Tuscia University of Viterbo
Faculty of Agriculture
Department of Plant Protection

PHD IN PLANT PROTECTION
AGR/12
- XXIII CYCLE -

***CURRICULUM*: "**Plant parasite control with minimal environmental impact**"**

TITLE

Antagonistic and plant growth promoting bacteria from organic growing substrates for controlling some soil borne fungal pathogens of melon

Ph.D. Dissertation of:
Claudine Sebaaly

Coordinator
Prof. Leonardo Varvaro

Supervisor
Prof. Leonardo Varvaro

Tutors:
Prof. Leonardo Varvaro
Dr. Anna Maria D'Onghia
Dr. Mariagrazia Antonelli

Academic Years 2007-2010

The PHD thesis "**Antagonistic and plant growth promoting bacteria from organic growing substrates for controlling some soil borne fungal pathogens of melon"** has been defended by **Claudine Sebaaly (Lebanon)** in February 2, 2011.
The thesis committee members are:

Prof. Antonio BELCARI, Università degli Studi di Firenze, Italy.
Prof. Lucio MONTECCHIO, Università di Padova, Italy.
Dott. Giorgio Mariano BALESTRA, Università degli Studi della Tuscia, Italy.

*Tutors***:** Prof. Leonardo Varvaro
Dr. Anna Maria D'Onghia
Dr. Mariagrazia Antonelli

Dedications

I will offer this work,

To my husband Chadi Adwan,

To my parents Ishaak & Najat,

To my brother and my sisters,

To all my family,

And

To all my friends and my colleagues,…

SEBAALY Claudine

Acknowledgments

I would like to express my cordial thanks and great gratitude to my supervisor Prof. Leonardo VARVARO and my tutors Dr. Anna Maria D'ONGUIA and Dr. Mariagrazia ANTONELLI for their helpful and precious advices, suggestions and guidance for the achievement of my work, and all the time that they dedicated me, but especially for their kindness, their encouragements and for the confidence that they had in me during all these months.

My sincere thanks and gratitude to MAI-Bari staff, with special mention to the Director Dr. C. LACIRIGNOLA, the Deputy Director Dr. M. RAELI and the Dr. Anna Maria D'ONGUIA for their financial support, for their suggestions, advices and help during the three years of studies.

My special recognition and deep thanks to Dr. Alfredo Fabi for his helpful advising for my thesis work.

And I would like to express my thanks to my colleagues in Tuscia University for their loving care, patience and support.

Finally, I would like to address my thanks to my friends who were always sustaining me by their encouraging, supporting and helping, also for their kindness and friendship.

The PhD thesis has been co-financed by the Mediterranean Agronomic Institute of Bari (MAIB).

TABLE OF CONTENTS

List of abbreviations

°C	Degree Celsius
Cfu	Colonies forming units
Cm	Centimeter
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra-acetic
G	Gram
H	Hour
ha	Hectare
L	Litre
M	Mole
m^2	Scare meter
Mg	Milligram
Min	Minute
Ml	Millilitre
Mm	Millimetre
mM	Millimole
Nb	Number
Ng	Nanogram
PCR	Polymerase Chain Reaction
Rpm	Rotation per minute
Sec	Second
µl	Micro litre

List of figures

List of tables

1) Introduction

1.1. Melon cultivation

Melon is generally thought to have originated in western Africa (Bailey, 1976; Purseglove, 1976; Whitaker and Bemis, 1976; Zeven and Zhukovsky, 1975), with China or India as possible secondary centers of diversity. Wild melons growing in natural habitats have been reported in desert and savanna zones of Africa, Arabia, southwestern Asia, and Australia. As Sauer (1993) notes, it is unclear where melon was domesticated and "it is conceivable that it was independently domesticated from different wild populations in Africa and southwestern Asia". Melon was an important food crop in ancient China, where archaeological data suggest that it has been cultivated for over 5,000 years (Robinson and Decker-Walters, 1997). Archaeological evidence also suggests that melon was cultivated in Iran some 5,000 years ago and in Greece and Egypt about 4,000 years ago (Zohary and Hopf, 1988). As with cucumbers, melons were cultivated in the Roman Empire and diffused throughout Europe by the Middle Ages where the "variety and quality of melon cultivars were evidently greatly increased by selection in Medieval gardens" (Sauer, 1993). As with cucumbers and watermelons, melons were introduced to the New World by Spanish colonial settlers in the late fifteenth and early sixteenth centuries and subsequently spread very rapidly among Native American horticultural groups. Later during the eighteenth century they reached the Pacific Islanders via British explorers.

Melon belongs to the Kingdom *Plantae*, and is classified as a fruit. Its genus is *Cucumis* and its species is *Cucumis melo* L. Within these groups, they are also distinguished by their Latin names into two groups, *Cucumis* (muskmelons) and *Citrullus* (watermelons). Aside from these classifications, they are also separated into many varieties. Exactly how many varieties are unknown. Some biblical accounts say that there are thousands, and others far fewer. The three most common are the watermelon, cantaloupe, and honeydew. The cantaloupe, also called a muskmelon, it has beige, netted skin and sweet orange flesh that is high in beta carotene. Melons are mostly andromonoecious and have annual trailing vines with nearly round stems bearing tendrils and circular to oval leaves with shallow lobes. Staminate flowers are borne in axillary clusters on the main stem, and perfect flowers are borne at the first node of lateral branches. Fruits vary in size, shape, rind characteristics, and flesh color depending on variety. Fruit quality is related to external appearance, thick, well-colored interior flesh with high (>10 percent) soluble solids, and a pleasant aroma and taste

(Maynard and Elmstrom, 1991). It is a common misconception that poor-quality melon fruit results from cross-pollination with cucumber because these species are incompatible. Rather, the poor-quality melon fruit sometimes encountered is due to unfavorable weather or growing conditions that restrict photosynthetic activity and, thereby, sugar content of the fruit. Seeds are cream-colored, oval, and on average 10 mm long.

Among horticultural species, melons (*Cucumis melo* L.) have been produced in large quantity, especially in countries with a temperate-warm climate. The main producers are China, Turkey, Iran, Mexico, Romania and Spain; also North Africa is a very important area, where the climatic conditions lead to early productions (Bignami, 2002). Asia dominates the world production of melon by producing more than 71% of the total tonnage (FAO, 2004) (table 1). Cultivation areas in Europe account for about 11% of the world areas (161,000 ha) with a production of 3.16 million of tons: about 70% of these are produced in E.U. and particularly Spain (39,000 ha), Italy (23,000 ha), France (17,500 ha) and Greece (7,900 ha).

Table 1. Melon cultivated area (hectares) and production quantity (tons) in the entire world and in the continents.

Raking	Continent/Country	Production (tons)	Cultivated Area (hectares)
	World	**27,371,268**	**1,313,272**
	By continent		
1	**Asia**	**19,537,759**	**902,582**
2	**Europe**	**3,160,000**	**161,210**
3	**North and central America**	**2,440,099**	**125,081**
4	**Africa**	**1,601,325**	**79,250**
5	**South America**	**565,400**	**42,800**
6	**Australia**	**64,150**	**2,635**
	By Nation		
2	**Turkey**	**1,700,000**	**115,000**
3	**U.S.A**	**1,240,000**	**46,000**
4	**Iran**	**1,000,000**	**70,000**
5	**Romania**	**1,000,000**	**52,000**
6	**Spain**	**1,000,000**	**39,100**

Cucurbits, in their complex, cover more than 40,000 ha in Italy; melon is the most diffused one, with two different kind of cultivation: melon in the open field mainly diffused in the South and islands; sheltered cultivation, forced (greenhouse or tunnel) or semi-forced (little tunnel), in the North and for early production in every area. In the last ten years, melon cultivation expanded constantly, and in the last five years the cultivated surface overcame 27,000 ha: 3,000 ha in greenhouse and about 24,000 in the field (Chilosi *et al*., 2008). In northern Italy are cultivated about 11,000 ha (in Lombardia, Emilia-Romagna and Veneto), in the South 8,000 ha and more than 1,000 ha on islands, in the central part more than 6,000 ha (Lazio, Toscana, Marche and Umbria); in particular, in the province of Viterbo the cultivation of melon (ISTAT, data last five years) increase to 256 ha.

1.2. Phytosanitary problems of melon plants

Like most other plants - ornamental and edible - melons are susceptible to a number of pests, viruses, phytoplasma, bacteria, nematodes and fungi, some of which may be more prevalent in one area of the country than another. A lot of parasitic adversities can attack cucurbits' crop both in sheltered cultivation and in the field. Some pests of Italian environments affect plant aerial part (*Pseudoperonospora cubensis, Erysiphe cichoracearum, Sphaerotheca fuliginea, Colletotrichum lagenarium, Alternaria cucumerina, A. Alternata*), while others develop on roots; these are particularly diffused and are very difficult to control. The main are: *Sclerotinia sclerotiorum, Didymella bryoniae*, *Fusarium oxysporum* f. sp. *melonis*, *Verticillium dahliae* and *V. albo-atrum*, *Rhizoctonia solani*. *Fusarium solani*, *F. oxysporum* f. sp. *radici-lycopeci*, *Macrophomina phaseolina*, *Pythium ultimum*, *P. aphanidermatum*, *P. myriotylum* and *Pyrenochaeta lycopersici*, can be also considered.

In recent years, the two main soil born fungal pathogens of melon causing a critical problem and economic losses in central Italy are *Monosporascus cannonballus* (MC) and *Fusarium oxysporum* f. sp. *melonis* (FOM) (Chilosi *et al.*, 2004; 2008). These pathogens are agents of collapse and vascular wilts of the melon plants, respectively. Thus, the suppression of these plant pathogens is considered an urgent need of present agriculture. The use of organic amendment to suppress soil borne pathogens of melon plant has been extensively reviewed, and several microorganisms, have been studied in different organic amended substrates (Antonelli *et al.*, 2009; Sebaaly *et al.*, 2010).

1.2.1. *Monosporascus cannonballus* (MC)

1.2.1.1. History of Monosporascus root rot

Monosporascus root rot and vine decline is an emergent disease all over the world (Cohen *et al*., 2000; Martyn and Miller, 1996) and one that only recently has gained attention among plant pathologists. The causal agent, *Monosporascus cannonballus*, was described as novel genus and species by Pollack and Uecker (1974) on the base of some melon necrotic roots isolated in Arizona. At that time no pathogenic tests were performed; later, the pathogenicity of this pest has been determined in Israel, in 1983, when it was correlated with adult plant collapse (Dias *et al.*, 2004; Martyn and Miller, 1996). Pathogenicity of isolates from the United States was first reported in 1991 (Mertely *et al.*) in Texas, and the disease was named Monosporascus root rot and vine decline (MRR/VD). In 1996, the disease was identified in Arizona and California (USA) and in other thirteen countries: Mexico, Guatemala, Honduras, Spain, Israel, Iran, Libia, Tunisia, Pakistan, India, Saudi Arabia, Japan and Taiwan (Martyn, 2002). It has since been reported in four additional countries (Korea, Italy, Brazil, Iraq) bringing the total number of countries reporting this disease in 2009 to seventeen. It is highly probable it will be detected in additional countries in the near future. In Italy it was signaled on water-melon near Bologna (Gennari *et al*., 1999) and on melon in Lazio and Puglia (Infantino *et al.*, 2002; Stravato and Vagnozzi, 2003), later on melon and cucumber near Imola and Ravenna (Montuschi, 2002).

This pathogen was cancelled from the EPPO Alert List in 2001, because of the repeated signallings. In consideration of the aspecific disease symptoms, it cannot be excluded that this pathogen could be more diffused than known.

1.2.1.2. Classification of *Monosporascus cannonballus* (Pollack & Uecker, 1974)

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Subclass: Sordariomycetidae

Order: Sordariales

Family: Incertae sedis

Genus: *Monosporascus*

Species: *M. cannonballus*

1.2.1.3. Morphological features

M. cannonballus is an Ascomycetes pirenomycetes; it's a homothallic fungus that produces abundant typical reproductive structures, perithecia, both on infected roots and on lab's artificial substrate. Perithecia contain a lot of ascus, mainly with only one ascospore. It's not known the production of asexual spores by *M. cannonballus*; therefore, pathogen survival and diffusion are ensured by ascospores (Waugh *et al*., 2003) and, maybe, by mycelium present on infected crop residual.

1.2.1.4. Symptoms

Aboveground symptoms: The symptoms are manifested at the end of the vegetative cycle, about 2-3 weeks before harvest. Affected plants initially show a state of suffering with extended yellow leaves followed, very soon, by drying and withering (figure 1). Fruits of these plants have a reduced dimension and they do not reach commercial maturation, therefore they have not suitable organolectic requirements for bestowal (lower degree sweetens and less resistant peel) (Mirotti *et al.*, 2003). Moreover, collapsed plants expose fruits to solar radiation, rendering them subject to burns.

Belowground symptoms: The most representative symptoms of the disease are localized on roots, with necrosis and lesions of different extension, reduced development and rots. Root lesions, root rot, loss of feeder roots and, in severely dry conditions, death of taproot are results of Monosporascus root rot and vine decline (figure 2). Lesions first develop as small areas of necrosis at the joints between secondary and tertiary roots or at the tips of young roots. These lesions are typically dry, however in the event of abundant soil moisture they may appear as a wet rot. Lesions are tan to red-brown. In severe cases of *M. cannonballus* infection, most of the root system may become necrotic and result in death of the plant. Large, black perithecia form on dead roots and are visible to the naked eye. The perithecia first appear on smaller feeder roots in the first few centimeters of soil and typically appear late in the season.

Researchers conducted in Spain on collapsed plant showed that the described symptomatology can be attributed also to *Acremonium cucurbitacearum* and *Plectosporium tabacinum* (Abad *et al*., 2000; Garcìa-Jiménez, 1994), while in USA Farr *et al*. (1997) have isolated *Rhyzopycnis vagum* from roots of a collapsed melon plant that in pathogenetic tests reproduced disease symptoms. Recent studies conducted in Italy on collapsed plant grown in

different regions showed the constant presence of *M. cannonballus*, *A. cucurbitacearum*, *P. tabacinum* and *R. vagum* (Montuschi, 2002; Infantino *et al*., 2002a, 2002b).

Fig 1. Yellowing and death of the melon leaves

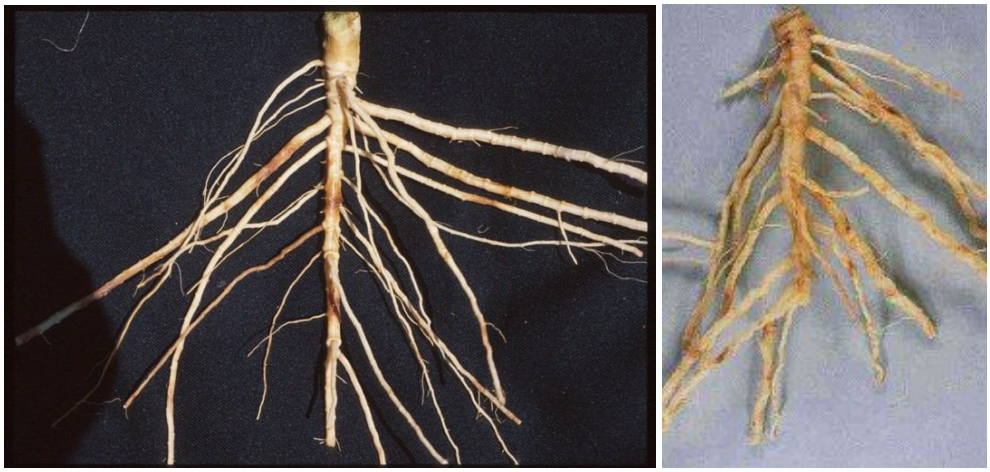

Fig 2. Necrosis. Lesions and loss of feeder melon roots

1.2.1.5. Life cycle

The disease cycle of *M. cannonballus* appears to be relatively simple. Infection of the roots can occur from either mycelium that survived in the soil or plant debris or from germinating ascospores. The fungus invades the fine feeder roots, colonizes the cortex and xylem, and kills the rootlet. The infection advances to the next root *via* the root junction or perhaps from new infections. Smaller roots die while lesions are formed predominantly on larger roots. The fungus continues to colonize the root tissue and invades the xylem causing the plant to form tyloses. While the fungus invades the xylem, it becomes systemic in the plant however it is not a true vascular wilt pathogen, such as *Fusarium oxysporum* or *Verticillium* spp. Infection is favored by warm soil temperatures (25 to 35°C). In climates where double

cropping of melons occurs (two crops per year), disease typically is more severe in the late-summer or autumn-planted crop than the spring or winter-planted crop. Perithecia are formed on the roots most abundantly late in the season but may form throughout the life cycle. When mature, the perithecia rupture, discharging the ascospores into the soil. Several hundred ascospores may form in a single perithecium and a single mature melon plant root system is capable of producing 400,000 or more ascospores.

1.2.1.6. Epidemiology

No aerial stage of the disease is known, and no asexual spore stage of the fungus has been observed. Thus, ascospores are considered the primary inoculum and can survive in the soil or plant debris until the next planting season or, in the absence of a host, may survive for many years in a dormant state. Mycelium from infected crop debris can serve as a source of inoculum, although it is not known how long mycelium survives in the soil. The disease is considered monocyclic.

Ascospore germination is favorites by the presence of exudates produced by roots. *M. cannonballus* adapted at temperate-warm climate, it has an optimum of development at 25-30°C (Pivonia *et al*., 2002), with pH of 6-7; its development is completely inhibited at pH 4. Moreover this pathogen seems favored by moderately alkaline and salt soil. Studies on variability of *M. cannonballus* are few and no one includes Italian isolates. Isolates coming from USA, Spain and Japan seem to be characterized by a low variability of morphology, cultural characters, virulence on melon and also in some sequences of DNA. Vegetative compatibility between isolates was not deeply studied, but it seems that there are meaningful differences between the American one and the others.

Ascospores can build up to high numbers in fields cropped successively to melons; however, the minimum threshold level for disease development is not known. Severe disease has occurred in fields with as few as one to two ascospores per gram of dry soil and as high as 15 spores per gram of dry soil. In the few cases studied, ascospores appear uniformly distributed throughout the fields, with the highest concentration in the upper 10 inches (25 cm). Thus, the epidemiology of this disease may be more complex than we currently understand.

1.2.1.7. Disease dissemination

The collapse has assumed a greater importance in the last 15 years, but the reasons are not completely known. Probably, several factors contributed to the increasing spread of the disease. The spread of the pathogen can be by any method that displaces contaminated soil or crop debris (*e.g.*, cultivation, flooding, wind, erosion, etc.). Airborne spread of the pathogen is not likely because of the large size of the ascospores and the lack of a known conidial stage. There is no evidence that the pathogen is seedborne or systemic in the plant. Colonization appears to be limited to the roots. Fruit and foliage are not infected directly, although they manifest symptoms related to root infection, water stress and plant decline.

Another aspect regards the changes of the tillage techniques recorded in the last 15-20 years, just contemporaneously with disease spreading. A strong variety change has been recorded, passing from cultivar with a wide genetic base, to hybrids that have plants with more premature and concentrated production. Moreover, the technique of the transplant in place of the direct sown, employing plastic film and localized irrigation diffused. These techniques carried remarkable agronomic and economic advantages, but they produced plants with superficial and little developed roots. Finally, growth of the investments and the production costs induced many farmer to a shorten crop alternation and, in extreme cases, to cultivate cucurbits on the same field for two or three years. This technique inevitably increased the inoculum presence in the soil. For this reason it's necessary to consider the fact that *M. cannonballus* produces a huge amount of ascospores, which are equipped of a very thick wall and can survive in the soil for several years. Moreover, the pathogen seems to be able to colonize the roots of several vegetable species, both dicotyledonous and monocotyledon, cultivated in succession to the cucurbits without causing noticeable disease, providing another means of survival and reproduction. Moreover, ascospores were found in soils never subjected to cucurbit cultivation and in native desert soils in Texas and Arizona in the U.S. and in Brazil; therefore, it can be argued that *M. cannonballus* is an indigenous fungus.

1.2.2. *Fusarium oxysporum* f. sp. *melonis* (FOM)

1.2.2.1. History of Fusarium Wilt

Separate pathogens or formae speciales (f. sp.) are responsible for Fusarium wilts of melon and cucumber. There are seven formae speciales attacking cucurbits, with *Fusarium oxysporum* f. sp. *melonis* is one of the most important pathogen on the major melon growing areas (Dutky *et al.*, 1986; Gonzalez *et al.*, 1988; Gubler and Grogan, 1976; Katan *et al.*,

1994, 1996; Martyn and Amador, 1987; Smith *et al*., 1992;) by producing serious economic problems. Fusarium wilt of melon (*Fusarium oxysporum* f. sp. *melonis* abbreviated as FOM) was first reported as a disease of melon in the United States in New York in 1930, although its pathogenicity was not confirmed until the disease was described in Minnesota in 1933 (Thomas, 1998). By 1945, the disease was described from states as far west as California and north into Ontario, Canada. Although the pathogen is not uniformly distributed throughout the melon producing regions of the world, it is very widespread.

Investigations over the years on pathogen variability (called races) have led to the description of as many as four races. The four races described for FOM are called races 0, 1, 2, and 1,2. Until recently, the only described race of FOM in the United States was race 2. In 1985, race 1 was reported from Maryland, and in 1987, race 0 was discovered in Texas. In 1992, race 1 was recovered from collapsed melon fields in New York. But race 2 still remains the most widely distributed race in the United States. In contrast, race 1 is the most common race in Europe and the Middle East. Until recently, race 1,2 had only been reported from France, but is now known to occur in Maryland (Villeneuve and Maignien, 2008).

