate surface were scraped with sterile scalpel to make the conidia or spores suspending into sterile distilled water poured into the plate, then the suspension was sieved through 75 μm mesh then counted using haemocytometer.

2.2 Techniques of Invert Emulsion Preparation and *Trichoderma harzianum* Introduction

The ingredients of the invert emulsion used in our experiments to formulate *T. harzianum* conidia (strain: Th2) were similar to the ingredients used in the research conducted by (Batta, 2004). Accordingly, it contains the following ingredients (w/w): sterile distilled water (45.25%), glycerine (4.00%), water – soluble wax or Dehymuls K® (0.75%), Tween 20 (2.50%), and a mixture of 19.00% coconut oil + 28.50% soybean oil (Batta, 2004). The fungus (*T. harzianum*) was introduced as conidia into the invert emulsion described above according to the technique developed by (Batta, 2004). The concentration of introduced *T. harzianum* conidia in the invert emulsion was titrated at 2.6×10^8 conidia / ml.

2.3 Biological Efficacy Evaluation Technique of *Trichoderma harzianum*

For testing biological efficacy of *T. harziannum* against *Rhizopus* soft rot on apple, pear and peach fruits, four types of treatments were used:

1. *Rhizopus* + *Trichoderma* (formulated in invert emulsion described above).
2. *Rhizopus* + *Trichoderma* (suspended in sterile distilled water),
3. *Rhizopus* + sterile distilled water as control,
4. *Rhizopus* + invert emulsion (blank formulation).

The effect of these four treatments on the development of typical lesion to *Rhizopus* soft rot on the three types of fruits (Figures 6, 7, and 8) was tested at the same time of pathogen inoculation on wounded and unwounded fruits. For this, 25 - µl droplet taken from formulated *T. harzianum* conidia in invert emulsion (concentration = 2.6×10^8 conidia/ml) or unformulated *T. harzianum* conidia (suspended in sterile distilled water at a concentration = 9.6×10^8 conidia/ml) was applied per fruit. The same droplet size (25 µl) was also applied from sterile distilled water (control treatment) or blank formulation of invert emulsion for comparison of treatment effect. Inoculation of *R. stolonifer* (strain RS1) on the different types of fruits was done by putting 25- µl droplet of the pathogen suspension (concentration = 4.5×10^6 sporangiospores /ml) per wound. Incubation of fruits after inoculation and treatment was carried out at 20 $\pm$ 2ºC or 30 $\pm$ 2ºC in closed plastic cans at a rate of 1 fruit / can. Assessment of treatment effect was done by measuring the disease lesion diameter formed around the wounds

after three or four days of inoculation and treatment. The means of disease – lesion diameter in each type of treatment was calculated.

Fig. no. 6: Typical symptoms of *Rhizopus stolonifer* on apple.

Fig. no. 7: Typical symptoms of *Rhizopus stolonifer* on peach.

Fig. no. 8: Typical symptoms of *Rhizopus stolonifer* on pear.

2.4 Determination of Protection Period from Infection with *Rhizopus* soft rot After *T. harzianum* Treatment

This is done on microwounded fruits of apple, pear, and Peach in comparison with the unwounded fruits.The microwounds were done on the fruits by sterile needles. Two types of treatments were used:

1. Formulated *T. harzianum* on microwounded fruits inoculated with *R. stolonifer*.

2. Control treatment with blank formulation of invert emulsion on microwounded fruits inoculated with *R. stolonifer*.

The same types of treatment were applied on unwounded fruits for comparison. To carry out these treatments, constant volume of 2 ml of formulated *T. harzianum* conidia (2.6×10^8 conidia / ml) was sprayed per fruit using small hand sprayer. The same volume (2 ml) was also sprayed per fruit in the control treatment with blank formulation of invert emulsion. Inoculation of *R. stolonifer* was carried out by spraying 1 ml of *R. stolonifer* spore suspension (4.5×10^6 sporangiospore / ml) per fruit. Microwounds were made by needle pricks. Incubation of fruits after inoculation and treatment was conducted at 20 ± 2ºC in closed plastic cans (one per can) until evaluation. The minimum protection period from infection with *R. stolonifer* on each fruit type after treatment with *T. harzianum* formulated in invert emulsion was determined by calculating the time from inoculation and treatment until appearance of first disease lesion on the fruit surface in each fruit type.