1.2.2.2. Classification of *Fusarium oxysporum* f. sp. *melonis*

Kingdom: Fungi
Subkingdom: Dikarya
Phylum: Ascomycota
Subphylum: Pezizomycotina
Class: Sordariomycetes
Subclass: Hypocreomycetidae
Order: Hypocreales
Family: Nectriaceae
Genus: *Fusarium*
Specific descriptor: *oxysporum*
Scientific name: *Fusarium oxysporum* f. sp. *melonis* (W.C. Snyder & H.N. Hansen).

1.2.2.3. Morphological features

Fusarium wilt of melon is caused by a seed and soil borne fungus that is specific to melon. *F. oxysporum* produce three types of asexual spores. These include the macroconidia, microconidia, and chlamydospores. Macroconidia spores have between three and five cells,

septate, that are larger, slightly curved at the ends. These spores are often found on the surface of the plants killed by *F. oxysporum*. Microconidia spores have between one or two cells, small, colorless, oval to elliptical. They are the type most often produced by the pathogen and are located within the actual vessel of infected plants. Chlamydospores are round spores that have between one and two cells. They have thick walls that are created in either older mycelium (vegetative part of the fungus) or in macroconidia.

1.2.2.4. Symptoms

Plants may be affected in any stage of development; those affected early in their development suffer greater injury than those infected later in the season. Infected plants are often stunted and yellowed. In seedlings, the cotyledons or seed leaves lose their healthy lustre and wilt, followed by the complete collapse of the plant. If affected at an early enough stage, plants will rot (or damp-off) at the soil line and die. In older plants, there is marginal yellowing progressing to a general yellowing of the older leaves, and wilting of one or more runners. In some cases, sudden collapse occurs without any yellowing of the foliage. Fruit from affected vines (if any is produced) tend to be small, with poor flavour and colour. Mature plants often wilt severely (collapse) late in the season because of the fruit load stress.
Fusarium wilt collapse should not be confused, however, with sudden wilt of melon. Sudden wilt is a complex disease associated with plant stress brought on by heavy fruit set, cool evening soil temperatures followed by warm and sunny days, feeder root loss caused by soil borne fungi (may include *Verticillium* and other species), and virus infection (primarily cucumber mosaic and watermelon mosaic, but also papaya ring spot W strain and zucchini yellow mosaic virus).

Leaves: Older plants can show wilting and yellowing of leaves near the crown (figure 3). As the disease progresses, the leaves show tip-browning. Leaves often have dead areas which can mimic nutrient deficiencies. In some cases, plants exhibit a temporary wilt which appears repeatedly in the middle of the day. Some recovery may occur at night, but the plant finally dies.

Stems and runners: On stems near the crown of the plant, a linear, necrotic lesion may develop (figure 4), extending up the plant and usually on one side of the vine. One runner on a plant may wilt and collapse, with the rest of the runners remaining healthy, and if environmental conditions change to those which do not favour the fungus, the plant may

recover and fruit normally. However, if conditions continue to favour FOM infection, the whole plant will eventually die.

Roots and vascular system: External lesions may develop on roots, accompanied by red gumming at or just below the soil surface, similar to that seen on stems (figure 5). Vascular discoloration should be evident and is very diagnostic. If the taproot and stem are split open, an orange-brown discoloration of the water conducting tissues can be seen.

Fig 3. Yellowing and wilting of leaves in melon plants

Fig 4. Necrotic lesion and red gumming on stems near the crown of the melon plant

Fig 5. External lesions on the melon roots

1.2.2.5. Disease life cycle

Once introduced into a field, *F. oxysporum* f. sp. *melonis* can remain indefinitely, even after rotation to nonsusceptible crops (Gordon *et al.*, 1989). These pathogens can exist time among susceptible crops (Burgess, 1981; Garret, 1970) with their persistence mainly attributed to the production of long-lived chlamydospores (Garret, 1970; McKeen and Wensley, 1961; Newcombe, 1960), which are thick-walled modifications of the mycelium. FOM may also be seed borne, both externally and internally. Internal infection is limited to the area immediately beneath the seed coat and does not infect the embryo. Infection of the host can also occurs by penetration the host through root tips, primarily in the elongation area, and aided by wounding (Martyn and Gordon, 1996). It can also invade the plant with its sporangial germ tube or the mycelium. After penetration, once inside the plant, the mycelium grows within the cells, through the root cortex. When the mycelium reaches the xylem, it invades the vessels. It remains in the vessels and moves up toward the stem and the top of the plant. Then the mycelium branches and produces microconidia, which is carried to the plant's sap stream. The mycelium can penetrate the upper wall of the xylem vessel when the microconidia germinate and produce the next vessel. After infected plants wilt, the *Fusarium* fungi produce masses of microscopic spores on and in dead vines. The conidia may be splashed and blown from diseased vines resulting in expansion of areas infested by the fungi. Disease development is favored by high nitrogen, low calcium, and low potassium levels in the soil.

Populations as high as 3,300 propagules/gram of soil have been reported in wilt-infested soil but decline more than fourteen-fold during a nine-month fallow interval between crops. Wilt-susceptible varieties tend to increase populations of the fungus. Unfortunately, FOM is saprophytically competitive with nonpathogenic strains of *F. oxysporum* and can survive on such nonhosts as tomato, alfalfa.

1.2.2.6. Epidemiology

Environmental and soil conditions are important for infection and in symptom expression. Both the incidence and severity of the disease increase during warm, dry weather. Disease severity is maximum at soil temperatures of 17-25 ^{o}C and declines dramatically above 30 ^{o}C. At high soil temperatures, plants become infected but may not wilt; rather they develop severe stunting. However, very high temperatures (32-38 ^{o}C) combined with increased humidity seem to reduce the disease levels. As with most Fusarium wilts, the following

apply: 1) low soil moisture favors the pathogen and accentuates the wilting symptom; 2) high nitrogen, especially NH_4-nitrogen, light, sandy, and slightly acidic soils (pH 5-5.5) favor disease development; 3) liming the soil to increase the pH to 6.5-7.0 decreases wilt severity.

1.2.2.7. Disease dissemination

Fungi is commonly spread from one field or garden to another by infested soil, compost and manure adhering to machinery, tools and the feet of humans and animals; surface-drainage water; and infected seeds. FOM can be seed-borne, but most transmission occurs by the movement of infected soil or plant parts. The fungus is well adapted to life in the soil, and can survive season to season by living (saprophytically) on dead plant material, or on the roots and stems of other plants such as tomatoes, alfalfa and weeds. Season to season survival also occurs through the formation of very robust resting spores (chlamydospores) which can remain viable in the soil for many years. Chlamydospores are stimulated to germinate by the growth of susceptible host plant roots nearby. Eventually water movement is reduced sufficiently to produce the wilting symptoms typically associated with the disease.

1.3. Disease management

Management strategies for these pathogen are focused on preventive measures; amongst those are the use of resistant rootstocks or attempts for biological control practices (Berg *et al*., 2001, 2005; Suarez-Estrella *et al*., 2007; Tjamos *et al*., 2004) including the use of compost soil amendments (Paplomatas *et al*., 2005; Suarez- Estrella *et al*., 2007; Termorshuizen *et al*., 2006; Yogev *et al*., 2006), solarization and integration between all these systems (Cohen *et al*., 2000). There are no chemical treatments to control these diseases (except for soil fumigation, which is applied only to high-value crops usually in greenhouse cultivations).

1.3.1. Physical control

Solarization is indicated as a basic agronomic technique for a correct and heal cultivation to ambient low impact, together with rotation, green manure and rationalization of irrigation. Its use, although obtaining good phytoiatric results, is strongly penalized because of its dependency on the climatic and seasonal fluctuations and on the necessity of an extended interruption of the normal cultural cycles (Materazzi *et al.*, 1987; Triolo *et al.*, 1991). Using transparent or semi-transparent plastic films, it is possible to obtain thermal increasing in the

soil that allows reaching temperatures necessary to control pathogens and to contain the development of the infesting grasses. In fact, unlike vapo-disinfection, it does not create biological empty but it perform a pasteurization with elimination of a large number of pathogens, respecting rate of useful microorganisms, that are ready to develop as antagonists. After elimination of vegetable residual, soil is tilled and irrigated until field capacity, it's covered for a certain period during summer, when solar radiation is maximum, with transparent plastic sheets in order to reduce dispersion of solar energy and to raise temperature. Wetting must increase soil thermal conductivity and sensitize quiescent shapes of pathogens (spores, sclerotia, seeds). If solarization is applied correctly, it's possible to reach soil temperatures of 50 °C at 15-30 cm depth. This technique is very effective against several telluric fungi (*Fusarium, Verticillium, Sclerotinia, Pythium*), nematode and infesting grass (Conway, 1983). Recently some studies were conducted in order to improve solarization efficacy, arranging it with a reduced dose of specific geo-disinfestants (Sbaraglia *et al.*, 2003), with biocontrol agents and above all with amending organic that produce composed flown that are accumulated under the plastic cloth (biofumigation). This appears a valid solution because the integration of physical (solarization) and chemical or biological disinfection could carry to better results (Sbaraglia *et al.*, 2003).

The causal agent of sudden wilt of melons (FOM) and of melon collapse (MC) is affected by solarization or by the combination of solarization and cabbage residues or chicken manure amendment. The role of these techniques for control of these diseases has not yet been verified in Italian environmental conditions.

1.3.2. Chemical control

Collapse control is very difficult: presence of lasting ascospores, lack of resistant or highly tolerant melon genotype and absence of effective fungicides for the chemical control contribute to this difficulty.

Chemical methods used to control vascular wilt are either not very efficient or imply negative effects on environmental and human health (Becker and Schwinn, 1993; Boutler *et al.*, 2000; Brimner and Boland, 2003; Hoitink and Boehm, 1999).

Fumigation, in particular bromide action, is the most effective control method (Martino, 1997; Montuschi, 2002; Serra *et al.*, 2004), but commercial retirement of methyl bromide as geosterilizer, from 1st January 2005 (Reg. CE 2037/2000), imposes the employment of

alternative control methods (Camponogara e Gasparrini, 2003; Camponogara *et al*., 2003; Gullino *et al*., 1999).

1.3.3. Resistant cultivars and rootstocks

As a result of the persistence of these pathogens, effective control of these diseases has generally been achieved by the use of disease-resistant cultivars (Latin, 1989; Mas *et al*., 1981; Ng and Kantzes, 1991; Zink *et al*., 1983; Zink, 1992). Nowadays many sources of resistance to FOM races 0, 1 and 2 are known (Alvarez *et al*., 2005; Pitrat *et al*., 1996) but it is not the same for race 1,2; resistance to this race has only been found in some Far Eastern accessions (Messiaen *et al*., 1962) which are organoleptically far from commercialized melon in the western countries.

Another mean of potentially effective control is represented by employment of grafted plants (*Cucurbita maxima* seems to be less susceptible to *M. cannonballus*) generally available for the control of other diseases (Mirotti *et al*., 2003). Herbaceous graft on cucurbits is a practice generally used for the control of telluric origin pathogens with more of 9 million melon plants produced in 2000 (Assenza *et al*., 2000; Minuto *et al*., 1999; Morra, 1997; Morra *et al*., 1997; Rappuoli, 1996; Serges *et al*., 2000). Although about 26 rootstocks exist, the commonest are melon's hybrid Jador, Dinero, Belimo and pumpkin's hybrid RS841, P360 and TZ148 (Morra *et al*., 2001). These differ each other for the resistance degree to several telluric pathogens; it is better to use pumpkin's hybrid, more thriving and resistant, in soil where not even melon's hybrid are able to give protection against pathogens (Ferrari *et al*., 1993; Morra, 1997; Morra *et al*., 2001; Temperini *et al*., 1999; Trionfetti Nisini *et al*., 2002, 2000).

Because of the great spread of graft, the behavior of rootstock against *M. cannonballus* must be estimated in a double view: to increase use of less susceptible rootstocks and, at the same time, to advice against use of those highly sensitive (at least in infected soils). As well, since grafting represents an usual procedure for controlling *Fusarium oxysporum* f. sp. *melonis* race 1,2, evaluation of rootstocks resistance was also carried out toward a local isolate of this fungus. Evaluations of rootstocks of melon for resistance to FOM were conducted with artificial inoculations on different melon and squash genotypes and grafted plants, the tested rootstocks confirmed to be sufficiently resistant to melon fusarium wilt (Chilosi *et al*., 2004).

1.3.4. Biological control

The reduction or elimination of synthetic fungicide applications in agriculture is highly desirable. One of the most promising means to achieve this goal is by the use of new tools based on biocontrol agents (BCAs) for disease control. To date, a number of BCAs have been registered and are available as commercial products, including strains belonging to bacterial genera such as *Agrobacterium, Pseudomonas, Streptomyces* and *Bacillus*, and fungal genera such as *Gliocladium, Trichoderma, Ampelomyces, Candida* and *Coniothyrium*. Also, it is well known that some soils are naturally suppressive to some soil borne plant pathogens such as *Fusarium oxysporum, Pythium* and *Phytophthora* species and this suppression relates to both physiochemical and microbiological features of the soil.

Recently, the adopted methodology for suppression of some soil borne pathogens is using the organic amendment, which is particularly important not only to improve plant growth but also to make the plant less susceptible to pathogen infection by postulating different organic amendment mechanisms such as competition for nutrients, antibiotic production by beneficial microorganisms or activation of disease resistance genes in plants. However, only a few organic amendments are currently on the market. Also, many microbial agents isolated from the organic amendments are reported as PGPR (plant growth promoting rhizobacteria), which are free-living soil borne bacteria that colonize the rhizosphere and, when applied to seed or crop, enhance the growth of the plant.

1.3.4.1. Plant growth promoting bacteria

Rhizobacteria have long been known for their role in promotion of plant growth and biological control of plant pathogenic microorganisms. It is believed that plant growth promoting rhizobacteria act by displacing or antagonizing plant pathogenic microorganisms. Various microorganisms in soil, including *Enterobacter aerogenes, Pseudomonas fluorescens, Pseudomonas cepacia*, and *Bacillus* species, has been reported to produce antibiotics and antifungal compounds that effectively control or suppress phytopathogenic fungi both *in vitro* and *in vivo*.

Bacteria of the genus Bacillus produce a variety of peptide antibiotics that are antibacterial and/or antifungal. Several Bacillus species, including *B. subtilis, B. pumilus, B. cereus* and *B. licheniformis* have been shown to be antagonistic to plant pathogenic fungi and bacteria (Neyra and Sadasivan, 1997). Phytopathogen antagonistic strains of *B. subtilis* have reported to produce two peptide antibiotics: bacilysin, a dipeptide that inhibits yeast and bacteria, and

fengycin (fengymycin), a lipopeptide antagonistic against phytopathogenic fungi such as *Rhizoctonia solani* (Loeffler *et al*., 1986) *B. cereus* produces mycocerein.

In addition to Bacillus species, suppression of soil borne diseases like Fusarium wilt and Monosporascus root rot has also been attributed to certain elements of the fluorescent pseudomonads community resident in these suppressive soils. Disease suppression appears to function *via* direct antagonism through competition for iron (Raaijmakers *et al*., 1995), direct inhibition of saprophytic fungal growth (Duijff *et al*., 1999) and induced systemic resistance (Duijff *et al*., 1998; Lemanceau *et al*., 1993). It is likely that these microbial elements, and other elements of the saprophytic microflora, act in concert rather than independently of each other, in the suppression of Fusarium wilts and Monosporascus root rot.

1.3.4.2. Fungi

The free-living fungi are ubiquitous in the soil and are being successfully used and commercialized to combat a broad range of phytopathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum* and *Botrytis cinerea* (Fravel, 2005). Filamentous fungi from the genus *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. Moreover, Djonović *et al*. (2006) demonstrated that using *Trichoderma virens* as biocontrol agent could induce plant defense mechanisms.

In addition, the role of non-pathogenic *Fusarium* spp. in disease suppression was first suggested as a result of the observations that large populations of these fungi were resident to suppressive soils, and that suppressiveness could be reestablished in a heat-treated soil through the introduction of a non-pathogenic *Fusarium* strain (Rouxel *et al*., 1979). Numerous studies have since demonstrated that non-pathogenic *Fusarium* spp. is functional elements of the resident microflora in Fusarium wilt suppressive soils from diverse geographic regions (Alabouvette *et al*., 1996). Suppression of Fusarium wilt diseases by non-pathogenic *Fusarium* spp. occurs *via* various mechanisms, including saprophytic competition for substrate (Couteaudier and Alabouvette, 1990), induction of systemic resistance in the plant host (Duijff *et al*., 1998) and parasitic competition for infection sites.

A recent study was carried out to determine the reduction of wilt disease severity in melons caused by FOM by applying *Penicillium oxalicum* in the growth chamber and glasshouse (De cal *et al*., 2009).

1.3.4.3. Organic amendment

The role of organic matter in crop protection is associated with increase microbial activity, reduced aggressiveness and infestation of pathogens, increased viral resistance and a reduction in soil tiredness or toxicity. Organic soil amendments, including animal and green manures and wastes from processed animal products such as blood meal, bone meal, have been used for centuries as soil supplements.

1.3.4.3.1. Nitrogenous soil amendments

Organic amendment containing high nitrogen, such as poultry manure, meat and bone meal, and soy meal, significantly reduced populations of a wide spectrum of soil borne plant pathogens. Addition of organic matter improves the vigor of the plant as a result of physical and chemical improvement of the soil also increases resistance of the individual plant as a result of uptake of phenols, phenolic and other compounds such as salicylic acid which have an antibiotic effect and also work directly on pathogens (Lampkin, 1999).

Applying organic manures makes a direct contribution to the anti-phytopathogenic potential of soils. This is particularly important in case of fungal damping-off diseases such as *Rhizoctonia*, *Fusarium* and *Pythium* (Lampkin, 1999). Various chemicals in the soil are known to contribute to the anti-phytopathogenic potential of the soil. The breakdown of organic manures results in the release of carbon dioxide, which is harmful to some pathogens in high concentrations. Toxins available in the crop residue which is produced against other biological agents such as weeds and are called allelochemicals, may also act against plant pathogens (Lampkin, 1999).

In many cases, the incorporation of organic matter in the soil showed beneficial alternative to chemicals for plant disease control. For example, Viana *et al.* (2000) reported that matured cattle manure and sugarcane husks are efficient alternatives for control of bean damping-off (*Sclerotinia sclerotiorum*). Farm yard manure applied at 5 t/ha, once every 3 years, reduced dry root rot (*Macrophomina phaseolina*) to 32 % in groundnut (*Arachis hypogaea* L.) compared with untreated plants (Harinath and Subbarami, 1996). Disease control with organic amendment occurs when soil and biological factors are conducive to activating the processes that reduce pathogen survival. Understanding the mechanisms allows prediction of efficacy based on analysis of the soil and the organic amendment (Lazarovits, 2001).

1.3.4.3.2. Green Manure effects

Green manure, defined by Pieters (1927) as "the practice of enriching the soil by turning under fresh plant material either *in situ* or brought from a distance" is a widely used practice in organic farming to maintain soil organic matter. It also adds nitrogen and other nutrients and offers several other physical and biological soil improvements.

From the microbiological point of view, green manure has two main positive effects: i) it provides nutrients rich in organic carbon for the microbial biomass which converts unavailable nutrients in plant residues to ones available for crops; ii) it enhances biodiversity of soil microorganisms. This positive effect on soil microbial populations can be increased by inserting different green manure selections in crop rotation programs (Bezdicek and Granatstein, 1989; Reeves, 1994).

Incorporation of plant tissues into the soil enhances the microbial biomass (Lynch and Pating, 1980) with an advantageous increase in microbial activity. This improves soil fertility and may suppress soil borne pathogens by increasing microbial competition and antagonism, while at the same time reducing the inoculum potential through germination and lysis of propagules in the soil (Lumsden *et al*., 1983).

1.3.4.3.3. Compost

Compost is the product obtained from the aerobic decomposition of organic matter. It is considered as organic wastes that have been degraded by termophilic and mesophilic microorganisms and possibly further during maturation (Day and Shaw, 2001). Composts differ according to the raw starting material and the nature of the process. Composts that serve as components of container media must be stable, in order to avoid competition for oxygen and nitrogen between microorganisms and plant roots. They must have low salinity, low concentrations of phytotoxic substances and be free of phytopathogenic organisms (Epstein, 1997; Inbar *et al*., 1993; Miller and Metting, 1992; Raviv, 2005). Composts serving as soilless media are produced from diverse organic wastes, such as sewage sludge, municipal solid waste, animal excreta and food industry wastes such as rice hulls and corn cobs.

Various types of composts are known to suppress diverse diseases caused by soil borne pathogens (Erhart *et al*., 1999; Gorodecki and Hadar, 1990; Hadar and Mandelbaum, 1986; Hoitink *et al*., 1993, 1997; Kwok *et al*., 1987; Trillas-Gay *et al*., 1986). The suppressive capacity of composts is clearly linked with their degree of maturity, although excessively

stabilized composts tend to lose this quality (Hoitink and Grebus, 1997). The suppressive agents are complexes of microbial populations, which invade the compost pile during the curing stage. Sterilization largely negates the disease suppressive capacity of composts (Larkin *et al*., 1993; Mandelbaum *et al*., 1988; Reuveni *et al*., 2002), which suggests that most of it is associated with microbial activity, although some residual activity is probably related to fungistatic compounds that are also present there (Hoitink and Fahy, 1986).

El Masry *et al*. (2002) have found that water extract from several composts were suppressive to several soil borne pathogens, but the extracts did not contain antibiotics or siderophores. The greater efficacy of composts in suppressing disease in greenhouses or growth rooms than in field may be due to enhanced activity of microbial antagonists at higher temperatures. Hoitink *et al*. (2001) state that *Bacillus* spp, *Enterobacter* spp., *Flavobacterium balustinum, Pseudomonas* spp., *Streptomyces* spp. and other bacterial genera have been identified as biocontrol agents in composted amended substrates. Several studies have addressed suppression of *Fusarium* pathogens by composts, but they have mostly focused on only one forma specialis (Chef *et al*., 1983; Cheuk *et al*., 2005; Kannangara *et al*., 2000; Raviv *et al*., 2005; Reuveni *et al*., 2002), such as coffee-waste composts for the control of Fusarium wilt in melon plants (Ros *et al*., 2005), pulp and paper mill (Pharand *et al*., 2002) or tomato residues (Cheuk *et al*., 2005) for the control of Fusarium crown and root rot in tomato plants, and separated cattle manure for the control of Fusarium root and stem rot in cucumber plants (Kannangara *et al*., 2004) and Fusarium crown and root rot in tomato plants (Raviv *et al*., 2005).

Other mechanisms of control include the presence of toxic or stimulatory volatile compounds in composts, or changes to the physical properties of the growing medium or soil, and changes to soil Conductivity and pH (Tilston *et al*., 2002).

Assaying for pathogen viability in compost requires a direct test capable of differentiating between pathogen suppression and pathogen elimination. Ros *et al*., (2001) think that the physicochemical characteristics of compost can affect the suppressive properties of the soil indirectly, through the improvement of soil structure and porosity, along with other factors; this fact is more important in compost than in sewage sludge. Numerous container-based studies in greenhouses or growth rooms have consistently demonstrated a suppressive effect of composts on soilborne diseases such as damping-off and root rots (*Pythium ultimum, Rhizoctonia solani, Phytophthora* spp.), and wilts (*Fusarium oxysporum* and *Verticillium dahliae*) (Noble and Coventry, 2005).

2) Objectives

Soil borne pathogens cause heavy economic losses to a wide range of crops. *Fusarium oxysporum* f. sp. *melonis* (FOM) and *Monosporascus cannonballus* (MC) are the major soil borne fungi of melon crops causing serious economic losses to farmers, as there are no chemical treatments to control them (except for soil fumigation, which is applied only to high value crops usually in greenhouse cultivations). These pathogens are considered as a critical problem in most melon crop zones of Central Italy. Management strategies, for both pathogens are focused on preventive measures; amongst those are the use of resistant rootstocks or attempts for biological control practices (Berg *et al.*, 2001, 2005; Suarez-Estrella *et al*., 2007; Tjamos *et al.*, 2004) including the use of compost soil amendments (Paplomatas *et al*., 2005; Suarez- Estrella *et al*., 2007; Termorshuizen *et al.*, 2006; Yogev *et al*., 2006).

For centuries, farmers have consciously and unconsciously manipulated the ecology of the soil by using organic matter. Organic amendments are known to affect soil aeration, structure, drainage, moisture holding capacity, nutrient availability and microbial ecology. These practices influence pathogen viability and distribution, nutrient availability and the release of biologically active substances from both crop residues and soil microorganisms. Furthermore, using of biocontrol agents will satisfy requirements in modern, sustainable agriculture of environmentally friendly disease control measures. However, only a few biocontrol products and organic amendments are currently available on the market (Fravel, 2005), and there is an urgent need to provide a knowledge base for developing new commercial bioproducts and application procedures of the alternative control means, either in the nursery and in the field.

The demands in introducing new techniques for pathogen control, leads to focus our research on the use of an organic medium amended or not with bacterial antagonists and with plant growth promoting rhizobacteria, for the control of the main soil borne pathogens affecting the melon crops production and for the improvement of the plant growth, respectively. Antagonists are naturally occurring organisms with traits enabling them to interfere with a pathogen's growth, survival, infection or plant attack.

The use of organic amendment to suppress soil borne pathogens of melon plants has been extensively reviewed and several microorganisms, especially those that belong to two cultural bacterial groups (fluorescent pseudomonads and aerobic spore forming bacteria), have been studied in different organic amended substrates.

Our attention was focused on those two bacterial groups in aim, that the pseudomonads are important nutrient cycling, assisting plant with phosphorous availability, and some have been linked to the biological control of plant pathogen. The aerobic spore-forming bacteria (*i.e.*, *Bacillus* spp.) are able to produce different type of antimicrobial compounds, to induce growth and defense responses in the host plant. Furthermore, *Bacillus* spores resist adverse environmental conditions and permit easy formulation for commercial purposes. Therefore, the objective of this study was to quantify and isolate the bacterial communities present in some organic amendment substrates. In addition, to investigate the isolated antagonistic bacteria *in vitro* and *in vivo* either for their antagonism toward FOM and MC or for their promoting plant growth activities. The overall objective was pursued by seven sets of experiments:

- The first one refers to the study of the microbiological communities present in the bulk soil of a commercial compost (ECOS), and in the bulk soil, rhizosphere and rhizoplane of melon plants grown in an organic growing medium (MAIB-ECOS) and of melon plants grown in an infected field by FOM.
- The second experiment was specifically set up to evaluate *in situ* and *in vitro* the suppressive effect of a commercial agricultural compost (ECOS) on some soil borne melon pathogen (*Fusarium oxysporum* f. sp. *melonis* (FOM) and *Monosporascus cannonballus* (MC)).
- The third experiment was performed to investigate *in vitro* the antagonist activities of the isolated bacteria against the two fungal pathogens of melon plants.
- The fourth experiment was designed to detect *in vitro* the plant growth promoting features of the isolated bacteria.
- The fifth experiment was designed to develop organic amendment substrate, that can serve as growing media for melon grown crops, by assessing the horticultural traits of six organic growing substrates and by evaluating the bacterial population density before and after sowing of melon seeds in those growing media.
- The sixth experiment was carried out to evaluate *in vivo* the antagonist activities of the microorganisms selected in the preliminary test (*in vitro* assays) against the soil borne pathogen of melon *Fusarium oxysporum* f. sp. *melonis* (FOM) through artificial inoculations.
- The last objective was performed to identify by the molecular methods the bacteria possessing the ability to suppress FOM in the nursery.

3) Materials and Methods

3.1. Isolation and quantification of bacteria from compost (ECOS), organic growing substrate (MAIB-ECOS) and infected field by FOM

3.1.1. Collection of samples

Different locations were used to prepare a collection of diverse bacterial strains. Bacteria were isolated from four different sites representing varied ecological situates, from bulk soil and from rhizosphere and rhizoplane of a melon seedling (cv. *Proteo*).

3.1.1.1.Commercial compost (ECOS)

The "ECOS" commercial compost, produced by BERCO Company (Milano), has an acceptable level of heavy metals. Because of that, ECOS compost is certified as organic compost. It is obtained from composting different organic fractions coming from the maintenance of green ornamental residues. The main characteristics of ECOS product are shown in table (2). Six replicates of bulk soil sample were collected randomly from the ECOS compost.

Table 2. The main characteristics of ECOS commercial product

Parameters	**Value**
Humidity	50%
Ph	7.5
EC	2.5 dS/m
Total N	12 g/kg
Organic carbon	250 g/kg
C/N ratio	16.6
P2O5	7.5 g/kg
Potassium	9.5 g/kg
Microelements	10 g/kg

3.1.1.2.Organic growing substrate (MAIB-ECOS)

Six bulk soil samples were collected randomly from an organic compost based substrate (MAIB-ECOS) before melon seeds sowing, also rhizosphere and rhizoplane samples were collected randomly from six melon seedlings grown in this organic substrate (MIAB-ECOS). Melon seedlings were removed from the substrate at first-leaf stage.

The MAIB-ECOS substrate is prepared at the Mediterranean Agronomic Institute of Bari (*CIHEAM/MAIB*) using the commercial compost (ECOS) and a bio-fertilizer (Guanito) with a percentage of 30 % and 0.5 %, respectively. The MAIB-ECOS mix is prepared by mixing all components together such as sand (20 %), pumice (20 %), perlite (15 %), peatmoss (15 %), and microelements (0.0 2%).

3.1.1.3.Infected field by FOM

Soil and melon plant samples were also collected from an infected field by *Fusarium oxysporum* f. sp. *melonis*, which is located in Montalto di Castro in the city border of Pescia Romana, representing various moisture and temperature levels, moreover characterized by a sandy fertile soil, melon cultivated intensely inside a protective tunnel. At the selected location, 3 soil samples, each about 200 g, were taken randomly over an area of about 100 m^2, from the top layer (2 – 15 cm) of melon field. All soil samples from the same location were mixed to produce one representative sample for the location, where soil samples were collected from six different locations. Six samples of rhizosphere and rhizoplane were taken from melon plant showing the fusarium wilt symptoms in the field. Soil and plant samples were stored at 4 °C until use.

3.1.2. Isolation of microorganisms and colony counting from soil samples

Microbiological analyses of bulk soil were performed using the dilution plate technique (Wakelin *et al*. 1998). Each 10 g sample was added to 90 ml of sterile distilled water inside 250 ml conical flask. The mixture was shaken vigorously for 1 h at 150 rpm to form uniform solution of 10^{-1} concentration. The stock was subjected to decimal dilution by taking 1 ml from each diluted solution, using sterile pipettes to form 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} and 10^{-7} concentrations.

For spore forming bacteria an improved method was developed in which 4 ml of original solution were placed inside a tube and incubated for 15 min at 80 °C in autoclave. The spore forming bacterial suspension was subjected to decimal dilution by taking 1ml from each diluted solution, using sterile pipettes to form 10^{-2}, 10^{-3} and 10^{-4} concentrations**.** A sterile pipette was used to transfer 100 µl of each dilution onto a sterile plate with different agar growth mediums. King's B (KB) medium for the fluorescent pseudomonad bacteria and for the total viable bacteria which contained the following ingredients (per liter): Pseudomonas

Agar-F 35 g, Agar 3 g, Glycerol 10 ml; and Tryptose soy agar (TSA) medium for spore forming bacteria isolation (per liter): 40 g Casein-peptone, soymeal, peptone agar.

Dilutions of each bulk soil sample were made on duplicate plates and then were incubated at 27±1 °C for 2 days. The numbers of colonies were counted after 48 h of incubation from the Petri dishes containing 30–300 colonies. Microbial colonies were counted as colony forming units (cfu) and the amount was determined as given: Direct counting x Dilution factors = Number/Soil Weight (g). Afterwards, the values of bacterial populations were referred to soil dry weight.

Representative bacteria of the predominant morphological types present on the plates were selected at random and purified on Nutrient agar (NA) medium (containing per liter: Agar technical 15 g, Lab-Lemco Agar 8 g) for spore forming bacteria and on KB medium for fluorescent bacteria. Representative taxa were further characterized using standard physiological tests. All purified bacteria were lyophilized for long-term storage.

3.1.3. Isolation of culturable rhizosphere and rhizoplane bacteria

The plant was selected at the growing stage as excretes more volume of root exudates and can bear more diversified rhizosphere and rhizoplane microbes in its roots. The rhizosphere is the soil found around the root and under the influence of the root. It is a site with complex interactions between the root and associated microorganisms (Sylvia *et al.* 1998). Approximately 1 g of the adhered soil samples were suspended in 100 ml of sterile distilled water and subjected to shaking for 1 hour at 150 rpm in a rotary shaker (Sonal *et al.*, 2006).

Rhizoplane bacteria were defined as cells adhering to the roots, that could be removed by following two experimental procedures in order to count and isolate the bacterial colonies loosely adhering to root surface (rhizoplane a) and the bacteria tightly adhering to the rhizoplane and/or endophyte (rhizoplane b). The cells of the rhizoplane (a) were removed from 1 g wet root tip material suspended in 65 ml sterile distilled water by shaking for 10 min at 150 rpm in a rotary shaker (Chave *et al.*, 2008). After 10 min shaking, the washed roots were then macerated with a mortar and pestle by adding a sterile distilled water to obtain the bacterial suspension adhering to the rhizoplane (b) (Sangeeta *et al.*, 2001). The rhizosphere and the two prepared rhizoplane suspensions were separately performed by serial dilution technique as describe in the previous paragraph, using King's B medium for fluorescent and total viable bacteria, and TSA for aerobic spore-forming bacteria. The dilutions of each rhizosphere and rhizoplane (a) and (b) samples were plated twice and the

plates were incubated at 27±1 °C. Colonies were observed regularly after every 24 h and counted by standard plate count method; its mean values were taken as cfu/g sample, and the microorganisms' amount isolated from the rhizosphere site was determined as given: Direct counting x Dilution factor = Number/Soil Weight (g). Afterwards, the values of bacterial populations were referred to soil dry weight.

While these isolated from the rhizoplane sites (a) and (b) were calculated as: Direct counting x Dilution factor = Number/Root Weight (g). Afterwards, the values of bacterial populations were referred to root fresh weight.

Prominent growing single colonies were picked up and re-streaked two to three times so as to obtain pure colonies in NA medium plates. The morphological characteristics of the colonies were noted and purified colonies were tested for the other physiological and biochemical characteristics as per methods described in the Bergey's manual of determinative systematic bacteriology (1986).

3.1.4. Physiological and biochemical test

The identification of the two isolated bacterial groups (fluorescent pseudomonad bacteria and spore forming bacteria) was carried out by physiological and biochemical determinative tests, in order to differentiate the species of the *Bacillus* and *Pseudomonas* genus, and to avoid the cultivation of human bacterial disease.

3.1.4.1.KOH test

The simple KOH technique can be used as a rapid test for identification the bacterial isolates belonging to gram negative or gram positive. The test consists of mixing a loopful bacterial culture with 2 drops of 3 % KOH. Gram negative bacteria will become gummy upon mixing with a loop while Gram positive bacteria will not.

3.1.4.2.Levan production

Culture was streaked onto 5 % (w/v) sucrose nutrient agar, in which containing the following ingredients (per liter): Lab-Lemco broth 8 g, Agar tecnico 15 g, sucrose 50 g. This test was used to identify the fluorescent pseudomonad bacteria that indicate a positive reaction by showing white mucoid, dome-shaped colonies after 3 to 5 days incubation. We used *P. syringae* pv. *syringae* as positive and *Erwinia carotovora* as negative control.

3.1.4.3.Liquefaction of gelatin

The reduction of a gelatin culture medium to the liquid state occurs by the presence of enzymes produced by bacteria in a stab culture. This test is used for the identification of a certain bacterial species. Stab inoculate tube contains per liter 0.4 % of gelatin (Defco) (40 g) and Lab-Lemco broth (8 g). The liquefaction is controlled after 15 days of incubation at 20±1 °C. Before determining liquefaction the tube is kept at 5 °C for 30 min. The positive reaction of the two isolated bacterial groups to the liquefaction of gelatin is seen when tubes failure to harden at 5 °C.

3.1.4.4.Lecithinase production

The test is used to differentiate and to detect the presence of some human pathogenic bacteria (*i.e., Bacillus cereus*). The lecithinase production was identified by streaking the fluorescent and the spore bacterial isolates on egg yolk agar that is prepared by diluting the egg yolk to 40 % with sterile distilled water. Then the diluted egg yolk was incorporated into molten nutrient agar cooled to 55 °C just before plates are poured at the rate of 10 ml / 100 ml of NA medium. The positive reaction of the test appears as opaque zone around bacterial growth after 7 days of incubation at 27±1 °C.

3.1.4.5.Growth at 41 °C

To avoid the cultivation of *Pseudomonas aeruginosa.* An inoculated slant of King's B medium were incubated in a water bath at 41 °C, and within 48 h the presence of growth was recorded, that indicate the presence of *Pseudomonas aeruginosa*.

3.2. Antagonistic effect of the matured compost (ECOS)

The main objective was to estimate, as an initial approximation, the potential suppressive effects of ECOS compost on a group of soil borne pathogenic fungi. The suppressive effect of the compost against the indicator fungi was examined using *in situ* and *in vitro* assays.

3.2.1. The indicator fungi

Two soil borne fungi of melon plants were used in this study: one *Monosporascus cannonballus* isolate (MC-R) and one *Fusarium oxysporum* f. sp. *melonis* (race 1-2) isolate (FOM F30) originated from infected melon plants. The fungi were kindly provided by Prof. G. Chilosi (Department of Plant Pathology, Faculty of Agriculture, University of Tuscia).

3.2.2. *In situ* suppressive effect of ECOS compost

The aim of this work is to investigate the usefulness and efficacy of using ECOS compost to suppress some fungal soil-borne melon plant pathogens *in situ*.

The indicator fungi (FOM and MC) were separately grown on sterile potato dextrose agar (PDA) plates containing per liter distilled water: 39 g of Sigma-PDA. Twenty-five milliliters of phosphate buffer (pH 7.2) were poured in the PDA plates cultured with 5 day-old MC-R isolate and 7 day-old FOM F30 isolate. The fungus was suspended into phosphate buffer by stirring with a sterile string. Then, aliquots of 0.1 ml of each indicator fungal suspension were spread on sterile fresh PDA plates. Sample (0.5 g) of autoclaved or unautoclaved compost was placed onto the centre of the seeded plate's surface. The experiments were conducted in quintuplicate for each autoclaved and unautoclaved sample. The plates were incubated for 7 days at 24±1 ºC and for 5 days at 30±1 °C for FOM and MC, respectively. The compost suppressiveness was determined by the presence of an inhibitory zone around the samples.

3.2.3. *In vitro* antagonism assay for bacterial extraction from water suspension of ECOS compost

The aim of this assay is to select microorganisms present in the water suspension of ECOS compost showing the antagonistic effect against fungal soil borne pathogens of melon plants.

Briefly, 1 g of ECOS compost was placed in 9 ml sterile distilled water. The mixture was blended for 30 sec at high speed. Fivefold dilution series were prepared with sterile distilled water, and then 0.1 ml of each suspension was aliquoted onto two replicate plates of PDA media. Discs 6.5 mm in diameter of 5 day-old MC-R isolate and 7 day-old FOM F 30 isolate were placed in the centre of each seeded PDA plate surface. The plates were incubated for 7 days at 24±1 ºC and for 5 days at 30±1 °C for FOM and MC, respectively. Plates were then assessed by the presence of inhibition on the growth of the mycelium. The bacterial colonies that show a positive inhibition zone were selected and purified to be identified by using the biochemical and physiological tests, then tested *in vitro* again for their antagonist activities and for their plant growth promoting (PGP) activities.

3.3. *In vitro* antagonism assays against two fungal pathogens

This step aimed to test the collected bacterial isolates (fluorescent and spore forming bacteria) that could have an antagonistic effect against the phytopathogenic fungi mentioned before (section 3.2.1). The collection of 150 different types of bacterial isolates was done by selecting the representative microbial morphologies from the bulk soil and the water suspension of ECOS compost and from the bulk soil, the rhizosphere and the rhizoplane of organic growing substrate (MAIB-ECOS) and infected field by FOM. The antifungal effects were performed with the following evaluation tests.

3.3.1. Dual culture-plate method on PDA

The object of the assay was to evaluate the capability of isolated microorganisms (table 3) to suppress the growth of the selected fungal plant pathogens (MC and FOM). Fungal pathogen isolates were transferred to potato dextrose agar (PDA) plates and grown for 7 days at 25±1 °C, for FOM F30 isolate, and for 5 days at 30±1 °C, for MC-R isolate. From the edge of each growing mycelia fungi, a 6.5 mm plug was transferred to PDA agar media. Pure cultures of fresh bacterial isolates were streaked in a straight line on the opposite side of the plate from the fungal plug at a distance of 3 cm. To ensure adequate bacterial and fungal growth, fungal isolates were initially grown on PDA plates one day before bacterial streaking and incubated at 25±1 °C for FOM and at 30±1 °C for MC. The percentage of antagonistic bacteria inhibiting the growth of FOM and MC mycelium, was assessed by the presence of inhibition zone (inhibit the growth of the mycelium) between pathogen and bacterial cultures (figure 6). This test was carried out in triplicate.