2.5 Experimental Design and Analyses of Data

The completely randomized design (CRD) was used in designing the experiments with four experimental treatments. Each treatment was replicated four times representing four fruits. Mean lesion diameter in each treatment was calculated for comparison and analysis. Data were analysed using statistical program for carrying out ANOVA, in addition to mean separation using Scheffee test.

Chapter Four

Results

1. Effect of Treatment with *Trichoderma harzianum* on *Rhizopus* soft rot on Peach Fruits

There were significant differences ($P \leq 0.05$) between mean lesion diameters of *R. stolonifer* in different treatments at 20 ± 2°C, whereas no significant differences between mean lesion diameters of *R. stolonifer* on the different treatments at 30 ± 2°C (Table 2). Treatment with *R. stolonifer* + formulated *Trichoderma* in invert emulsion was significantly different from treatments with *R. stolonifer* + sterile distilled water as control treatment. The mean lesion diameter decreased significantly from 51.75mm to 36.50mm. This demonstrated the efficacy of treatment with formulated *Trichoderma* in invert emulsion. However, no significant differences were observed between other treatments at the same temperature. This demonstrated that non formulated *Trichoderma* (*Trichoderma* in sterile distilled water) did not decrease significantly the mean lesion diameter compared to the control. So, no effect of treatment with blank formulation of invert emulsion, therefore the effectiveness of treatment effect was attributed to the formulated *Trichoderma* in invert emulsion formulation (Table 2).

Table no. 2: *Rhizopus* soft rot – lesion diameter in mm developed on peach fruit 3 days after inoculation and treatment.

Treatment	Temperature	
	20 ± 2°C	30 ± 2°C
Rhizopus + *Trichoderma* (formulated in IE)	36.50 a*	0.00 a*
Rhizopus + *Trichoderma* (suspended in water)	40.00 ab	0.00 a
Rhizopus + Sterile distilled water as control	51.75b	10.50 a
Rhizopus + IE (blank formulation as control)	49.50 ab	6.75 a

* means in each column followed by different letters are significantly different at $P \leq 0.05$ using ANOVA and Scheffe test, IE: invert emulsion.

2. Effect of Treatment with *Trichoderma harzianum* on *Rhizopus* soft rot on Pear Fruits

There were significant differences ($P \leq 0.05$) between mean lesion diameters of *R. stolonifer* in different treatments at 20 ± 2°C, whereas no significant differences ($P \leq 0.05$) between mean lesion diameters of *R. stolonifer* on the different treatments at 30 ± 2°C (Table 3). Treatment with *Rhizopus* + formulated *Trichoderma* in invert emulsion was significantly different from treatment with *Rhizopus* + sterile distilled water as control treatment. The mean lesion diameter decreased significantly from 26.25mm to 8.0 mm. This demonstrated the efficacy of treatment with formulated *Trichoderma* in invert emulsion. However, no significant differences were observed between other treatments at the same temperature. This demonstrated that non – formulated *Trichoderma* (*Trichoderma* in sterile distilled water) treatments had no significant reduction in mean lesion diameter compared to the control (blank formulation of invert emulsion). So, no effect of treatment with blank formulation of invert emulsion. Therefore the effectiveness of treatment was attributed to the formulated *Trichoderma* in invert emulsion formulation (Table 3).

Table no. 3: *Rhizopus* soft rot – lesion diameter in mm developed on pear fruit 3 days after inoculation and treatment.

Treatments	**Temperatures**	
	20 ± 2°C	30 ± 2°C
Rhizopus + *Trichderma* (formulated in IE)	8.00 a*	4.00 a*
Rhizopus + *Trichderma* (suspended in water)	9.75 ab	4.50 a
Rhizopus + S.D water as control	26.25 b	7.00 a
Rhizopus + IE (blank formulation as control)	22.00 b	6.75 a

*means in each column followed by different letters are significantly different at $P \leq 0.05$ using ANOVA and Scheffee test, IE: invert emulsion.