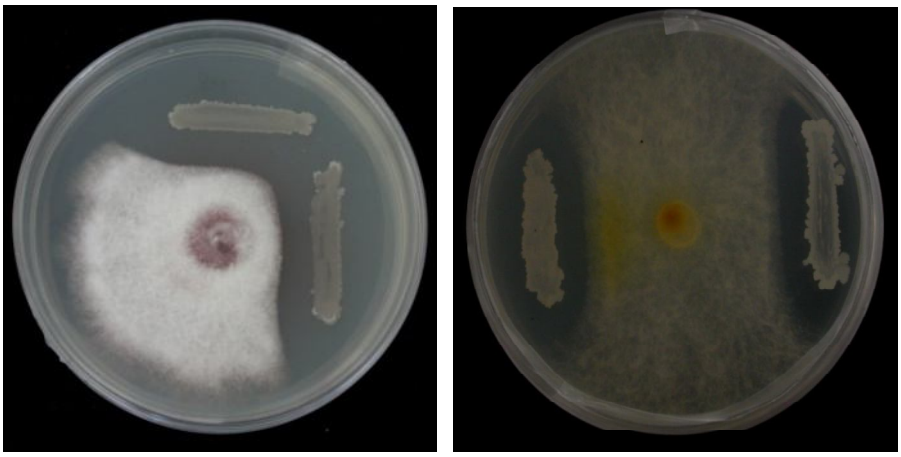

Fig 6. Dual culture plate method on PDAmedium.
The left and right plate show respectively the FOM F30 and MC-R isolates

Table 3. The number of bacterial isolates selected from the different ecological sites of the collected samples (ECOS-compost, Organic growing substrate (MAIB-ECOS) and infected field by FOM)

Origin of samples	Isolated sites	Bacterial groups	Nb. of isolates
ECOS compost	Bulk soil	FPB	15
		SFB	6
	Water suspension	SFB	18
Organic growing substrate (MAIB-ECOS)	Bulk soil	FPB	13
		SFB	13
	Rhizosphere	FPB	6
		SFB	7
	Rhizoplane (a)	FPB	5
		SFB	6
	Rhizoplane (b)	FPB	5
		SFB	4
Infected field by FOM	Bulk soil	FPB	8
		SFB	8
	Rhizosphere	FPB	6
		SFB	6
	Rhizoplane (a)	FPB	5
		SFB	7
	Rhizoplane (b)	FPB	6
		SFB	6

3.3.2. Inhibition of fungal mycelium growth

Each isolated bacterium showed an inhibition zone in the previous antagonism assay (table 4) was selected to evaluate its antagonistic effect by measuring the growth of FOM F30 and MC-R isolates on the Petri dish. The selected bacteria were grown on nutrient broth (NB; Oxoid, Basingstoke, UK) by fermentation process using 5 ml of NB, in cylinder tube. Each tube was inoculated with 24 h old bacterial culture grown on NA and incubated at 27±1 °C on constant rotary shaker at 150 rpm for 48 h. Biomass of bacteria was harvested by centrifugation of the culture broth at 10,000 rpm for 20 min. The bacterial cell dilution was made in 500 µl sterile distilled water to obtain a population size large enough to confirm growth for all the isolates. Fifty microliters of each isolated bacterial suspension was dropped in the middle of PDA plates (9 cm) drawing a line from one side of the plate to the opposite (4.5 cm); after 3 days of incubation at 27±1 °C, a disc (5 mm) of 7 day-old mycelium of FOM and 5 day-old mycelium of MC was placed on the opposite side of the grown bacterial suspension line. The plates were incubated for 15 days at 25±1 °C for FOM and for 7 days at 30°±1 C for MC. The antagonistic activity of the bacteria was measured by recording the

growth of FOM and MC mycelium (figure 7). The antagonistic activity was assessed establishing: a high suppression when FOM and MC growth on the plate was between 0 and 2.2 cm, medium suppression when FOM and MC growth was between 2.2 and 3.2 and no suppression when FOM and MC growth in the plate was higher than 3.2 cm. The measures of fungus growth were also recorded at intervals of 7, 10 and 15 days for FOM F30 isolate, and 5 and 7 days for MC-R isolate. These tests were conducted in six replicates.

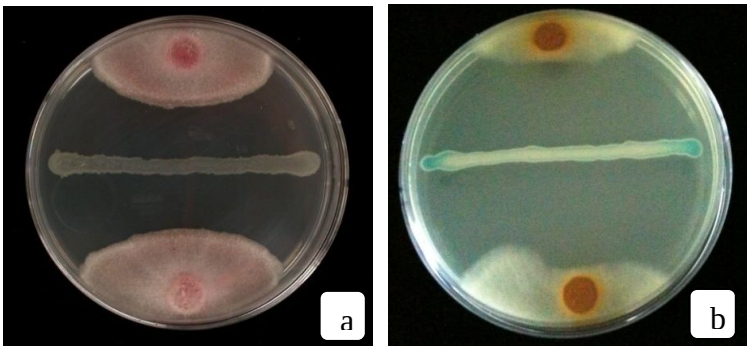

Fig 7. Qualitative evaluation of bacterial antagonism
by recording the mycelial growth of FOM F30 (a) and MC-R (b) isolates

Table 4. The bacterial isolates that were selected to be tested for their antagonistic effect against the pathogenic fungi (FOM F30 and MC-R isolates)

Origin of samples	Isolated sites	Bacterial groups	Bacterial isolates code
ECOS compost	Water suspension	SFB	B1
			B2
			B3
			C6
			M2
			M3
Organic growing substrate (MAIB-ECOS)	Bulk soil	FPB	I.2
			I.4
			II.1
			II.2
	Rhizosphere	FPB	S.2.1
			S.2.2
	Rhizoplane (a)	FPB	R.2.1
	Rhizoplane (b)	FPB	R.4.2
			R.4.3
Infected field by FOM	Bulk soil	SFB	P8
	Rhizoplane (a)	SFB	P16
			P18
			P22
	Rhizoplane (b)	SFB	P24
			P25
			P27

3.3.3. Assay for bacterial volatile inhibitors

Volatile fungistatic activity of bacteria was identified following Fernando *et al.* (2005) method with some modifications. Briefly, in a three-compartment Petri dish, an individual bacterial isolate from a fresh colony was streaked onto one compartment filled by the bacterial medium contained per liter: peptone from casein 5 g, peptone from meat 2.5 g, peptone from gelatin 2.5 g, yeast extract 1.5 g, NaCl l.5 g and agar-agar 15 g (pH 7.2); while the second compartment were poured with a layer of the fungal medium contained per liter: peptone 20 g, glucose 10 g and agar-agar 20 g (pH 6.8); and the third compartment was supplemented with or without an active carbon source (charcoal) to trap the volatiles, in order to prove that bacterial volatiles were the principal component impeding mycelial growth.

For those candidates producing volatile inhibitors (table 5), a 5 ml bacterial culture incubated at 27±1 °C under agitation for 2 days into nutrient broth (NB) were washed by centrifugation and adjusted to a concentration of 10^8 ml^{-1} by adding sterile distilled water. Then, 50 µl of the prepared bacterial suspension, or an equal volume of NB as control, was placed on the bacterial medium compartment. The plate was wrapped in Parafilm, so that volatile compounds could diffuse into the fungal medium. After incubation for 48 h, a fungal plug (Ø 6 mm) was taken from the margin of a 5 and 7 days old MC-R and FOM F30 mycelium respectively and placed onto the fungal medium side. The plate was re-sealed with Parafilm and incubated at 25±1 °C and 30±1 °C for FOM and MC, respectively (figure 8). Five replicates were used for each treatment. The intensity of antifungal activity by volatile compounds produced by the selected bacteria was recorded by observing the inhibition of mycelial growth of FOM F30 and MC-R isolates from the ninth day after pathogen inoculation.

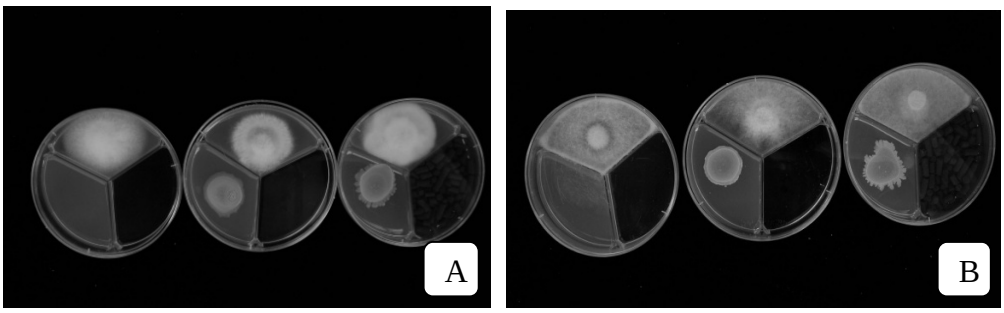

Fig 8. Co-cultivation of antagonsitic bacteria with pathogenic fungi in the presence or absence of charcoal. Nutrient broth (control, left plate) and overnight bacterial cultures were plated in one compartment (left panels) of a tripartite Petri dish. After 2 days of incubation, a fungal mycelium plug (FOM F30 [A] or MC-R [B]) with (right plate) or without charcoal (middle plate) were placed in the other compartments (middle and right panels, respectively)

3.3.4. Hydrocyanic acid (HCN) production

Hydrogen cyanide is produced by a number of bacteria and was recognized as a biocontrol factor against plant pathogenic fungi (Brimecombe *et al.*, 2001). This assay aimed to investigate the capability of the selected bacterial isolates (table 5) in the production of volatile compounds such as hydrogen cyanide.

HCN production of the isolates was detected by the method of Bakker and Schippers (1987). The bacterium was inoculated individually on Petri dishes containing tryptone soya agar supplemented with 4.4 g l^{-1} glycine. Filter paper discs soaked in 0.5 % picric acid and in 2 % sodium carbonate were placed in the lid of each Petri dish. Uninoculated controls were used for comparison. The plates were sealed with parafilm and incubated at 27±1 °C for 4 days. Change in color from yellow to light brown and reddish brown was indicative of moderate and strong production of HCN by the bacterium, respectively (figure 9).

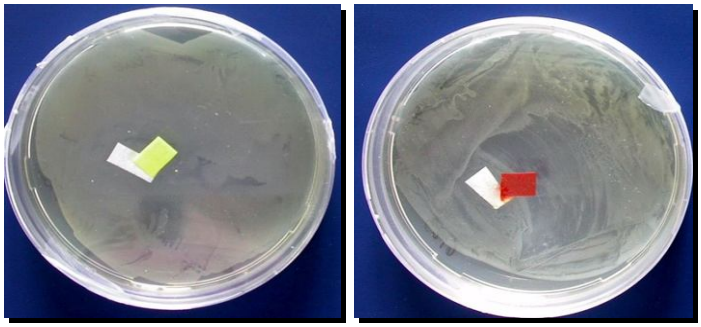

Fig 9. HCN production with a change in color from yellow (left plate) to reddish brown (right plate)

Table 5. The bacterial isolates that were selected to identify their volatile fungistatic activity

Origin of samples	Isolated sites	Bacterial groups	Bacterial isolates code
ECOS compost	Water suspension	SFB	B1
			B2
			B3
			C6
			M2
			M3
Organic growing substrate (MAIB-ECOS)	Rhizosphere	FPB	S.2.1
			S.2.2
	Rhizoplane (a)	FPB	R.2.1
	Rhizoplane (b)	FPB	R.4.3
Infected field by FOM	Bulk soil	SFB	P8
	Rhizoplane (a)	SFB	P16
			P18
			P22
	Rhizoplane (b)	SFB	P27

3.4. *In vitro* detection of bacterial plant growth promoting features

Some bacterial species, mostly those associated with the plant rhizosphere, are able to exert beneficial effects on their host**.** Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers for a number of years (Suslov, 1982; Glick, 1995). This group of bacteria has been termed "plant growth promoting rhizobacteria" (PGPR). The mechanisms by which PGPR can exert a positive effect on plant growth can be of two types: direct and indirect (Glick, 1995). Indirect growth promotion is the decrease or prevention of deleterious effect of pathogenic microorganisms, mostly due to the synthesis of antibiotics (Sivan *et al.*, 1992) or siderophores (Leong, 1986) by the bacteria. Direct growth promotion can be through the synthesis of phytohormones (Xie *et al.*, 1996), N2 fixation (Christiansen-Weneger, 1992), reduction of membrane potential of the roots (Bashan, 1991), synthesis of some enzymes (such as ACC deaminase) that modulate the level of plant hormones (Glick *et al.*, 1998), as well as the solubilization of inorganic phosphate and mineralization of organic phosphate, which makes phosphorous available to the plants (Krasilnikov, 1961; Subba Rao, 1982). The occurrence of these mechanisms in several PGPRs (table 3) and their possible role in the overall effects on plant growth promotion and antagonism activity will be discussed by studying the following mechanisms.

3.4.1. Siderophore production

Under iron stress, bacteria produce low-molecular-weight ligands called siderophores; these siderophores play an important role in the extracellular solubilization of iron from minerals or organic substances. Many plant growth promoting bacteria, especially pseudomonads, produce high affinity Fe^{3+}-binding siderophores under conditions of low iron concentration. This may result in severe iron limitation in the rhizosphere, which could limit the growth of other rhizosphere bacterial and fungal pathogens. Many authors have demonstrated the importance of siderophores in the inhibition of both fungal and bacterial pathogens.

The siderophore production was detected by using chrome azurol-S (CAS). The assay is based on the use of Petri dishes in which the surface was split using half for the cultivation medium and half for the CAS detection medium. The isolated bacteria were grown on MM9 growth medium that was derived from M9 medium, containing per liter: $Na_2HPO_4.7H_2O$ 64 g, KH_2PO_4 15 g, NaCl 2.5 g, NH_4Cl 5 g. The MM9 medium growth was prepared by taking 200 ml of M9 salts (5x) added to $MgSO_4$ (1M) 2 ml, $CaCl_2$ (1M) 0.1 ml, 20 ml of appropriate

carbon sources solution (20 % glucose); for solid medium, just before autoclaving, add 15 g/l Bacto Agar.

CAS medium was prepared according to Schwyn and Neilands (1987). The medium for a liter of overlay was as follows: Chrome azurol S (CAS) 60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 ml; Agar (0.9 %, w/v) was used as gelling agent.

Siderophore detection was achieved after 15 ml (standard, 90 mm diameter Petri dishes) overlays of this medium were applied over those 24 h incubated agar plates containing cultivated microorganisms to be tested for siderophore production. After a maximum period of 15 min, a change in color will be observed in the overlaid medium, exclusively surrounding producer microorganisms, from blue to purple (as described in the traditional CAS assay for siderophores of the catechol type) or from blue to orange (as reported for microorganisms that produce hydroxamates) (figure 10 “a”). These experiments were made with three replicates for each isolate.

3.4.2. Production of Indole-3-acetic acid (IAA)

One of the direct mechanisms by which rhizobacteria promote plant growth, is the production of plant growth regulators or phytohormones (IAA) which, when applied to plants help in increasing plant yield and growth. Moreover, the IAA hormone is known to have dual role in influencing plant growth, by involving in the biocontrol together with glutathione-s-transferases in defense-related plant reactions and inhibits the germination of spore and growth of mycelium of different pathogenic fungi.

Production of IAA was determined by growing the bacterial colony onto LB medium amended with L-tryptophane and sodium dodecyl sulfate, and the production of IAA was identified by a color change of the Salkowski’s reagent from yellow to red-orange.

Production of IAA was determined following the standard Bric *et al.* (1991) method with some modifications. Briefly, overnight grown single colony was streaked onto LB medium agar, which contains (per liter): tryptone 10 g, yeast extract 5 g, NaCl 5 g, agar 15 g amended with 0.06 % sodium dodecyl sulphate and 1 % glycerol. After incubation for 24 h at 27±1 °C the single grown colony was allowed to grow for 3 days at 27±1 °C in a LB liquid medium with some modifications to LB medium agar by adding 5 mM L-tryptophan (1g/1l) and elimination of agar. After incubation in the agitator for 3 days, 1 ml of the bacterial

suspension was centrifuged for 10 min at 10,000 rpm, and then the surnatant was added to Salkowski's reagent (Gordon *et al.*, 1951) having the formulation of: 2 % of 0.5M ferric chloride in 35 % perchloric acid. After 1 h of incubation at room temperature, the production of IAA was identified by a color change of the Salkowski's reagent from yellow to red-orange (figure 10 "b").

3.4.3. Mineral phosphate solubilization assays

Phosphorus is one of the major plant nutrients limiting plant growth. Most of the essential plant nutrients, including phosphorus, remain in insoluble form in soil **(**Abd-Alla, 1994; Yadav *et al.*, 1997). A large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application by its conversion into calcium or magnesium salts in soils and becomes unavailable to plants (Yadav *et al.*, 1997). Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability. Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus. Soil microorganisms involve transforming the insoluble forms of phosphorus into soluble forms and thus influence the subsequent availability of phosphate to plant root. Phosphate solubilizing microorganisms have been employed in agriculture and horticulture and have been considered very important due to their potential of ecological amelioration.

Phosphate-solubilizing bacteria (PSB) were tested by plate assay using NBRIP growth medium (National Botanical Research Institute's phosphate) (Nautiyal, 1999) based on AT salts which contained per liter the following ingredients: glucose 20 g, $Ca_3(PO_4)_2$ 5 g, $MgCl_2$ $6H_2O$ 10 g, $MgSO_4$ $7H_2O$ 0.25 g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.1 g, The pH of the media was adjusted to 7.0 before autoclaving, the media was supplemented with 15 g agar. A fresh culture of the tested bacteria was placed on the NBRIP medium plates and the presence of transparent halo around the colony (figure 10 "c"), observed after 3 days of the plates incubated at 27±1 °C, indicates the solubilzation of phosphate.

Fig 10. *In vitro* detection of bacterial Plant Growth Promoting (PGP) features: Siderophores production (a), Indole-3-acetic acid (IAA) production (b), Phosphate-solubilization (c)

3.5. Standardization of organic growing media for melon crops

3.5.1. Experimental site

The present study is designed to develop organic amendment substrate that can serve as growing media for organically melon grown crops. The specific goals were to assess the horticultural traits of six organic growing substrates in terms of their suitability to serve as a growing medium. Compost, coconut fiber and peatmoss were tested as a based growing media using as indicator plant the melon (cv. *Proteo*) grown under organic conditions. The prepared volume of each medium was mixed with sea-bird guano at a rate of 0.25 % medium, as N source, before planting.

The experiment was run at two different growing seasons in the greenhouse at the department of plant protection of Tuscia University of Viterbo. The first experiment was done during June – July 2009, while the second one during April – May 2010. Both experiments were carried out with six trials composed of six substrate types:

T1 - MAIB growing media (MAIB) (control);

T2 – 50 % peatmoss + 50 % (MAIB);

T3 – 30 % coconut fiber (CF) + 70 % (MAIB);

T4 – 20 % commercial compost (CC) + 80 % (MAIB);

T5 – 40 % (CC) + 60 % (MAIB);

T6 – 30 % (CF) + 20 % (CC) + 50 % (MAIB).

The material's characteristics used as MAIB substrate are reported in table (6). The MAIB mix is prepared by mixing all components together and adding a water solution of micronutrients. The volume of each substrate was prepared in the Integrated Pest Management Department of the Mediterranean Agronomic Institute of Bari, then labeled and stored into big bags in order to be sent to the plant protection department of Tuscia

University. Sowing was done in expanded polystyrene trays, containing 5 cells, a seed being put in the center of each tray cell. The experiment was conducted in randomized block design, with four repetitions of each trial. The experimental unit was made up of 20 plants, mean germination time (MGT), germination percentage (G), the height of the plant (H), the length of the root (L), shoot (SFW) and root (RFW) fresh weight, plus the shoot (SDW) and root (RDW) dry weight were appraised.

Moreover, in order to evaluate some microbial communities present in those organic substrates, fluorescent pseudomonads and aerobic spore-forming bacteria were isolated and quantified from the bulk soil, the rhizosphere and the rhizoplane of melon plants grown in those substrates.

Table 6. The main components of MAIB growing media

Component	**Percentage (%)**
Sand	20
Pumice	20
Perlite	10
Sulfur	0.04
Poultry manure based (Guanito)	0.25
Micronutrients	0.02

3.5.2. Media ingredients

3.5.2.1.Inert materials

The major inert materials used in the trial are peatmoss (VIGORPLANT Company, Italy), sand, pumice (Mongelli Company) and perlite (AGRI LIT Company). In the case of perlite, the chemical characteristics are reported on their bags (table 7), while for the peatmoss originated from the acid sphagnum peat was based on organic carbon and nitrogen of biological origin.

Table 7. Characteristics of perlite

Elements	**Value**
pH	6.5-7.5
Toxicity	Un-toxic for plants and animals
Source	Natural
Type	Inert
The odor	Hasn’t odor
Degradability	Stable (un-degradable)
Sterilization	Sterilized

3.5.2.2.Organic fertilizer

Guanito is a commercial product produced by ITAL POLLINA Company. Its source is poultry manure (table 8), N source. Guano was selected as fertilizer as it is commonly used by organic farmers and is readily available in most parts of the world.

Table 8. Characteristics of Guanito

Elements	**Value**
pH	6.5
Humidity	7 %
Total Nitrogen	6 %
Total organic carbon	32 %
Organic matter	52 %
Phosphorus	15 %
Potassium	3 %
Calcium	10 %
Magnesium	2 %
Humic and Fulvic Acid	11 %
Microelements (Fe, B, Zn, Mn, Mo)	1 %

3.5.2.3.Coconut fiber and commercial compost

The good physical properties of the coconut fiber, its non-reaction with the nutrients from fertilization, its long durability without alteration of physical characteristics, the sterilization possibility, the abundance of the raw material that is renewable and the low cost for the producer makes green coconut fiber a substrate difficult to replace with other material types, mineral or organic, in vegetable cultivation.

The main characteristics of ECOS commercial compost used in this study was mentioned before (section 3.1.1.1).

3.5.2.4.Micronutrients

The micronutrients used in this study are a commercial product (Micro-Z G) produced by VALAGRO Company and its components are shown in table (9).

Table 9. The components of micronutrients commercial product (Micro-Z G)

Elements	MgO	SO3	B	Cu	Fe	Zn
Percentage	10 %	26 %	0.2 %	0.1 %	6 %	1 %

3.5.3. Parameters and measurements

Parameters of this study are divided into two groups. The first one was the evaluation of the effect of the studied substrates on the plant growth during the trial period. At the first growing phase mean germination time (MGT), germination percentage (G) and chlorophyll

level (C) were assessed; at the end of the experiment (50 days after sowing) height of the plant (H), length of the root (L), shoot (SFW) and root (RFW) fresh weight, plus shoot (SDW) and root (RDW) dry weight were estimated. The second one was the quantification of bacterial population isolated from bulk soil, rhizosphere and rhizoplane ("a" and "b") of melon seedlings grown in those growing media.

3.5.3.1.Plant growth parameters

3.5.3.1.1. Germination percentage (G) and mean germination time (MGT)

Germination was evaluated for 20 days, and the germinated seeds were examined every 2 days. Germinated seeds being considered those that emitted the caulicle.