3. Effect of Treatment with *Trichderma harzianum* on *Rhizopus* soft rot on Apple Fruits

There were significant differences ($P \leq 0.05$) between mean lesion diameters of the different treatments at 30 $\pm$ 2°C, whereas no significant differences ($P \leq 0.05$) between mean lesion diameters of the different treatments at 20 $\pm$ 2°C (Table 4). Treatment with *Rhizopus* + formulated *Trichoderma* in invert emulsion which has 9.75 mm as mean lesion diameter was significantly different from all other treatments especially the treatment with *Trichoderma* suspended in water which has 49.5 mm as mean lesion diameter, treatment with sterile distilled water as control (73.25 mm) and treatment with blank formulation (Rhizopus + IE) as control treatment (75.75 mm) (Table 4). This demonstrated the efficacy of treatment with formulated *Trichderma* in invert emulsion compared to other treatments. No significant differences were observed between *Rhizopus* + *Trichderma* suspended in water and *Rhizopus* + sterile distilled water as control although *Rhizopus* + *Trichderma* suspended in water decreased the mean lesion diameter from 49.50 mm to 73.25 mm. However, there were significant differences between *Rhizopus* + *Trichderma* suspended in water and blank formulation of IE as control, and also *Rhizopus* + *Trichderma* suspended in water significantly decreased the mean lesion diameter from 75.75 mm to 49.50 mm (Table 4).

Table no. 4: *Rhizopus* soft rot – lesion diameter in mm developed on apple fruits 3 days after inoculation and treatment.

Treatments	Temperatures	
	20 ± 2°C	30 ± 2°C
Rhizopus + *Trichderma* (formulated in IE)	7.75 a*	9.75 a*
Rhizopus + *Trichderma* (suspended in water)	10.75 a	49.50 b
Rhizopus + S.D water as control	26.00 a	73.25 cb
Rhizopus + IE (blank formulation as control)	19.75 a	75.75 cd

* Means in each column following by different letters are significantly different at $P \leq 0.05$ using ANOVA and Scheffee test, IE: invert emulsion.

4. Protection Period from Infection of *Rhizopus* on Different Types of Fruits after Treatment with *T. harzianum*

The longest minimum protection period against *Rhizopus stolonifer* infection was obtained on unwounded apple fruits treated with formulated *Trichoderma* in invert emulsion. It was 100 days, but it was the shortest on wounded apple fruits treated with blank formulation of invert emulsion (28 days). This indicates that the fungus protected the fruits 72 days more than the control (Table5). Also, the longest minimum protection period against *R. stolonifer* infection was obtained on unwounded peach fruits treated with formulated *Trichoderma* in invert emulsion. It was 14 days, but it was the shortest on wounded peach fruits treated with blank formulation of invert emulsion (3 days). This indicates that the fungus protected the fruits 11 days more than the control (Table 5). The longest minimum protection period against *R. stolonifer* infection was obtained on unwounded pear fruits treated with formulated *Trichoderma* in invert emulsion. It was 18 days, but it was the shortest on wounded pear fruits treated with blank formulation of *Trichoderma* in invert emulsion (3 days). This indicates that the fungus protected the fruits 15 days more than the control.

Comparison of three types of fruits indicated that the biggest minimum protection period was obtained on apple (72 days) and the smallest minimum protection period was on peach (11 days) (Table5).

Table no. 5: Minimum protection period in days for the treatment of *Rhizopus* soft rot on apple, peach, and pear after inoculation and treatment at 30 ± 2°C.

Fruit type	**Wounded fruits (1)**		**Unwounded fruits (1)**	
	R. stolonifer + formulated *Trichoderma* (2)	*R. stolonifer* + Blank formulation of IE (2)	*R. stolonifer* + formulated *Trichoderma* (2)	*R. stolonifer* + Blank formulation of IE (2)
Apple	87 days	28 days	100 days	80 days
Peach	5	3	14	11
Pear	8	3	18	16

(1): No of replicates = 4 represent 2 treatments of (wounded, unwounded) on 2 fruits.
(2): Lesions appeared at end of protection period range from 10.7 mm – 25 mm according to fruit type.

Chapter Five

Discussion and Conclusion

The control of *Rhizopus* soft rot is very important since it is one of the most serious postharvest diseases. Chemical fungicides which can control the disease are very few but effective such as Iprodione, Thiabendazole, Dichloron, Imazalil,and Benomyl. Many of the former products that were used to control postharvest diseases are no longer permitted to be used because of concerns with residues and possible toxic effects (Homles and Eckert, 1999). Large efforts are now underway to locate the appropriate biological control agents including antagonists. Biological control including use of bacteria (Wilson et al., 1987; Bonaterra et al., 2003; Nunes et al., 2001; Holmes, 2005), fungi and yeasts (Mercier and Jiménez, 2004; Qing et al., 2000; Conway, 1984). In 1982 Papavizas has begun to select fungicide – resistant strains of *Trichoderma* fungi for possible use in integrated control programmes, since these fungi are effective against a wide range of plant pathogenic fungi including: *Verticillium, Botrytis, Pythium*, *Fuzarium* and others (Monte, 2001; Harman, 2000; Sawant et al., 1995). *Trichoderma spp*. have evolved numerous mechanisms for attacking other fungi, these processes known as modes of action which are summarized in mycoparasitism, nutrient or site competition, antibiosis, production of volatile compounds, solubilization and sequestration (Agrios, 1997; Vinãs, 2004; Monte, 2001). In this study, *T. harzianum* was used to assess its biological effectiveness against *Rhizopus* soft rot caused by the fungus *Rhizopus stolonifer* on three types of fruits (apple, pear, peach) at two temperatures: 20 $\pm$ 2ºC, and 30 $\pm$ 2ºC under laboratory conditions. The laboratory experiment indicated that when using formulated form of *T. harzianum* in invent emulsion, the mean lesion diameter of the disease on the three types of infected fruits with *Rhizopus* soft rot decreased

significantly after 3 days following inoculation and treatment.This demonstrated the efficacy of treatment with formulated *Trichoderma* in invent emulsion. A similar significant effect was obtained in previous study in controlling *Penicillium expansum* on apples through studying the effect of treatment with *T. harzianum Rifai* formulated in invert emulsion on postharvest decay of apple blue mold (Batta, 2004). Significant differences were obtained between means of percent reduction in decay – lesion diameter treated with formulated and non – formulated conidia of *T. harzianum* relative to sterile distilled water (control treatment). This could be explained by the disruption of the host fungus cell wall by direct parasitism of *Trichoderma* (Goldman and Goldman, 1998; Monte, 2001), or by competing on the site or nutrient of the host fungus cell (Filonow, 1998), or by producing toxic substances or volatile compounds as ethylene, released by the metabolic activities of the antagonist, that may change the radial growth, spore formation and CFU's of the target fungi (Vinãs, 2004). The present study also measured the minimum protection period from infection with *Rhizopus* on the three types of fruits after treatment with *T. harzianum*. The longest minimum protection period was obtained on unwounded apple (100 days), but it was the shortest on unwounded peach (14 days) and it was intermediate on unwounded pear (18 days). This may be explained by that *Rhizopus* is a strictly wound – parasite, so it can penetrate host tissues only through bruises and fresh wounds, especially in the fields through harvesting, handling, insects, and rodents (Barnes, 1979; Lisker et al., 1996). The smallest minimum protection period that was obtained in the present study on peach was attributed mainly to its soft fleshy nature. This is in agreement with the results of a pervious study carried out on *P. expansum* infection on unwounded apple fruits (Batta,

2004) when these fruits were dipped for 30 – second period in formulated *T. harzianum* conidia befor being inoculated by *P. expansum* compared to the wounded fruits. This indicates the importance of this type of treatment in protecting apple fruits from blue mold infection for long time at postharvest stage without refrigeration (Batta, 2004).

In conclusion, since the present study constitutes the first trial to use the antagonistic fungus *T. harzianum* (especially in formulated from using invert emulsion) against *R. stolonifer*, it may be considered as the first step towards using *T. harzianum* in biocontrol of *R. stolonifer* commercially or, at least, in the disease management programs. However, further experiments are recommended to be conducted before this commercial use such as confirmation of the fungus efficacy against *R. stolonifer* under natural conditions of fruit storage and marketing; the side – effects (if any) of the formulation when applied under natural conditions should be also investigated.

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Appendices

Appendix A

Table: *Rhizopus* soft rot – lesion diameter in mm developed on peach fruit 3 days after inoculation and treatment at 20 ± 2°C.

Treatments	Replicates (Lesion diameter in mm)				Mean
	R_1	R_2	R_3	R_4	
1.*Rhizpous*+*Trichoderma* (formulated in IE).	40	42	29	35	36.5^{a*}
2.*Rhizpous*+*Trichoderma* (suspended in water)	44	47	35	34	40^{ab}
3.*Rhizpous*+ S.D.W as control	50	54	48	55	51.75^{b}
4.*Rhizpous*+IE(blank formulation as control)	54	60	39	45	49.5^{ab}

* Means followed by different letters are significantly different at P≤0.05 using ANOVA and scheffee test, IE: invert emulsion.

$C = Y^2../rt$

$$\frac{(40+42+...45)^2}{4\times4} = \frac{505521}{16} = 31595.0625$$

$$SS\ \ total = \sum y_{ij^2} - C = (40)^2 + ...(45)^2 - C = 32763 - 31595.0625 = 1167.93$$

$$SS\ \ treatment = (\sum Y_{ij})^2 / r - C = \frac{128969}{4} - 31595.0625 = 647.1875$$

$$SS\ error = SS\ \ total - SS\ treatment = 1167.9 - 647.187 = 520.742$$

H_0: $M_1 = M_2 = M_3 = M_4$

H_1: at least two means are different.