Mean germination time (MGT) in days was calculated according to Ellis and Roberts (1980):

MGT = Sum(D*n)*/Sum*(n)*

n: number of seeds germinated on day (D),

D: number of days counted from the beginning of the germination test.

3.5.3.1.2. Chlorophyll level

Minolta SPAD 502 chlorophyll meter was used to measure the chlorophyll level of the seedling leaves with a unit of Soil Plant Analysis Development (SPAD) values. Number of leaves measured was around 3 leaves/plant, with a time interval of 1 week after 22 days of seeds sowing.

3.5.3.1.3. Plant height (H)

The height of the plant was measured from the base of the hypocotyls to the seedling's apex. Ruler is used to measure the plant height of each seedling with unit of centimeter (cm).

3.5.3.1.4. Root length (L)

Development of root system for each seedling in different growing media is determined using a ruler to measure the root length. The length of the roots was measured from the root tip to the base of the hypocotyls.

3.5.3.1.5. Fresh and dry weight of the plant

Scale is used to measure fresh and dry weight of the plant: shoot (SFW) and root (RFW) fresh weight plus shoot (SDW) and root (RDW) dry weight. The samples needed to put in oven at 70±1 °C for 72 hours in order to take the dry weight of seedlings.

3.5.3.2.Colonization of bulk soil, rhizosphere and rhizoplane by bacterial communities

A total six soil and root samples were randomly collected from each trials of the first experiment, in order to evaluate the allocation of the studied bacterial population density before and after sowing of melon seeds in each organic substrates. The bulk soil samples were collected from each organic substrate before seed sowing while the rhizosphere and rhizoplane samples were collected at the end of the experiment.

Isolation and counting of bacteria from bulk soil, rhizosphere and rhizoplane "a" and "b" were performed by serial dilution technique as described in the section (3.1.2 – 3.1.3), using King's B medium for fluorescent and total viable bacteria and TSA medium for aerobic spore-forming bacteria.

3.6. *In vivo* antagonism assay against *Fusarium oxysporum* f. sp. *melonis*

The main objective of this experiment was to estimate *in vivo* the antagonist activities of the isolated bacteria against the soil borne pathogen of melon (*Fusarium oxysporum* f. sp. *melonis* (FOM)) through an artificial inoculation.

Four *Bacillus* spp., two fluorescent bacterial isolates and two *Bacillus* spp. isolated, respectively, from compost, MAIB-ECOS substrate and infected field by FOM, were tested *in vivo* by applying two treatment techniques. The first by coating the melon seeds with the selected bacterial isolates and the second by drenching the soil around the crown of the plants with bacterial suspension, to determine if they possessed the ability to suppress Fusarium wilt of melon plants.

3.6.1. Experimental site

The antagonist effect of eight microorganisms, selected in the preliminary test *in vitro* assays (table 10), against FOM F30 isolate was investigated under greenhouse conditions at Plant protection Department of Tuscia University, by applying two separately experimental designs (figure 11 "A"). The first experiment was carried out during the months of April – May 2010. The second was carried out during the months of May – June 2010.

Table 10. The characteristics of the microorganisms selected through a previous *in vitro* assays.

Isolated sites	Bacterial groups	Bacterial isolates code	Date
Water suspension of ECOS compost	SFB	B1	April – May 2010
		B2	
		B3	
		C6	
Rhizosphere of MAIB-ECOS	FPB	S.2.2	May – June 2010
Rhizoplane (a) of MAIB-ECOS	FPB	R.2.1	
Bulk soil of infected field by FOM	SFB	P8	
Rhizoplane (a) of infected field by FOM	SFB	P18	

According to the results obtained in the previous experiment concerning the standardization of organic growing media for melon crops (section 3.5) and the recently published study that described the use of peat for enhancing fusarium wilt development (Cohen *et al.*, 2008), melon seeds (cv. *Proteo*) were grown in plastic pots (5cm x 5cm) containing peat based soil mixed with vermiculite at a volumetric ratio of 2:1. The mixture was sterilized in an autoclave at 121 °C for 30 min, then dried to be ready for plantation. The peat used in the present study was manufactured by the VIGORPLANT Company mentioned in the section (3.5.2.1).

The seeds corresponding to each assay were pregerminated in a humid room at 25±1 °C in darkness, and then transplanted to pots. Four replicate pots were set up with six seedlings each for each trial, where they were placed in a random design in a growth chamber bench at 25± 5 °C.

At the first leaf stage, a 15 day-old FOM culture grown in potato dextrose broth (PDB) was added by irrigation to the pots containing plants and antagonistic microorganisms. Pathogen inoculum consisted of a mixture of conidia and chlamydospores was added to the potting mix at a rate of 10^5 conidia / g of substrate.

Concerning the microorganism treatment, the selected bacterial isolates were tested separately by applying two different techniques (i) coating the melon seeds by the bacterial isolates (bacterization of seed) (figure 11 “B”), and (ii) drenching the soil with bacterial suspension around the melon seedlings (figure 11 “C”). Treatment of the coated seed trials was done 1 day before sowing by coating the melon seeds with a CMC bacterial suspension at a concentration of 10^8 cfu / 1 ml CMC, a second bacterial inoculation for these trials was carried out 5 days after pathogen inoculation by drenching the soil with a bacterial suspension at a concentration of 10^8 cfu / 1 ml sterile distilled water (SDW). The drenching

bacterial trials were as well inoculated twice; the first inoculation was added one week prior to pathogen inoculation and the second one week after the pathogen inoculation, by irrigating the top of the potting mix around the crown of the plant with a bacterial suspension at a concentration of 10^8 cfu / 1 ml SDW.

In these test, a commercial bio-product formulation "Sublic" used as positive control and based on *B. licheniformis* and *B. subtilis* (produced by ELEP company) were drenched to the soil using the recommended concentration (5 ml of Sublic / 1 L SDW). The prepared Sublic solutions were added to the soil following the approach applied for the drenching bacterial trials (one week before and after the pathogen inoculation).

Plants without antagonistic microorganisms and infected with FOM were used as negative control, and one more control trial was consisted of plant not inoculated by the pathogen and not treated by the antagonistic microorganisms.

Trials of the first experiment (table 11) were consisted of: (A) plants not inoculated with pathogen and not treated with antagonistic microorganisms; (B) plants inoculated with *F. oxysporum* f. sp. *melonis* (FOM) and not treated with any antagonistic microorganisms; (C) plants inoculated with FOM F30 isolate and treated with "*Sublic*" formulation; (D), (E), (F) and (G) plants inoculated with FOM F30 isolate and treated by coating the melon seeds with B1, B2, B3 and C6 isolate, respectively; (H), (I), (J) and (K) plants inoculated with FOM F30 isolate and treated by drenching the soil with B1, B2, B3 and C6 bacterial suspension, respectively.

Trials of the second experiment (table 12) consisted of: (I) plants not inoculated with pathogen and not treated with antagonistic microorganisms; (II) plants inoculated with FOM F30 isolate and not treated with any antagonistic microorganisms; (III) plants inoculated with FOM F30 and treated with "*Sublic*" formulation; (IV), (V), (VI) and (VII) plants inoculated with FOM F30 and treated by coating the melon seeds with S.2.2, R.2.1, P8 and P18 isolate, respectively; (VIII) (IX) (X) and (XI) plants inoculated with FOM and treated by drenching the soil with S.2.2, R.2.1, P8 and P18 bacterial suspension, respectively.

3.6.2. Production of spore forming bacteria (SFB)

The spore forming bacteria preparation were developed following the procedures similar to El-Hassan and Gowen (2006). Fresh cells of SFB were grown on nutrient broth (NB; Oxoid, Basingstoke, UK) by a fermentation process using 150 ml of NB plus 2 % glucose, in 250 ml Erlenmeyer flasks, until sporulation was completed. Each flask was inoculated with 24 h old

bacterial culture grown on NA plates and incubated at 25±1 °C on constant rotary shaker at 150 rpm for 5 days. Biomass spores of SFB were harvested by centrifugation of the culture broth at 4,100 *g*, 20 °C for 30 min in 150 ml sterile universal graduated conical plastic tubes. The pelleted bacterial spores were washed twice with cold 1M NaCl and thrice with sterilized 0.05M phosphate buffer saline (PBS, pH 7.0) solution, re-suspended and centrifuged again under similar conditions. The pellet was resuspended in a small amount of sterile carboxymethyl cellulose (CMC) for the coated seed trials and of sterile distilled water (SDW) for the drenching suspension trials and then diluted with an adequate amount of CMC and of SDW to obtain a bacterial suspension of 0.2 optical density (10^8 cfu ml^{-1}).

3.6.3. Production of fluorescent pseudomonads bacteria

The production of fluorescent pseudomonads was done following Ramamoorthy *et al.* (2002) method with some modifications. The bacterial isolates were grown in Erlenmeyer flasks (250 ml) containing 150 ml of NB broth with 2 % of glucose for 48 h on a rotary shaker at 27±1 °C. Cells were removed by centrifugation at 8,000 *g* for 10 min at 4 °C in 150 ml sterile universal graduated conical plastic tubes and then washed in sterile distilled water. The pellet was resuspended in a small amount of sterile CMC for the coated seeds trials and of SDW for the drenching suspension trials and then diluted with an adequate amount of CMC and of SDW to obtain a bacterial suspension of 0.2 density (10^8 cfu ml^{-1}).

3.6.4. Seed preparation

Seeds of melon for the coated and drenched trials were surface sterilized by 2.5 % (vol/vol) of household bleach (NaOCl), washed three times in sterile distilled water (SDW) with the final wash lasting 5 min. The seeds of the drenched trials were dried on filter paper under a laminar airflow cabinet for at least 2 h.

Whereas, the seeds of the coated trials were immersed in the CMC bacterial suspension for 10 min, (approximately 10 g seeds were moistened with 0.2 ml of a 1 % solution of CMC, as a sticking agent), the entire mixture was shaken in a plastic box for 1 min to form an even coating of the seeds. The seeds of the (A) and (I) trials were immersed in the CMC solution without the presence of bacterial isolates. Then the coated seeds were dried to be putted after that with the other seed trials in a humid room at 25 °C in darkness for a pregermination.

3.6.5. Preparation of inocula (FOM)

In collaboration with the researcher group of Prof. Chilosi, fungal inocula were prepared by growing in potato dextrose broth (PDB) containing 7 g of PDB per 1 liter. One hundred ml of sterile PDB in an Erlenmeyer flask was inoculated with agar plugs (5 mm) from 7 day-old FOM F30 isolate grown on PDA medium. Cultures in liquid medium were incubated for 15 days at 24±1 °C on a rotary shaker at 150 rpm and then the supernatant was filtrated to remove spores and mycelium. Then fungi conidial suspensions were prepared in sterile distilled water at a rate of 10^5 propagules g^{-1} of substrate. Pathogen inoculum, consisted of a mixture of conidia and chlamydospores, was directly used and added to each pot at a rate of 20 ml of FOM inoculum. The pathogen suspension was added by irrigating the top of the potting mix around the crown plant which contain the melon seedlings at the first-leaf stage and antagonistic microorganisms. The inoculated and the uninoculated pots were placed in a random design in greenhouse.

3.6.6. Data analysis

Each treatment was consisted of four replicate pots and each pot was dived by six sub-pots (5cm x 5cm) that every sub-pot contains one plant. Disease severity and area under the disease progress curve were monitored for 2 to 3 weeks after inoculation. Plants were harvested at 40 – 45 days (figure 11 ”D”) and plant height, root length, root and shoot weight (wet and dry) were measured.

3.6.6.1.Disease severity (DS) and area under the disease progress curve (AUDPC)

Symptom severity of the areal parts of the plants was assessed (8, 10, 12, 14 days after pathogen inoculation) using the following index (table 13): 0, no symptoms; 1, yellowing of the cotyledons or the first leaf; 2, yellowing of two leaves; 3, yellowing of three or more leaves; 4, died plant. The disease severity index was calculated using the formula (Soriano-Martin *et al.*, 2006):

$$\text{Disease severity index (\%)} = \frac{\sum ni\, si}{4N} \text{ x } 100$$

Where *n*i is the number of plants affected by each degree of severity, *s*i the degree of severity of the attack (0 – 4) and *N* the total number of plants used for each energy level applied.

The area under the disease progress curve (AUDPC) for melon plants was calculated for 8, 10, 12, 14 days using the method that was described by Shaner and Finney (1977). Each AUDPC was calculated using the formula:

$$AUDPC = \sum_{i=1}^{n-1} \left[\frac{(t_{i+1} - t_i)(d_i + d_{i+1})}{2} \right]$$

where t is days after transplanting, d is the percentage of disease severity caused by *F. oxysporum* f. sp. *melonis* at each recording date (i), and n is the number of estimations. The AUDPC was calculated for each trial using an Excel spreadsheet.

3.6.6.2.Growth measures

The growth measures (plant height, root length, fresh and dry weight of plants and roots) were assessed following the same methods described in the previous section (3.5.3.1). The plant and root parameters were recorded separately after 45 days of the experiment, where the plants were uprooted and washed under running tap water.

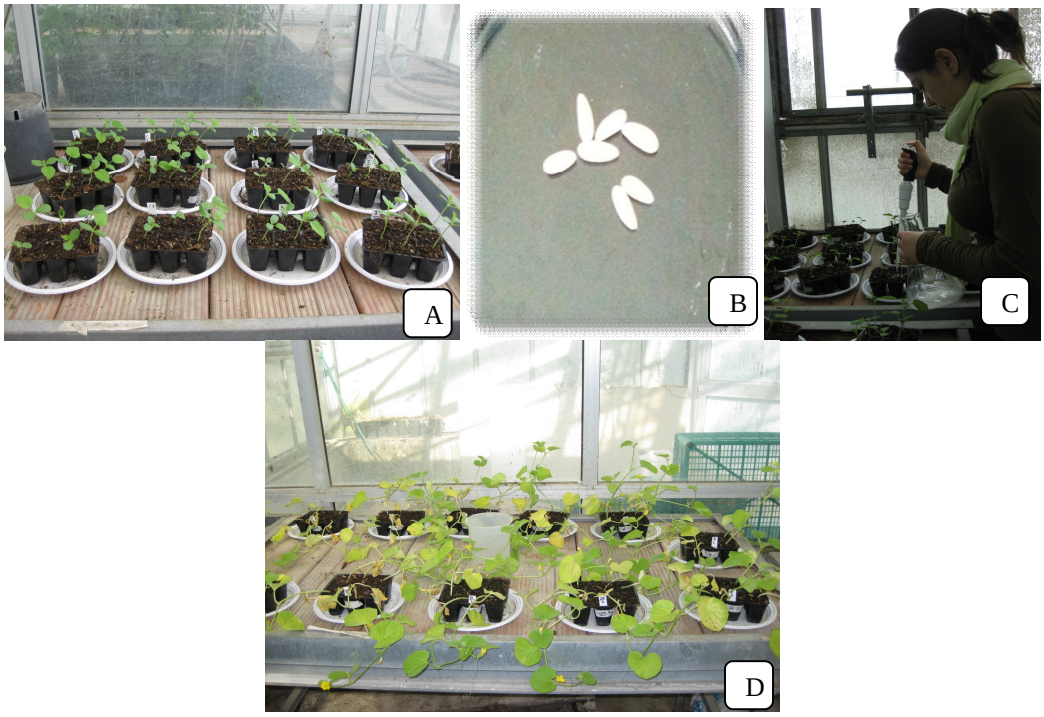

Fig 11. Fusarium wilt caused by FOM F30 isolate on melon in greenhouse experiment (A). The bacterial isolates were tested separately by applying two different techniques of treatment: (B) melon seeds coated by the bacterial isolates and (C) drenching a bacterial suspension in the soil around the melon plants. Plants at the end of the experiment (D)

Table 11. Characteristics of the bacterial and pathogen inoculation techniques of the first experiment

Trials code	Treatment of the experiment	Bacterial and pathogen inoculation techniques	First inoculation	Second inoculation
A	Control	-	-	-
B	FOM (Pathogen)	Irrigating the pots by the pathogen inoculums	At the first-leaf stage of melon seedlings	-
C	FOM + Sublic	Drenching Sublic suspension	One week before pathogen inoculation	One week after pathogen inoculation
D	FOM + B1 isolate	Bacterization of melon seed	Seed coated by CMC bacterial suspension one day before sowing	Drenching bacterial suspension 5 days after pathogen inoculation
E	FOM + B2 isolate			
F	FOM + B3 isolate			
G	FOM + C6 isolate			
H	FOM + B1 isolate	Drenching bacterial suspension	Bacterial suspension drenched one week before pathogen inoculation	Bacterial suspension drenched one week after pathogen inoculation
I	FOM + B2 isolate			
J	FOM + B3 isolate			
K	FOM + C6 isolate			

Table 12. Characteristics of the bacterial and pathogen inoculation techniques of the second experiment

Trials code	Treatment of the experiment	Bacterial and pathogen inoculation techniques	First inoculation	Second inoculation
I	Control	-	-	-
II	FOM (Pathogen)	Irrigating the pots by the pathogen inoculums	At the first-leaf stage of melon seedlings	-
III	FOM + Sublic	Drenching Sublic suspension	One week before pathogen inoculation	One week after pathogen inoculation
IV	FOM + S.2.2 isolate	Bacterization of melon seed	Seed coated by CMC bacterial suspension one day before sowing	Drenching bacterial suspension 5 days after pathogen inoculation
V	FOM + R.2.1 isolate			
VI	FOM + P8 isolate			
VII	FOM + P18 isolate			
VIII	FOM + S.2.2 isolate	Drenching bacterial suspension	Bacterial suspension drenched one week before pathogen inoculation	Bacterial suspension drenched one week after pathogen inoculation
IX	FOM + R.2.1 isolate			
X	FOM + P8 isolate			
XI	FOM + P18 isolate			

Table 13. Severity of symptoms was assessed using the following empirical scale with five classes of severity

Classes	**Symptom severity**	**Photo**
0	Symptomless	
1	Yellowing of the cotyledons or the first leaf	
2	Yellowing of two leaves	
3	Yellowing of three or more leaves	
4	Death of the plants	

3.7. Molecular characterization of antagonistic bacteria

According to the previous *in vitro* and *in vivo* assays two bacterial isolates (B2 and B3), belonging to spore forming bacteria group (Gram-positive), possessing the ability to suppress Fusarium wilt of melon plants were selected. In order to characterize these selected bacterial isolates, molecular analyses have been used for genetic identification studies.

3.7.1. DNA extraction

The genomic DNA of bacteria was extracted using a bacterial genomic DNA extraction kit (Pure link^{TM}, Genomic DNA Mini Kit, Invitrogen Srl., USA, Cat#:K1820-01). For this purpose, the bacterial isolates were grown in Petri dishes containing nutrient Agar (NA). After 48 h of incubation at 27±1 °C, the bacterial pellet of each isolates were collected and suspended in 180 µl of lyzozyme solution prepared by mixing 200 µl of lyzozyme digestion buffer (LDB) (25 mM Tris HCl; 2.5 mM EDTA; 1 % Triton X-100) with lyzozyme enzyme to reach a concentration of 20 mg/ml. After an incubation in thermocycler for 30 min at 37 °C, 20 µl of the proteinase K solution were added to each sample, followed by 200 µl of Genomic Lysis / Binding buffer, then vortexed thoroughly (about 15 seconds) and incubated at 55 °C for 30 min. After this step 200 µl of ethanol (95-100 %) were added to the lysate and mix thoroughly by vortexing for 5 - 10 seconds.

The purification phase was completed by transferring the entire contents of the tube into the Genomic Spin Columns, centrifuge for 1 min at 10000 rpm, then discards the collection tube containing the eluate and place the column in a new 2 ml collection tube. A wash phase was made by adding 500 µl of wash solution 1 to the column and centrifuge for 1 min at 10000 rpm, the collection tube containing the eluate was discarded and the column was placed in a new 2 ml collection tube. Second wash was proceeded by adding 500 µl of wash solution 2 to the column and centrifuge for 3 min at 14600 rpm, the collection tube containing the eluate was discarded and the column was placed in a new 2 ml collection tube. Last phase was done by adding 200 µl of elution solution, to increase the elution efficiency incubate the tube for 1 min at room temperature then centrifuge for 2 min at 14600 rpm to elute the DNA. The eluate containing pure genomic DNA was stored at -20 °C, and the DNA was quantitated by using an optical fluorimeter instrument (Qubit).

3.7.2. DNA quantification

The concentration and the quality of the genomic DNA was determined by optical fluorimeter instrument (Qubit), calibrated with an appropriate reagent kits which are used in the samples preparation for their reading.

Selected quantification program BR was proceeded with the preparation of standards for the calibration of the Qubit instrument and the quantification of the samples.

In a 1.5 µl Eppendorf tube is prepared an initial solution composed of 1 µl of component A (ds DNA BR reagent) and 199 ul of component B (ds DNA BR buffer) for each standard and sample.

STANDARD 1: in a Qubit Eppendorf 190 µl of the initial solution are mixed with 10 µl of component C (ds DNA BR standard 1).

STANDARD 2: in a Qubit Eppendorf 190 µl of the initial solution are mixed with 10 µl of component D (ds DNA BR standard 2).

Samples: in a Qubit Eppendorf 190 µl of the initial solution are mixed with 10 µl of each extracted DNA.

After vortexing, the standards and samples are incubated at room temperature for 2 minutes, currently the cleaned eppendorf was quantified with at least three readings. Quantification will be done in ng/µl.

3.7.3. Identification by polymerase chain reaction (PCR) technique

Bacterial 16S rRNA genes were amplified by PCR using a combination of bacterial primer NOC1F (AGA GTT TGA TCA TGG CT CAG) and NOC3R (ACG GTT ACC TTG TTA CGA CTT). The amplification were conducted in a volume of 25 µl containing, 1 µl of the extracted genomic DNA, 13 µl of master mix (Promega, Madison WI-U.S.A), 8 µl of RNAase free water (Promega, Madison WI-U.S.A), 1.5 µl each of forward and reverse primers (Primm company).

The amplification was carried out in the thermal cycling (Mastercycler). PCR amplification and sequencing conditions were consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation step at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A final extension for 5 min at 72 °C was done at the end of the amplification.

To visualize the amplification of the expected size fragment, 5 µl of each PCR sample was analyzed by agarose gel stained with Gel Red colorant (Sigma, St. Louis, Mo.). Amplicons

were separated by electrophoresis in 1.5 % agarose gels submarine with TAE buffer and visualized by UV light.

3.7.4. DNA sequencing and phylogenetic analysis

The clones from each unique amplified genomic DNA were submitted to the Macrogen Genomics Institute for sequencing. The resulting sequences were compared with those available in GenBank by use of the BLAST network service to determine their phylogenetic affiliation. Finally, the 16S rRNA gene sequences have been deposited in the NCBI nucleotide sequence database to identify the analyzed strains. (http://www.macrogen.com/eng/macrogen/macrogen_main.jsp/, http://www.ncbi.nlm.nih.gov/).

4) Results

4.1. Isolation and quantification of bacteria from compost (ECOS), organic growing substrate (MAIB-ECOS), and infected field by FOM

4.1.1. Analysis of bacterial population density isolated from the bulk soil of ECOS compost substrate

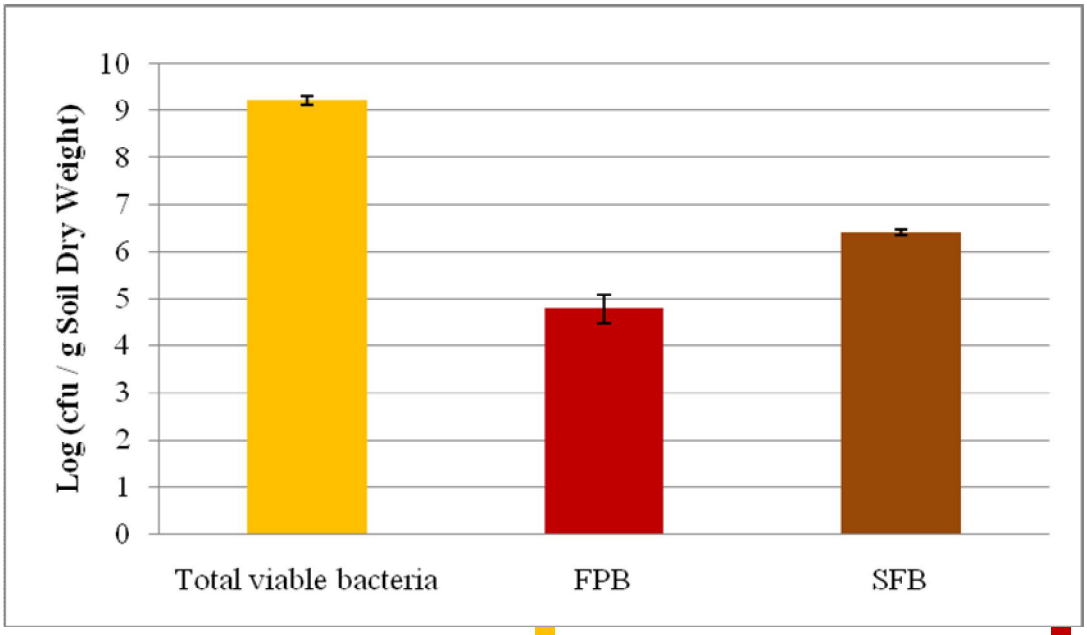

Fig 12. Total numbers of viable bacteria ( ), fluorescent pseudomonads (FPB) ( ) and aerobic spore-forming bacteria (SFB) ( ) isolated from bulk soil of ECOS compost substrate. The bars chart indicate the confidence limits (P = 95 %)

Microorganisms in ECOS compost that may have a suppressive effect were isolated, in which the obtained results indicated a variation in bacterial populations as well as in the number of cfu / g of soil dry weight.

After 48 h of incubation at 27±1 °C, fluorescent pseudomonads (FPB) and spore forming bacterial (SFB) groups were counted respectively from plated KB and TSA medium. The compost appears to be highly colonized by the bacterial population, where the total number of viable bacteria was around 1.67 x 10^9 cfu/g of soil dry weight (figure 12). The predominant bacterial taxa isolated from compost were belonging to SFB bacterial group with a value of 2.59 x 10^6 cfu/g of soil dry weight, while the FPB group represents a value of 6.23 x 10^4 cfu/g of soil dry weight.

4.1.2. Analysis of bacterial population density isolated from Bulk soil and rhizosphere of MAIB-ECOS substrate and infected field by FOM

The analysis of bacterial populations was carried out on bulk soil of MAIB-ECOS substrate and of infected field by FOM and on rhizosphere of melon seedlings grown in this substrate and in the infected field.

Results of bacterial population isolated from both sites (bulk soil and rhizosphere) of MAIB-ECOS substrate show a variation in bacterial number of cfu/g of soil dry weight. The larger amount of total viable bacteria was isolated from the bulk soil (1.75×10^8 cfu/g of soil dry weight), while the values of total viable bacteria were found slightly lower in those samples taken from the rhizosphere (6.13×10^7 cfu /g of soil dry weight) (Figure 13). The same amount of the studied bacterial groups (FPB and SFB) was obtained from bulk soil and rhizosphere of MAIB-ECOS substrate, with a value around 2.56×10^5 for FPB and 4.44×10^5 for SFB, no significant differences in number of isolates were observed.

Like as the MAIB-ECOS substrate, the number of the total viable bacteria isolated from bulk soil (4.54×10^9 cfu/g of soil dry weight) of infected field by FOM was significantly more than those isolated from rhizosphere (1.31×10^7 cfu/g) (figure 14). In addition, the larger amount of FPB (1.32×10^8 cfu/g) and SFB (3.30×10^9 cfu/g) groups were isolated from the bulk soil of infected melon plant, while it was lower in those samples taken from the rhizosphere (1.37×10^6 cfu/g of FPB and 4.56×10^6 cfu/g of SFB). As well, a slight difference in number of cfu between the FPB and SFB groups isolated from bulk soil site of infected field was appreciated.

The number of bacterial population were noticeable different between the MAIB-ECOS substrate and the infected field by FOM, especially those isolated from bulk soil site; the infected field samples have shown the higher number of cfu/g soil dry weight.

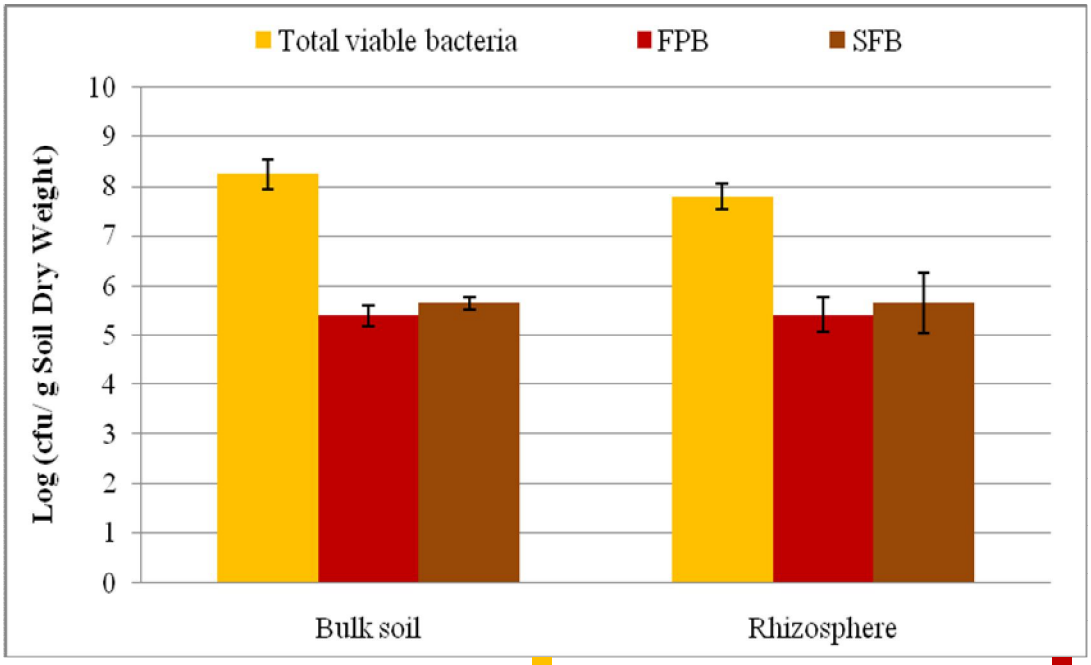

Fig 13. Total numbers of viable bacteria ( ), fluorescent pseudomonads (FPB) ( ) and aerobic spore-forming bacteria (SFB) ( ) isolated from bulk soil of MAIB-ECOS and rhizosphere of melon seedlings grown in MAIB-ECOS substrate. The bars chart indicate the confidence limits(*P* = 95 %)

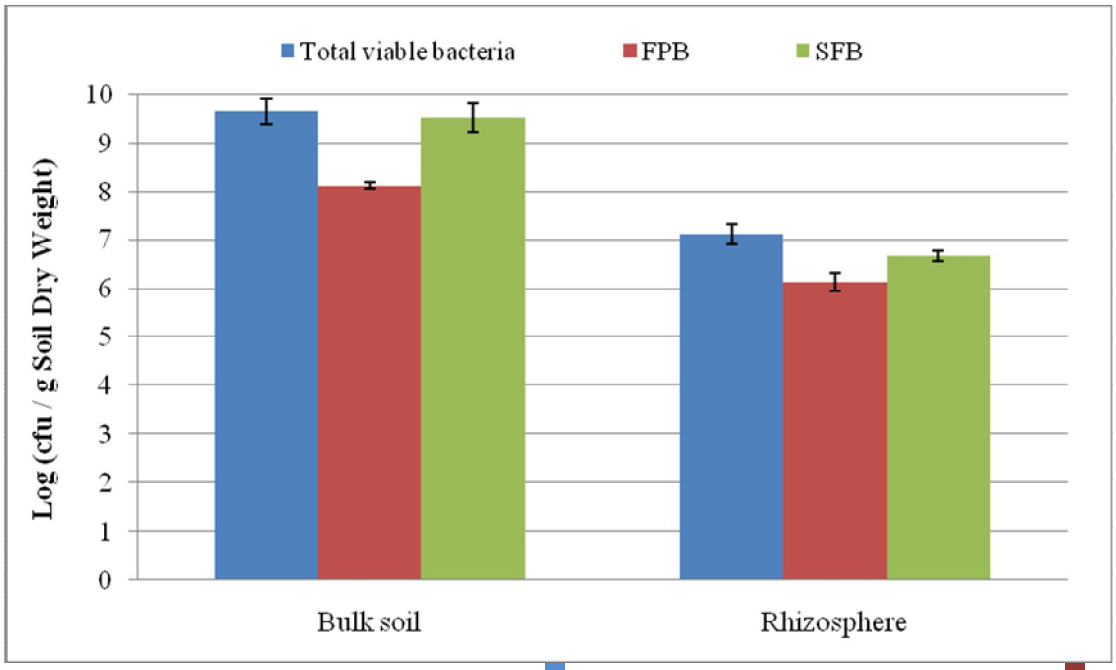

Fig 14. Total numbers of viable bacteria ( ), fluorescent pseudomonads (FPB) ( ) and aerobic spore-forming bacteria (SFB) ( ) isolated from bulk soil of infected field and rhizosphere of melon seedlings grown in infected field by FOM. The bars chart indicate the confidence limits (*P* = 95 %)

4.1.3. Analysis of bacterial population density isolated from rhizoplane “a” and “b” of MAIB-ECOS substrate and infected field by FOM

To analyze bacteria from rhizoplane, two experimental procedures were utilized in order to count and isolate bacteria loosely adhering to root surface (rhizoplane a) and bacteria tightly adhering to the rhizoplane and/or endophyte (rhizoplane b).

The number of culturable bacteria present on the surface of 1 g root (rhizoplane a) of melon plant grown in MAIB-ECOS substrate was higher than that endophyte bacteria (rhizoplane b), in which the total number of viable, fluorescent and spore forming bacteria isolated from rhizoplane “a” was respectively 5.01×10^7, 1.14×10^6 and 4.71×10^4 cfu/g of root fresh weight. While a further reduction in number of viable, fluorescent and spore forming bacteria present in rhizoplane “b” was observed with a value of 2.16×10^6; 6.78×10^5 and 3.24×10^2 cfu/g of root fresh weight, respectively (figure 15). The population density of viable and fluorescent bacteria isolated from both isolate sites was elevated than those of SFB.

Unlike the MAIB-ECOS samples, the viable and fluorescent bacteria present in the infected field samples were highly quantified from the rhizoplane “b” that shown a number of 9.65×10^8 and 4.20×10^7 cfu/g of root fresh weight respectively, while the aerobic spore-forming bacteria were highly quantified from the rhizoplane “a” (1.73×10^5 cfu/g of root fresh weight) (figure 16). As for the MAIB-ECOS samples, the viable and fluorescent bacteria isolated from rhizoplane “a” and “b” of melon seedlings grown in infected field represent the highest quantity of population density.

The obtained results showed that the aerobic spore-forming bacteria isolated from MAIB-ECOS substrate and infected field by FOM were extremely observable in the rhizoplane “a” of melon seedlings.

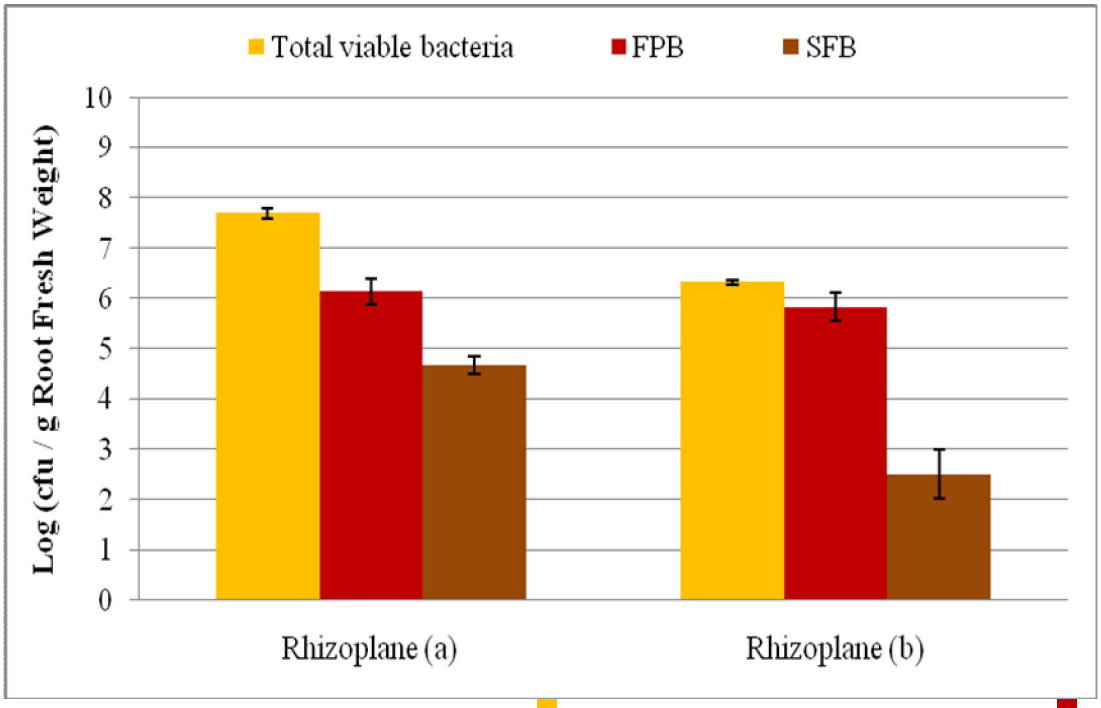

Fig 15. Total numbers of viable bacteria ( ), fluorescent pseudomonads (FPB) ( ) and aerobic spore-forming bacteria (SFB) ( ) isolated from rhizoplane ("a" and "b") of melon seedlings grown in MAIB-ECOS substrate. The bars chart indicate the confidence limits (*P* = 95 %)

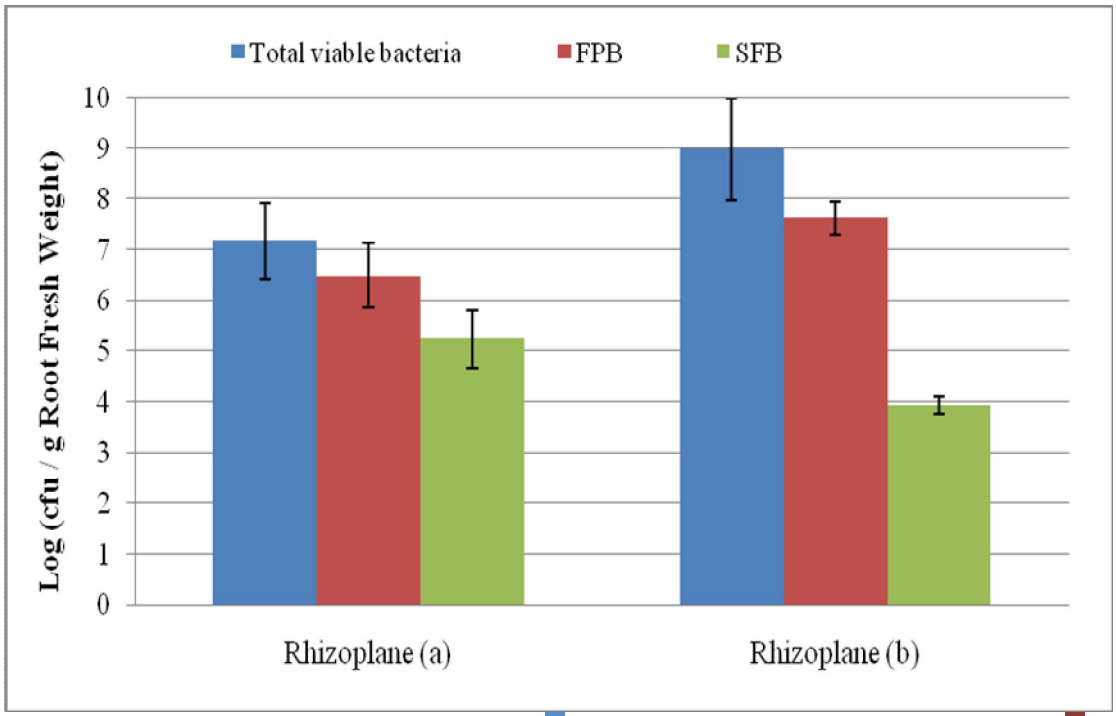

Fig 16. Total numbers of viable bacteria ( ), fluorescent pseudomonads (FPB) ( ) and aerobic spore-forming bacteria (SFB) ( ) isolated from rhizoplane "a" and "b" of melon seedlings grown in infected field by FOM. The bars chart indicate the confidence limits (*P* = 95 %)

4.2. Antagonistic effect of the matured compost (ECOS)

4.2.1. *In situ* antagonistic effect of matured compost

The qualitative results taken after 5-days incubation of *Monosporascus cannonballus* (MC-R) and 7-days incubation of *F. oxysporum* f.sp. *melonis* (FOM F30) showed that the mycelial growth of the indicator fungi was faster and larger in the case of using autoclaved compost than using unautoclaved compost. The figure also indicated that FOM F30 (figure 17 “b”) was less affected with ECOS matured compost than MC-R (figure 17 “a”) that was highly affected. The antagonistic effect appeared as an inhibition zone around the location of the compost on the medium surface that was inoculated with the fungus.