ANOVA table

Source of Variation	SS	dF	Ms	Fc
Treatment	647.1875	3	215.729	4.97
Error	520	12	43.39	
Total	1160	15		

F, 05(3.12) = 3.49. Since Fc > Ftabulated, we rejest H_0 so at least two means are different and it is significant.

According to Scheffee test:

1. H_0: $M_1 = M_2$, H_1: $M_1 \neq M_2$. We reject H_0 if:

$$/\overline{X_1} - \overline{X_2}/ \geq \sqrt{MSE.(K-1)\ .\ F\alpha\ (K-1\ .\ n-k)\ .\ \frac{1}{n_1}+\frac{1}{n_2}}$$

$$/63.5 - 40/ \geq \sqrt{43.39 \times 3 \times 3.49 \times \frac{1}{4}+\frac{1}{4}}$$

$$3.5 \geq 15.07\ [We\ don't\ reject\ H_0,\ so\ M_1 = M_2]$$

2. H_0: $M_1 = M_3$

H_1: $M_1 \neq M_3$

We reject H_0 if:

$$/\overline{X_1} - \overline{X_3}/ \geq \sqrt{MSE\ .(k-1)\ .\ Fx\,(k-1)\ .F\alpha\ (k-1,\ n-k)\ .\ \frac{1}{n_1}+\frac{1}{n_2}}$$

$$/36.5 - 51.75/ \geq \sqrt{43.39 \times 3 \times 3.49 \times 0.5}$$

$15.25 \geq 15.07,$ *we reject* H_0, *So* $M_1 \neq M_3$

3. H_0: $M_1 = M_4$, H_1: $M_1 \neq M_4$

We reject H_0 if:

$$/\overline{x_1} - \overline{x_4}/ \geq 15.07$$

$/ 36.5 - 49.5 / \geq 15.07$

$13 \geq 15.07$ we don't reject H_0, so $M_1 = M_4$.

4. H_0: $M_2 = M_3$, H_1: $M_2 \neq M_3$. We reject H_0 if:

$$/\overline{x_2} - \overline{x_3}/ \geq 15.07$$

$/40 - 51.75/ \geq 15.07$

11.75 ≥ 15.07, we don't reject H_0, so $M_2 = M_3$.

5. H_0: $M_2 = M_4$, H_1: $M_2 \neq M_4$. We reject H_0 if:

$$/\overline{x_2} - \overline{x_4} / \geq 15.07$$

/40 – 49.5/ ≥ 15.07

9.5 ≥ 15.07. We don't reject H_0, so $M_2 = M_4$.

6. H_0: $M_3 = M_4$, H_1: $M_3 \neq M_4$. We reject H_0 if:

$$/\overline{x_3} - \overline{x_4} / \geq 15.07$$

/51.75 – 49.5/ ≥ 15.07

2.25 ≥ 15.07. We don't reject H_0, so $M_3 = M_4$.

Appendix B

Table: *Rhizopus* soft rot – lesion diameter in mm developed on peach fruit 3 days after inoculation and treatment at 30 ± 2˚C.

Treatments	Replicates (Lesion diameter in mm)				Mean
	R_1	R_2	R_3	R_4	
1.*Rhizpous*+*Trichoderma* (formulated in IE).	0	0	0	0	0^a
2.*Rhizpous*+*Trichoderma* (suspended in water)	0	0	0	0	0^a
3.*Rhizpous*+ S.D.W as control	0	17	0	25	10.5^a
4.*Rhizpous*+IE(blank formulation as control)	0	0	10	17	6.75^a

* Means followed by different letters are significantly different at P≤0.05 using ANOVA and scheffee test, IE: invert emulsion.

$C = Y^2 .. / rt$

$$= \frac{(17+25+17)^2}{4\times 4} = \frac{4761}{16} = 297.56$$

$$SS\ totla = \sum Y_{ij}^{\ 2} - C = (17)^2 + (25)^2 + (17)^2 - 297.56 = 1303 - 297.56 = 1005.44$$

$$SS\,treatment = (\sum Y_{ij})^2 - C \Rightarrow \frac{2493}{4} - 297.56 = 325.69$$

SS error = SS total – SS treatment = 1005.44 – 325.69 = 679.75.

H_0: $M_1 = M_2 = M_3 = M_4$

H_1: $M_1 \neq M_2 \neq M_3 \neq M_4$

ANOVA table

Source of Variation	SS	dF	Ms	Fc
Treatment	325.69	3	108.56	1.916
Error	679.75	12	56.64	
Total	1005.44	15		

F 0.05(3.12) = 3.49. Since Fc < Ftabulated, we don't reject H_0, So $M_1 = M_2 = M_3 = M_4$ and there is no significant difference.

Appendix C

Table: *Rhizopus* soft rot – lesion diameter in mm developed on pear fruit 3 days after inoculation and treatment at 20 ± 2˚C.

Treatments	Replicates (Lesion diameter in mm)				Mean
	R_1	R_2	R_3	R_4	
1.*Rhizpous*+*Trichoderma* (formulated in IE).	6	7	8	11	8^{a*}
2.*Rhizpous*+*Trichoderma* (suspended in water)	8	7	12	12	9.75^{ab}
3.*Rhizpous*+ S.D.W as control	34	20	18	33	26.25^{b}
4.*Rhizpous*+IE(blank formulation as control)	37	26	13	12	22^{b}

* Means followed by different letters are significantly different at P≤0.05 using ANOVA and scheffee test, IE: invert emulsion.

$$C = \frac{(6+7+...12)^2}{16} = \frac{69696}{16} = 4356$$

$$SS\ \ total = \sum Y_{ij}^{\ 2} - C = (6)^2 + ...(12)^2 - C \Rightarrow 5998 - 4366 = 1642$$

$$SS\ treatment = (\sum Y_{ij})^2 / r - C \Rightarrow \frac{21314}{4} - 4356 = 5328.5 - 4356 = 972.5$$

SS error = SS total – SS treatment = 1642 – 972.5 = 669.5

H_0: $M_1 = M_2 = M_3 = M_4$

$H_{0:}$ At least two means are different.

ANOVA table

Source of Variation	SS	dF	Ms	Fc
Treatment	972.5	3	324.1	5.8
Error	669.5	12	55.79	
Total	1642	15		

F 0.05(3.12) = 3.49. Since Fc > Ftabulated, we don't reject H_0, So at least

According to Scheffee test:

1) H_0: $M_1 = M_2$, H_1: $M_1 \neq M_2$. We reject H_0 if:

$$/\overline{X_1} - \overline{X_2} / \geq \sqrt{55.79 \times 3 \times 3.49 \times \frac{1}{4} + \frac{1}{4}}$$

$$1.75 \geq 17 \left[We\ don't\ reject\ H_0,\ so\ M_1 = M_2\right]$$

2) H_0: $M_1 = M_3$, H_1= $M_1 \neq M_3$. We reject H_0 if:

$$/\overline{X_1} - \overline{X_3} / \geq \sqrt{MSE\,.\,(k-1)\,.\,Fx\,(k-1)\,.FXL}$$

$$18.25 \geq 17,\ we\ reject\ H_0\,,\ So\ M_1 \neq M_3$$

3) H_0: $M_1 = M_4$, H_1: $M_1 \neq M_4$. We reject H_0 if:

$$/\overline{x_1} - \overline{x_4} / \geq 17$$

$14 \geq 17$ .We don't reject H_0, so $M_1 = M_4$.

4) H_0: $M_2 = M_3$, H_1: $M_2 \neq M_3$. We reject H_0 if:

$$/\overline{x_2} - \overline{x_3} / \geq 17$$

$16 \geq 17$.We don’t reject H_0, so $M_2 = M_3$.

5) H_0: $M_2 = M_4$, H_1: $M_2 \neq M_4$. We reject H_0 if:

$$/\overline{x_2} - \overline{x_4} / \geq 17$$

$12.25 \geq 17$. We don't reject H_0, so $M_2 = M_4$.

6) H_0: $M_3 = M_4$, H_1: $M_3 \neq M_4$. We reject H_0 if:

$$/\overline{x_3} - \overline{x_4} / \geq 17$$

$4.25 \geq 17$.We don't reject H_0, so $M_3 = M_4$.

Appendix D

Table: *Rhizopus* soft rot – lesion diameter in mm developed on pear fruit 3 days after inoculation and treatment at 30 ± 2˚C.