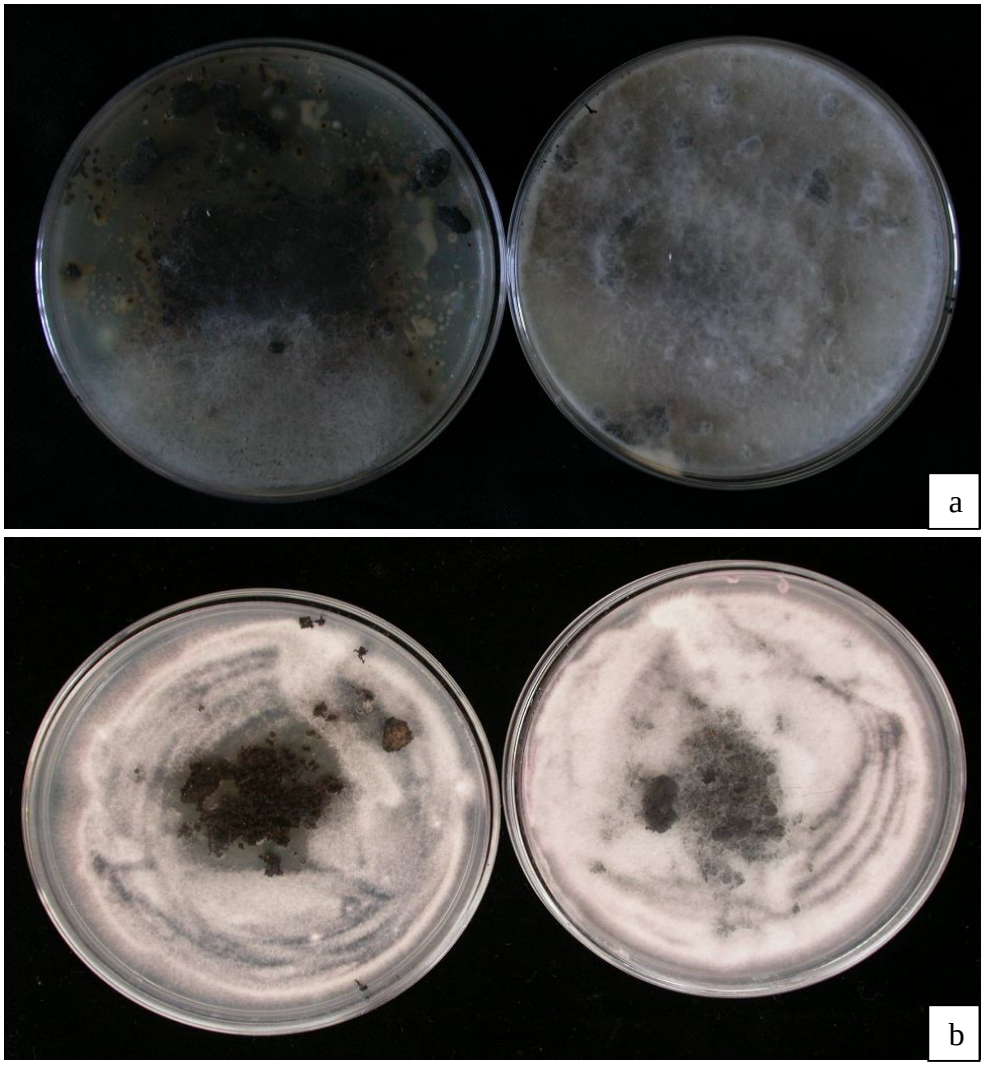

Fig 17. The suppression effect of ECOS compost on the hyphal growth of MC-R (a) and of FOM F30 (b). The left and the right plate in each photo, represent respectively the unautoclaved and autoclaved compost. The unautoclaved plates showed clear suppressive zone and weak fungal growth

4.2.2. *In vitro* antagonism assay for bacterial extraction from water suspension of ECOS compost

The antagonistic effect of the bacterial isolates, present in the water suspension of ECOS compost, against the indicator fungi was examined using the *in vitro* assay. The test indicated that water suspension of ECOS compost contains large varied population of bacteria that play a significant role in disease control. The results of the *in vitro* assay assist to screen the bacterial colonies possessing the suppressive effect against the phytopathogonic fungi.

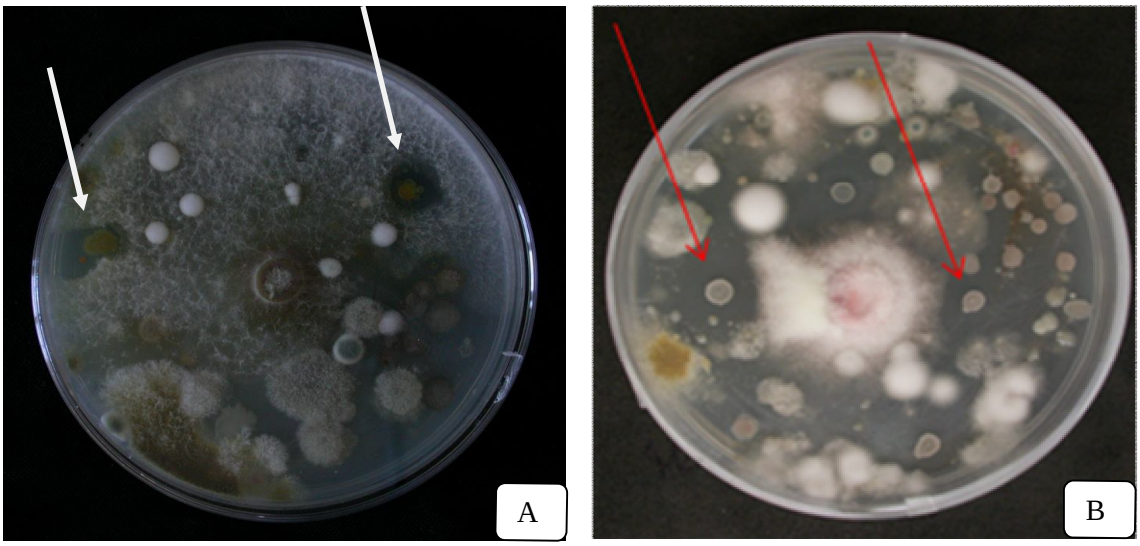

Fig 18. The arrows indicate the bacterial colonies present in the water suspension of ECOS compost possessing an antagonistic effect (shown as inhibition zone) against MC-R (A) and FOM F30 (B)

This method was extremely promising to select the bacterial strains present in the compost that have an important role in inhibiting the hyphal growth of MC-R (figure 18 “A”) and of FOM F30 (figure 18 “B”), where 18 bacterial colonies showing an inhibition zone (in which 5 of them were collected from the MC-R plates and 13 isolates were collected from FOM F30 plates) were selected and purified to be tested *in vitro* for their antagonistic effect by applying the dual culture-plate method. These selected bacteria belong to spore forming bacterial group (*Bacillus* spp.). The results of dual culture-plate method are presented in Table 14. In case of *F. oxysporum* f. sp. *melonis* (FOM F30) only four bacterial isolates collected from FOM F30 plates (B2, B2, B3, C6) gave an antagonistic effect against this fungus. Instead 6 bacterial isolates have shown antifungal activities against *Monosporascus cannonballus* (MC-R): 4 of them were collected from FOM F30 plates (B1, B2, B3, C6) and the two others were collected from MC-R plates (M2, M3).

Table 14. Antifungal activities of spore forming bacterial isolates collected from MC-R and FOM F30 plates against *F. oxysporum* f.sp. *melonis* (FOM F30) and *Monosporascus cannonballus* (MC-R).

Origin of samples	Collection of isolates from	Bacterial isolates code	FOM F30	MC-R
Water suspension of ECOS compost	FOM F30 plates	B1	+	+
		B2	+	+
		B3	+	+
		C1	-	-
		C2	-	-
		C3	-	-
		C4	-	-
		C5	-	-
		C6	+	+
		C7	-	-
		C8	-	-
		C9	-	-
		C10	-	-
	MC-R plates	M1	-	-
		M2	-	+
		M3	-	+
		M4	-	-
		M5	-	-

4.2.3. Physiological and biochemical test for bacterial identification

Following the morphological description of the bacterial colonies obtained from the dilution technique of the MAIB-ECOS substrate and infected field samples and from the water suspension of ECOS compost, 150 different types of bacteria were selected.

These bacterial isolates were divided into two groups based on their ability to produce fluorescent pigments on King B (fluorescent bacteria) or to produce spores after treatment at 80 °C for 15 minutes (spore-forming bacteria). Inside the two groups, it was decided to identify exclusively those that tested positive in the antagonism assays when confronted with the two fungal pathogens of melon plants (FOM and MC). Twenty-two bacterial isolates were chosen: 9 bacterial isolates, belong to fluorescent group, were isolated from the 4 different sites of MAIB-ECOS substrate (table 15); 13 bacterial isolates, belong to spore forming bacterial group, were collected from water suspension of compost and from the 3 different sites of the infected field by FOM (table 16).

These identifications were based on series of morphological and biochemical tests as well as physiological characteristics using the standard characterization definitions of Bergey's

manual of determinative bacteriology (1986). The fluorescent pseudomonad bacteria (Gram negative) were characterized by carrying out levan formation, gelatin liquefaction and lechitinase production tests in which all the bacterial isolates have shown a positive reaction of the studied tests, beside 1 isolate (R.2.1) gave a negative result of gelatin liquefaction test (table 15). The spore forming bacteria (Gram positive) were identified by applying gelatin liquefaction and lechitinase production tests, that all the bacterial isolates gave a negative reaction of the lechitinase production test while 5 isolates shown a positive reaction of the gelatin liquefaction test (table 16).

Table 15. General characteristics of fluorescent pseudomonad bacteria isolated from the four different sites of the organic growing substrate (MAIB-ECOS)

Origin of samples	Isolated sites	Bacterial isolates	KOH test	Levan formation	Gelatin liquefaction	Lecithinase production	Growth at 41°C	Fluorescent color under UV
MAIB-ECOS substrate	Bulk soil	I.2	Gram -	+	+	+	-	Blue
		I.4	Gram -	+	+	+	-	Blue
		II.1	Gram -	+	+	+	-	Blue
		II.2	Gram -	+	+	+	-	Blue
	Rhizosphere	S.2.1	Gram -	+	+	+	-	Green
		S.2.2	Gram -	+	+	+	-	Green
	Rhizoplane (a)	R.2.1	Gram -	+	-	+	-	Blue
	Rhizoplane (b)	R.4.2	Gram -	+	+	+	-	Blue
		R.4.3	Gram -	+	+	+	-	Blue

Table 16. Differential characteristics of spore forming bacteria isolated from the water suspension of ECOS compost and from three different sites of infected field by FOM

Origin of samples	Isolated sites	Bacterial isolates	KOH test	Gelatin liquefaction	Lecithinase production
ECOS compost	Water suspension	B1	Gram +	+	-
		B2	Gram +	+	-
		B3	Gram +	+	-
		C6	Gram +	+	-
		M2	Gram +	-	-
		M3	Gram +	-	-
Infected field by FOM	Bulk soil	P8	Gram +	-	-
	Rhizoplane (a)	P16	Gram +	+	-
		P18	Gram +	+	-
		P22	Gram +	+	-
	Rhizoplane (b)	P24	Gram +	-	-
		P25	Gram +	-	-
		P27	Gram +	+	-

4.3. *In vitro* antagonism assays against two fungal pathogens

4.3.1. Dual culture-plate method on PDA

The dual culture-plate assay was performed with agar plugs on PDA agar media on Petri dishes for all 132 microbial morphologies isolated from ECOS compost, MAIB-ECOS substrate and infected field by FOM.

In the *in vitro* assays, the 21 bacterial isolates collected from the bulk soil of ECOS compost have not shown any antagonistic activity toward *Fusarium oxysporum* f. sp. *melonis* (race 1-2) isolate (FOM F30) and *Monosporascus cannonballus* isolate (MC-R).

A total of 59 antagonist strains (29 FPB and 30 SFB) isolated from four different sites of MAIB-ECOS substrate were assayed for *in vitro* suppression of FOM F30 and MC-R isolates. The mycelial growth values obtained from the various antagonisms were transformed into mean inhibition percentages compared to growth in the control dishes. It has been observed a different response of the MC-R and FOM F30 isolates to the same FPB and SFB bacterial isolates. The highest percentage of FPB having an antagonistic activity against FOM F30 and MC-R were respectively isolated from rhizoplane “b” (40 %) and bulk soil (77 %), while all SFB isolated from “rhizoplane b” (100 %) were expressed an antagonistic activity towards FOM F30 (figure 19) and MC-R (figure 20). A lower percentage of SFB isolated from bulk soil (15 %) and rhizoplane ”b” (25 %) were shown an antifungal activities toward FOM F30, the same as for the FPB isolated from bulk soil (8 %), rhizosphere (16 %), rhizoplane “a” (20 %) and rhizoplane “b” (40 %) had an antagonistic effect against FOM F30. More than 77 %, 50 %, 60 % and 40 % of FPB isolated respectively from bulk soil, rhizosphere, rhizoplane “a” and rhizoplane “b” comprised a suppressive effect against MC-R.

As a result of the microbial quantification of the infected field by FOM, a total of 52 bacterial morphologies (25 FPB and 27 SFB isolates) were identified. In this first *in vitro* selection, remarkable differences were detected between the various microbial groups. No antagonistic activity of the fluorescent bacterial isolates (FPB) collected from the four different sites of infected field samples has been detected towards the two studied fungi, whereas antagonistic activity was only detected in the SFB strains isolated from three sites (bulk soil, rhizoplane “a” and “b”) when confronted with the FOM F30 (figure 21) and MC-R (figure 22). The highest number of antagonistic microorganisms against FOM F30 and MC-R isolates was obtained in SFB isolated from rhizoplane “a” (60 %), and the lesser

extent in SFB isolated from bulk soil (13 %). The SFB isolated from rhizoplane "b" shown a different response of antagonistic activities against FOM F30 and MC-R, the highest number (50 %) was those against MC-R. In both bacterial groups (FPB and SFB) isolated from rhizosphere were not manifested any antagonistic activities against the studied melon pathogens.

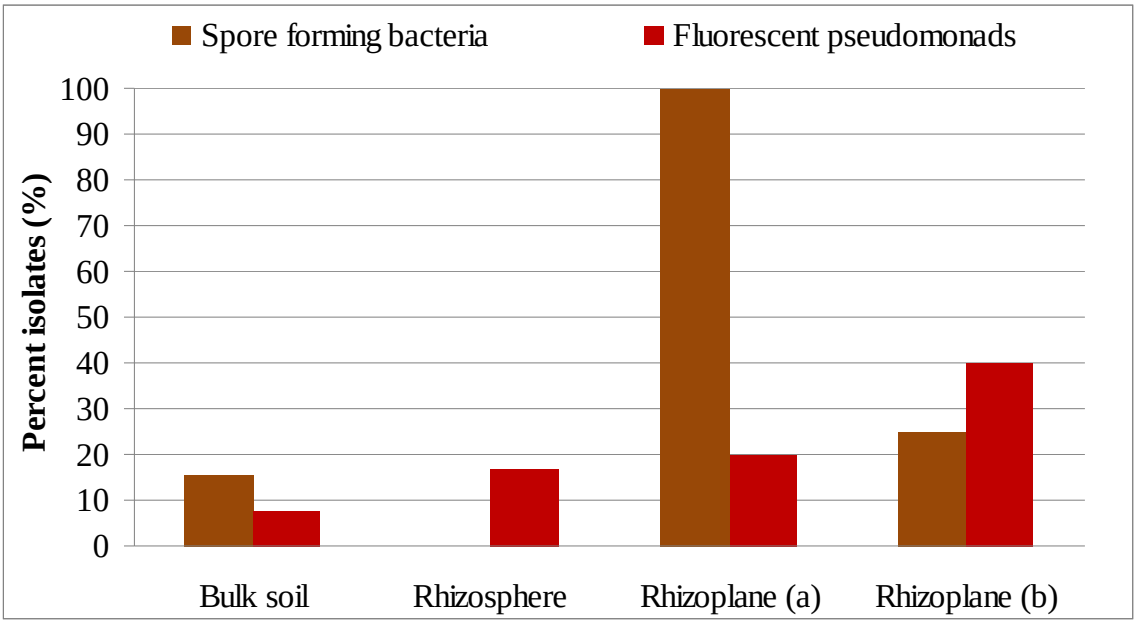

Fig 19. Antifungal activities of fluorescent pseudomonads and aerobic spore forming bacteria, isolated from the four different sites of MAIB-ECOS substrate, against *Fusarium oxysporum* f. sp. *melonis* (race 1-2) isolate (FOM F30)

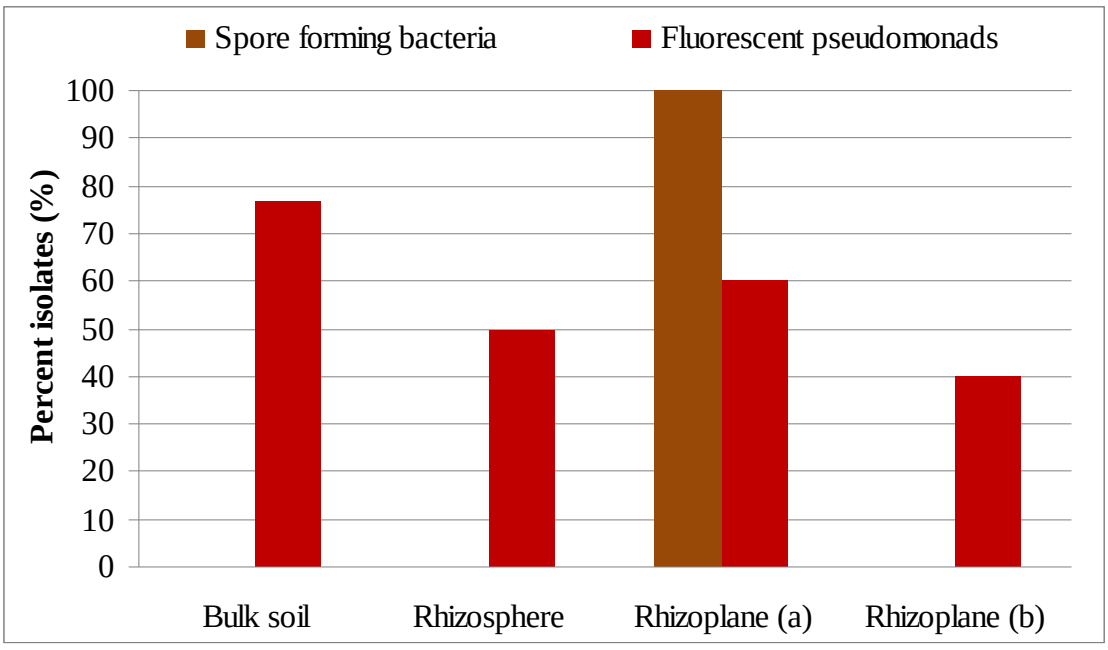

Fig 20. Antifungal activities of fluorescent pseudomonads and aerobic spore forming bacteria, isolated from the four different sites of MAIB-ECOS substrate, against *Monosporascus cannonballus* isolate (MC-R)

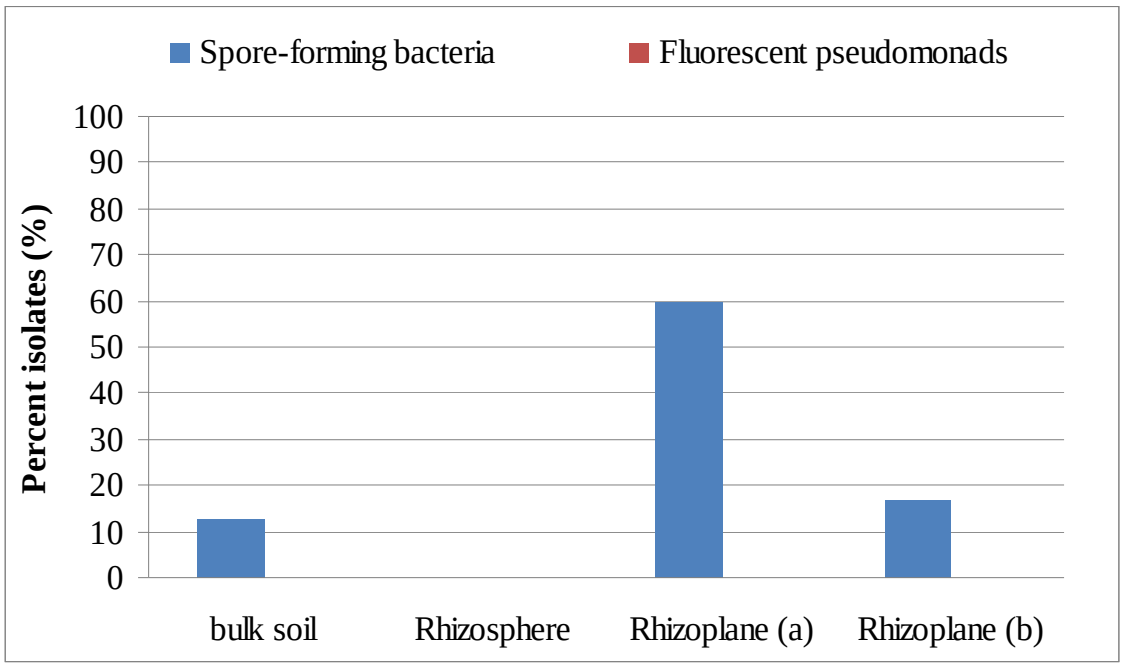

Fig 21. Antifungal activities of fluorescent pseudomonads and aerobic spore forming bacteria, isolated from the four different sites of infected field by FOM, against *Fusarium oxysporum* f. sp. *melonis* (race 1-2) isolate (FOM F30)

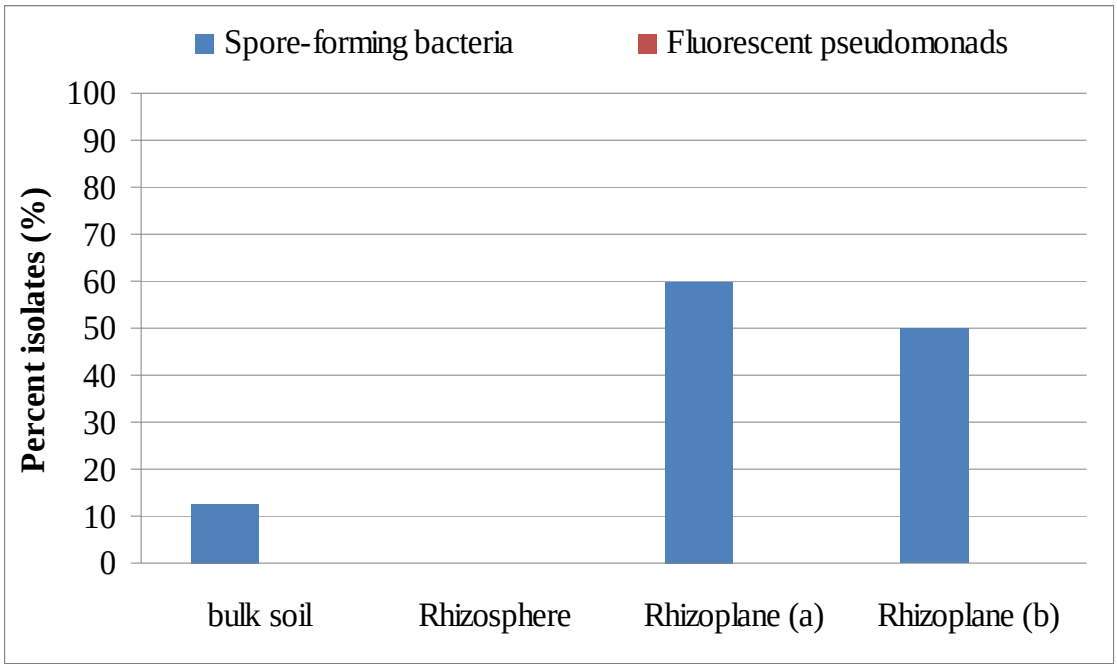

Fig 22. Antifungal activities of fluorescent pseudomonads and aerobic spore forming bacteria, isolated from the four different sites of infected field by FOM, against *Monosporascus cannonballus* isolate (MC-R)

4.3.2. Inhibition of fungal mycelium growth

The main bacterial isolates showing an inhibition zone in the previous antagonism assay were selected to evaluate their antagonistic effect by measuring the mycelial growth of *F. oxysporum* f. sp. *melonis* (FOM F30) and *Monosporascus cannonballus* (MC-R).