Treatments	Replicates (Lesion diameter in mm)				Mean
	R_1	R_2	R_3	R_4	
1.*Rhizpous*+*Trichoderma* (formulated in IE).	0	0	9	7	4^a
2.*Rhizpous*+*Trichoderma* (suspended in water)	0	0	8	10	4.5^a
3.*Rhizpous*+ S.D.W as control	0	8	10	10	7^a
4.*Rhizpous*+IE(blank formulation as control)	0	7	10	10	6.75^a

* Means followed by different letters are significantly different at P≤0.05 using ANOVA and scheffee test, IE: invert emulsion.

$C = Y^2 .. / rt$

$$= \frac{(9+7...10)^2}{4\times 4} = \frac{7921}{16} = 495$$

$$SS\ totla = \sum Y_{ij}^{\ 2} - C = (9)^2 + ...(10)^2 = 807 - 495 = 312$$

$$SS\,treatment = (\sum Y_{ij})^2 / r - C \ = \frac{2093}{4} - 495 \Rightarrow 523.25 - 495 = 28.25$$

SS error = SS total – SS treatment = 312 – 28.25 = 283.75

H_0: $M_1 = M_2 = M_3 = M_4$

H_1: at least two means are different.

ANOVA table

Source of Variation	SS	dF	Ms	Fc
Treatment	28.25	3	9.41	0.398
Error	283.75	12	23.6	
Total	312	15		

F 0.05(3.12) = 3.49. Since Fc < Ftabulated, we don't reject H_0, So $M_1 = M_2 = M_3 = M_4$, and there is no significant difference.

Appendix E

Table: *Rhizopus* soft rot – lesion diameter in mm developed on apple fruit 3 days after inoculation and treatment at 20 ± 2˚C.

Treatments	Replicates (Lesion diameter in mm)				Mean
	R_1	R_2	R_3	R_4	
1.*Rhizpous*+*Trichoderma* (formulated in IE).	23	8	0	0	7.75[a]
2.*Rhizpous*+*Trichoderma* (suspended in water)	0	0	7	36	10.75[a]
3.*Rhizpous*+ S.D.W as control	26	35	22	21	26[a]
4.*Rhizpous*+IE(blank formulation as control)	27	10	17	25	19.75 [a]

* Means followed by different letters are significantly different at P≤0.05 using ANOVA and scheffee test, IE: invert emulsion.

$C = Y^2 .. / rt$

$$= \frac{(23+...25)^2}{16} = \frac{66049}{16} = 4128$$

$$SS\ totla = \sum Y_{ij}^{\ 2} - C = (23)^2 + ...(25)^2 - C = 6507 - 4128 = 2379$$

$$SS\,treatment = (\sum Y_{ij})^2 / r - C = \frac{19867}{4} - 4128 \Rightarrow 4966.75 - 4128 = 838.75$$

SS error = SS total – SS treatment = 2379 – 838.75 = 1540.25 .

H_0: $M_1 = M_2 = M_3 = M_4$

H_1: at least two means are different.

ANOVA table

Source of Variation	SS	dF	Ms	Fc
Treatment	838.75	3	279.5	2.17
Error	1540.25	12	128.3	
Total	2379	15		

F 0.05(3.12) = 3.49. Since Fc < Ftabulated, we don't reject H_0, So $M_1 = M_2 = M_3 = M_4$, and there is no significant difference.

Appendix F

Table: *Rhizopus* soft rot – lesion diameter in mm developed on apple fruit 3 days after inoculation and treatment at 30 ± 2˚C.

Treatments	Replicates (Lesion diameter in mm)				Mean
	R_1	R_2	R_3	R_4	
1.*Rhizpous*+*Trichoderma* (formulation in IE).	32	7	0	0	9.75*[a]
2.*Rhizpous*+*Trichoderma* (suspended in water)	65	60	7	66	49.5[b]
3.*Rhizpous*+ S.D.W as control	73	70	72	78	73.25[cb]
4.*Rhizpous*+IE(blank formulation as control)	75	74	76	78	75.75 [cb]

* Means followed by different letters are significantly different at P≤0.05 using ANOVA and scheffee test, IE: invert emulsion.

$C = Y^2 .. / rt$

$$= \frac{(32+...78)^2}{16} = \frac{693889}{16} = 43368$$

$$SS\ totla = \sum Y_{ij}^{\ 2} - C = (32)^2 + ...(17)^2 - C = 57761 - 43368 = 14393$$

$$SStreatment = (\sum Y_{ij})^2 / r - C = \frac{218383}{4} - 43368 \Rightarrow 5459575 - 43368 = 1122775$$

SS error = SS total – SS treatment = 14393–11227.75 =3165.25.

H_0: $M_1 = M_2 = M_3 = M_4$

H_1: at least two means are different.

ANOVA table

Source of Variation	SS	dF	Ms	Fc
Treatment	11227.75	3	3742.5	14.19
Error	3165.25	12	263.7	
Total	14393	15		

F 0.05(3.12) = 3.49. Since Fc < Ftabulated, we don't reject H_0, So $M_1 = M_2 = M_3 = M_4$, and there is no significant difference

1) H_0: $M_1 = M_2$, H_1: $M_1 \neq M_2$. We reject H_0 if:

$/\overline{X_1} - \overline{X_2} / \geq \sqrt{263.7 \times 3 \times 3.49 \times 0.5} = 37.15$

$39.75 \geq 37 \left[We\ don't\ reject\ H_0,\ so\ M_1 \neq M_2\right]$

2) H_0: $M_1 = M_3$, H_1= $M_1 \neq M_3$. We reject H_0 if:

$/\overline{X_1} - \overline{X_3} / \geq 37.15$

$63.5 \geq 37.15\ \ we\ reject H_0,\ So\ M_1 \neq M_3$

3) H_0: $M_1 = M_4$, H_1: $M_1 \neq M_4$. We reject H_0 if:

$/\overline{x_1} - \overline{x_4} / \geq 37.15$

$66 \geq 37$ .We don't reject H_0, so $M_1 \neq M_4$.

4) H_0: $M_2 = M_3$, H_1: $M_2 \neq M_3$. We reject H_0 if:

$/\overline{x_2} - \overline{x_3} / \geq 37.15$

$23.75 \geq 37.15$.We don't reject H_0, so $M_2 = M_3$.

5) H_0: $M_2 = M_4$, H_1: $M_2 \neq M_4$. We reject H_0 if:

$/\overline{x_2} - \overline{x_4} / \geq 37.15$

$26.25 \geq 37.15$. We don't reject H_0, so $M_2 = M_4$.

6) H_0: $M_3 = M_4$, H_1: $M_3 \neq M_4$. We reject H_0 if:

$/\overline{x_3} - \overline{x_4} / \geq 37.15$

$2.5 \geq 37.15$ we don't reject H_0, so $M_3 = M_4$.

جامعة النجاح الوطنية
كلية الدراسات العليا

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قدمت هذة الاطروحة استكمالا لمتطلبات درجة الماجستير في العلوم البيئية بكلية الدراسات العليا في جامعة النجاح اتلوطنية في نابلس، فلسطين.

2005

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الملخص

يهدف هذا البحث الى تقييم فعالية الفطر المضاد (ترِيكوديرما هارزيانم) ضد مرض التعفن الطري (ريزوبس سوفت روت) في ثمار التفاح والاجاص و الكمثري الذي يسببه فطر (ريزوبس ستولونيفير). وأيضاً تحديد فترة الوقاية من الإصابة بهذا المرض على الأنواع الثلاثة من الفاكهة. لقد تم استعمال الفطر بشكل رئيسي كمستحلب منعكس بعد إدخاله إلى المستحلب بشكل كونيديا، بالإضافة إلى استعمال الفطر بشكل محلول مائي يحتوي على الكونيديا. تم إجراء تجربة (تقييم الفعالية) في المختبر في درجات حرارة مختلفة (20 ± 2م°, 30 ± 2م°). أثبتت النتائج التي حصلنا عليها أن الفطر (ترِيكوديرما هارزيانم) بصيغة المستحلب المنعكس كان فعالاً في تقليل قطر الإصابة لمرض التعفن الطري مقارنة بغيره من المعاملات. لقد وُجد أن هناك فروقات معنوية (الإحتمالية < 0.05) عند مقارنة متوسط قطر الإصابة للمرض في المعاملات بالمستحلب المنعكس المحتوي على الفطر والشاهد. كذلك أشارت النتائج الى أن فطر (تريكوديرما) بصيغة المستحلب المنعكس يعطي في ثمار التفاح المجروحة أطول فترة حماية ممكنة ضد مرض التعفن الطري وهذا يثبت الفعالية البيولوجية لفطر (ترِيكوديرما هارزيانم). ومع ذلك فإنه ينصح بإجراء مزيد من التجارب لزيادة التأكد من فعالية الفطر ضد مرض التعفن الطري (ريزوبس سوفت روت) لغرض الاستعمال في ظروف طبيعية تتعلق بخزن و تسويق الفواكة وقبل الإستعمال التجاري للفطر.