Nine bacterial isolates belonging to FPB group isolated from the four different sites of MAIB-ECOS substrate were assayed for *in vitro* suppression of FOM F30 and MC-R. Some of these microorganisms had a suppressive effect (table 17). The tested bacteria showed a high suppression (I.4, S.2.1, S.2.2, R.2.1 and R.4.3) and medium suppression (I.2, II.1, II.2 and R.4.2) toward MC-R, while only 4 fluorescent isolates (S.2.1, S.2.2, R.2.1 and R.4.3) demonstrated a medium suppression against FOM F30, and the 5 other fluorescent isolates (I.2, 1.4, II.1, II.2 and R.4.2) did not express any suppressive effect against FOM F30. The greater suppressive effect for MC-R was correlated to the strains R.4.3 (0.13 cm) and S.2.2 (0.3 cm) isolated respectively form rhizoplane "b" and rhizosphere, moreover the highest suppressive effect for FOM F30 was detected to the strains R.2.1 (2.68 cm) and S.2.2 (2.78 cm) isolated respectively from rhizoplane "a" and rhizosphere. We noticed that the suppressive effect of some bacterial isolates did not vary in correspondence with time as for I.2, I.4, II.2 and R.4.3 isolates toward MC-R isolate.

Thirteen spore forming bacterial (SFB) isolates were selected for *in vitro* suppression of the studied pathogens, in which 7 and 6 SFB isolates were collected respectively from infected field by FOM and water suspension of ECOS compost. The selection criterion was based on the valuation of *in vitro* antagonism assay (section 4.3.1.1). The mycelial growth inhibition of the selected bacterial isolates can be observed in table 18. The bacterial isolates collected from infected field have shown a high (P8, P18), medium (P16, P22, P27) and no suppression (P24, P25) toward both pathogens (FOM F30 and MC-R). However, all the collected bacteria from ECOS compost had a high suppression toward MC-R, while against FOM F30 the suppression reaction differ among isolates that the high suppression was shown for B1, B2, B3 and C6 isolates, medium suppression for M2 isolate, and no suppression for M3 isolate. The lowest mycelial growth of FOM F30 isolate was observed in case of the bacterial strains isolated from compost (B1; 1.41 cm), (B2; 1.31 cm), (B3; 1.48 cm), (C6; 1.31 cm), and of those isolated from the bulk soil (P8; 1.53 cm) and rhizoplane "a" (P18; 1.57 cm) of infected field. The higher suppressive effect toward MC-R was associated to the strains P8 and P18 (0.93 cm) isolated from infected field samples, and to the isolate B3 (0.94 cm) selected from water suspension of compost.

Table 17. *In vitro* effect of fluorescent bacteria (FPB) isolated from four different sites of MAIB-ECOS substrate on mycelial growth of *F. oxysporum* f. sp. *melonis* (FOM F30) and *Monosporascus cannonballus* (MC-R)

The mycelial growth diameter (cm)						
Isolated sites	FPB	Inhibition period (days)				
		FOM F30			**MC-R**	
		7 days	10 days	15 days	5 days	7 days
Bulk soil	I.2	2.03 ± 0.08	2.95 ± 0.14	3.03 ± 0.05	2.9 ± 0.14	2.9 ± 0.14
	I.4	1.8 ± 0.13	2.62 ± 0.08	3 ± 0.11	2.1 ± 0.25	2.1 ± 0.25
	II.1	1.92 ± 0.21	2.87 ± 0.37	3 ± 0.17	2.8 ± 0.38	2.94 ± 0.41
	II.2	2.1 ± 0.15	3.03 ± 0.08	3.15 ± 0.08	2.5 ± 0.37	2.5 ± 0.37
Rhizosphere	S.2.1	1.57 ± 0.10	1.97 ± 0.72	2.83 ± 0.15	0.5 ± 0.15	1.08 ± 0.23
	S.2.2	1.5 ± 0.09	2.23 ± 0.16	2.78 ± 0.21	0.2 ± 0.13	0.3 ± 0.28
Rhizoplane “a”	R.2.1	1.52 ± 0.04	2.15 ± 0.12	2.68 ± 0.17	1.22 ± 0.26	1.5 ± 0.29
Rhizoplane “b”	R.4.2	1.92 ± 0.39	2.72 ± 0.29	3.12 ± 0.10	2.26 ± 0.17	2.6 ± 0.24
	R.4.3	1.53 ± 0.08	2.28 ± 0.15	2.85 ± 0.14	0.13 ± 0.05	0.13 ± 0.05
Control		1.95 ± 0.07	2.8 ± 0.14	3.05 ± 0.21	2.50 ± 0.30	3.13 ± 0.27

Table 18. *In vitro* effect of spore forming bacteria, isolated from three different sites of the infected filed and from the water suspension of ECOS compost, on mycelial growth of *F. oxysporum* f. sp. *melonis* (FOM F30) and *Monosporascus cannonballus* (MC-R)

The mycelial growth diameter (cm)							
Origin of isolates	Isolated sites	SFB	Inhibition period (days)				
			FOM F30			**MC-R**	
			7 days	10 days	15 days	5 days	7 days
Infected field by FOM	Bulk soil	P8	1.35 ± 0.10	1.43 ± 0.15	1.53 ± 0.12	0.68 ± 0.32	0.93 ± 0.32
	Rhizoplane "a"	P16	1.72 ± 0.15	2.15 ± 0.14	2.67 ± 0.12	2.03 ± 0.43	2.20 ± 0.35
		P18	1.33 ± 0.08	1.47 ± 0.08	1.57 ± 015	0.93 ± 0.28	0.93 ± 0.28
		P22	1.75 ± 0.10	2.17 ± 0.10	2.75 ± 0.10	2.72 ± 0.35	2.77 ± 0.33
	Rhizoplane "b"	P24	1.93 ± 0.16	2.47 ± 0.18	3.08 ± 0.23	2.77 ± 0.10	3.07 ± 0.10
		P25	2.37 ± 0.08	3.08 ± 0.13	3.23 ± 0.12	3.10 ± 0.17	3.17 ± 0.10
		P27	1.77 ± 0.20	2.13 ± 0.21	2.72 ± 0.16	2.73 ± 0.14	2.87 ± 0.12
ECOS compost	Water suspension	B1	0.93 ± 0.22	1.17 ± 0.23	1.41 ± 0.33	0.57 ± 0.15	1.60 ± 0.42
		B2	1.00 ± 0.16	1.18 ± 0.21	1.31 ± 0.35	0.63 ± 0.22	1.33 ± 0.33
		B3	1.15 ± 0.05	1.4 ± 0.18	1.48 ± 0.13	0.93 ± 0.02	0.94 ± 0.02
		C6	1.04 ± 0.14	1.31 ± 0.22	1.31 ± 0.23	0.85 ± 0.07	1.30 ± 0.14
		M2	0.95 ± 0.10	2.17 ± 0.14	2.95 ± 0.30	2.08 ± 0.13	2.17 ± 0.29
		M3	0.87 ± 0.08	2.20 ± 0.26	3.02 ± 0.43	1.87 ± 0.25	1.93 ± 0.27
Control			2.50 ± 0.30	3.13 ± 0.27	3.28 ± 0.25	2.50 ± 0.30	3.13 ± 0.27

4.3.3. Assay for bacterial volatile inhibitors and HCN production

Fifteen isolates from the strain collection of antagonistic microorganisms were selected, comprising 6 SFB isolates collected from water suspension of ECOS compost, 4 FPB isolates collected from MAIB-ECOS substrate and 5 SFB isolated collected from infected field by FOM (Table 19). The criteria for selection of these strains were the high suppression of mycelial growth of the melon pathogens.

No more than nine bacterial isolates differed considerably in volatile fungistasis action toward the two phytopathogenic fungi, as well volatile fungistasis action of the same bacterial isolate differed in front of each studied fungi. Only volatiles of the P18 isolates exhibited strong fungistatic effects against MC-R in terms of mycelial growth inhibition. However, moderate volatile fungistasis was displayed from B2 and S.2.2 isolates against FOM F30 and from B1 and P27 isolates toward MC-R, and the antifungal activity was significantly less for B1, C6, R.2.1 and P8 against FOM F 30 and for C6, P8 and P16 toward MC-R.

One of the volatile compounds, hydrogen cyanide (HCN) is produced by a number of rhizobacteria and is reported to play an important role in biocontrol. We noticed that only the fluorescent bacteria isolated from the three different sites of MAIB-ECOS substrate have the capacity to produce HCN.

Table 19. The intensity of antifungal activity by volatiles produced by the selected bacteria on inhibition of mycelial growth of *F. oxysporum* f. sp. *melonis* (FOM F30) and *Monosporascus cannonballus* (MC-R) and the hydrogen cyanide (HCN) production

Origin of samples	Isolated sites	Bacterial groups	Bacterial isolates code	Growth inhibition		HCN production
				FOM F30	MC-R	
ECOS compost	Water suspension	SFB	B1	+	++	-
			B2	++	-	-
			B3	-	-	-
			C6	+	+	-
			M2	-	-	-
			M3	-	-	-
MAIB-ECOS substrate	Rhizosphere	FPB	S.2.1	-	-	+++
			S.2.2	++	-	++
	Rhizoplane (a)	FPB	R.2.1	+	-	+++
	Rhizoplane (b)	FPB	R.4.3	-	-	++
Infected field by FOM	Bulk soil	SFB	P8	+	+	-
	Rhizoplane (a)	SFB	P16	-	+	-
			P18	-	+++	-
			P22	-	-	-
	Rhizoplane (b)	SFB	P27	-	++	-

Strong (+++), Moderate (++), Low (+), Slight (-).

4.4. *In vitro* detection of bacterial plant growth promoting features

4.4.1. Quantitative screening of plant growth promoting rhizobacteria

The preliminary screening protocols of plant growth promoting features were used for identification, selection and establishing of efficient plant growth promoting rhizobacteria (PGPR) isolated from bulk soil of ECOS compost and from bulk soil, rhizosphere and rhizoplane "a" and "b" of MAIB-ECOS substrate and infected field samples. In the present study a quantitative screening of the selected bacterial isolates (FPB and SFB) was carried out. Our results show that several bacterial colonies were able to produce siderophore, IAA and phosphate solubilisation, however the FPB have the highest capacity to produce PGP activities than those bacteria belonging to SFB group.

Just the fluorescent bacteria isolated from bulk soil of ECOS compost manifested the PGP activities (figure 23). All the isolated bacteria have shown the siderophore production activity, as well as the 93 % of bacteria have presented phosphorus solubilisation activities, while a lower percent of FPB (60 %) possessed the auxin (IAA) production activity.

Responses of the bacterial strains isolated from MAIB-ECOS substrate to PGP tests are shown in Figures 24 and 25. The PGP assays demonstrated that all the FPB (100 %) isolated from the four studied sites of MAIB-ECOS substrate were able to produce siderophore and solubilize phosphate, whereas only those isolated from the bulk soil (23 %) have shown the production of the phytohormones (IAA). We noticed that the presence of melon root in the MAIB-ECOS substrate seems to have enhanced the growth of FPB with siderophores production and P-solubizing activities. The SFB isolated from the four different sites of the organic substrate showed only the siderophore production activity, and the higher production was observed for those colonies isolated from rhizoplane "a" (67 %).

The PGP activities of the fluorescent and spore forming bacteria isolated from the four sites of infected field by FOM exposed more or less the same results of those bacteria isolated from MAIB-ECOS substrate. Where, all the FPB (100 %) isolated from the four studied sites showed the siderophore production activity (figure 26), and the siderophore production was the only PGP activity performed by the SFB colonies specially those isolated from bulk soil (13 %) and rhizoplane "b" (33 %) (figure 27). Regarding the other PGP activities, the IAA production was detected in the FPB isolated from bulk soil (100 %), rhizosphere (83 %), rhizoplane "a" (60 %) and rhizoplane "b" (33 %); also the phosphate solubilisation activity

was present in 50 %, 80 %, 83 % and 50 % of FPB isolated respectively from bulk soil, rhizosphere, rhizoplane “a” and rhizoplane “b”.

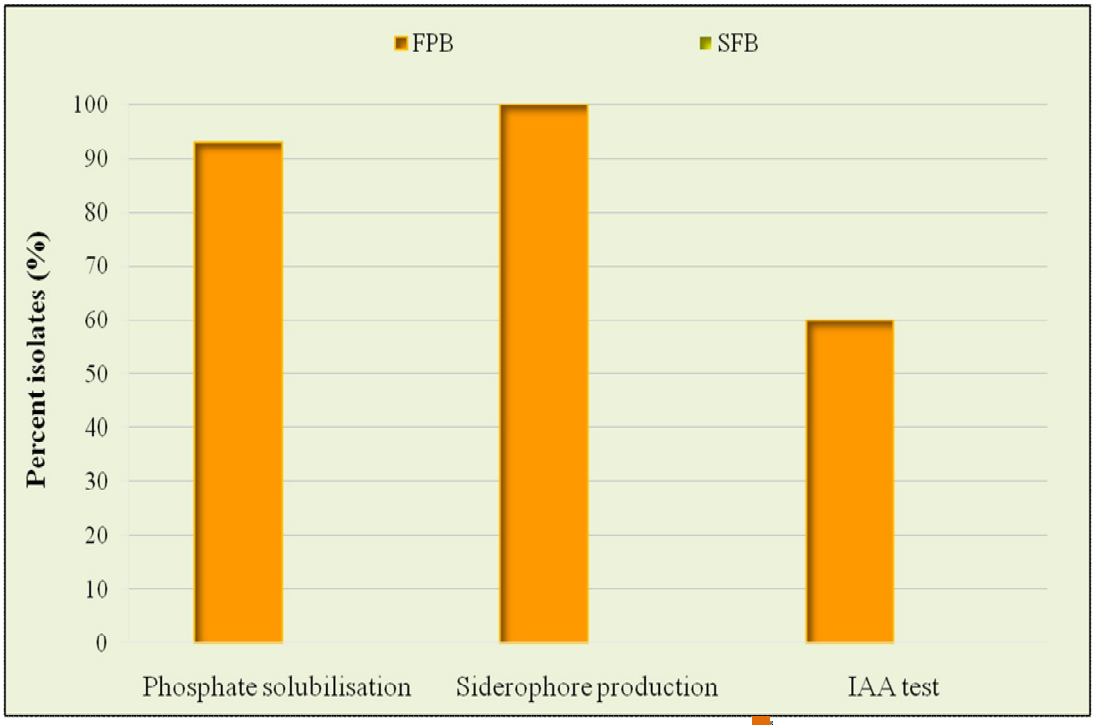

Fig 23. Percent of fluorescent pseudomonad bacteria (FPB) ( ) and spore forming bacteria (SFB) ( ) isolated from bulk soil of ECOS compost showing the plant growth promoting activities: phosphate solubilisation, IAA test and siderophore production

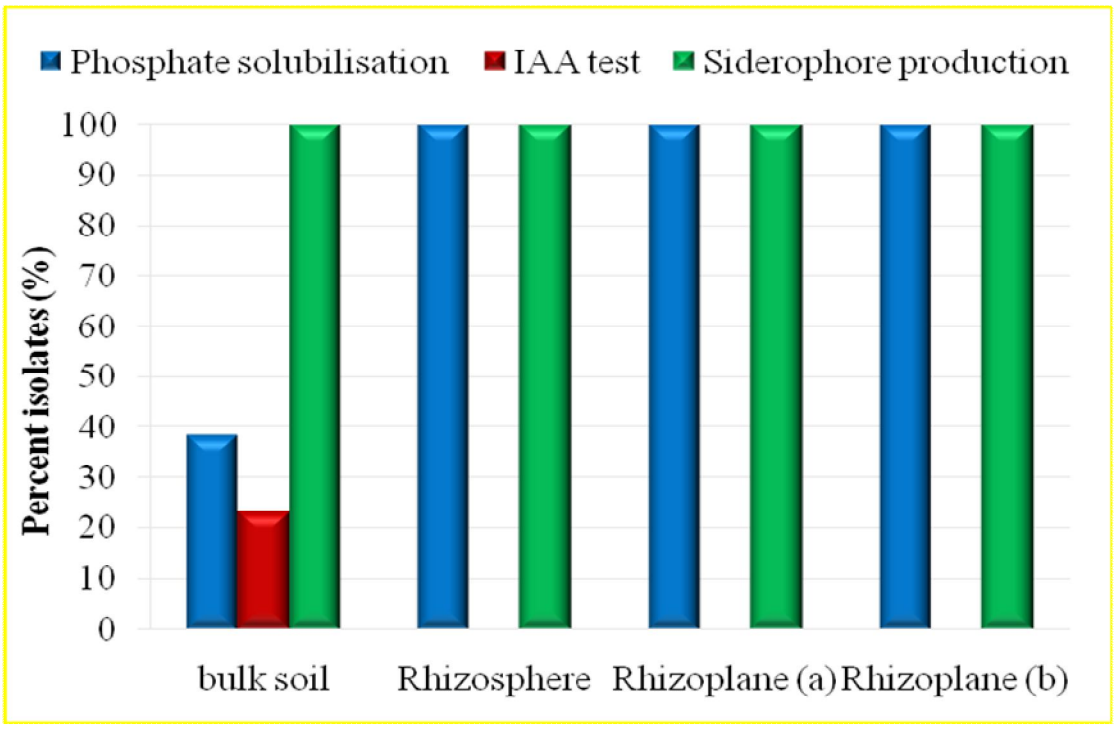

Fig 24. Percent of fluorescent pseudomonad bacteria (FPB) isolated from four different sites of MAIB-ECOS substrate samples showing the plant growth promoting activities: phosphate solubilisation ( ), IAA test ( ) and siderophore production ( )

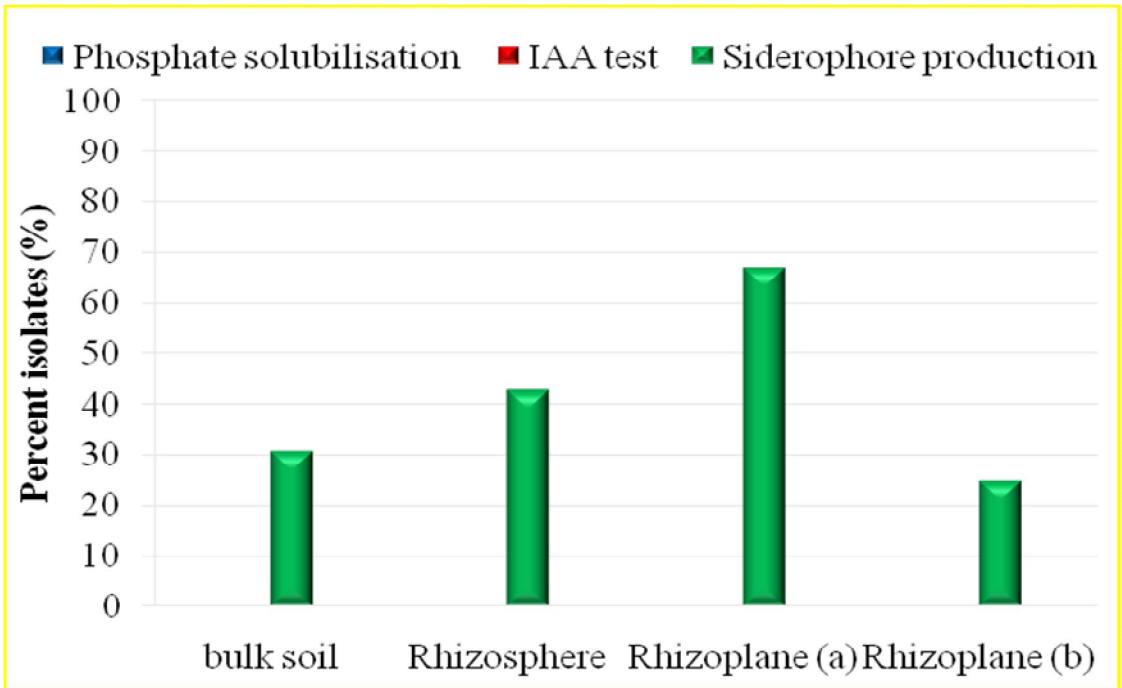

Fig 25. Percent of aerobic spore forming bacteria (SFB) isolated from four different sites of MAIB-ECOS substrate samples showing the plant growth promoting activities: phosphate solubilisation ( ), IAA test ( ) and siderophore production ( )

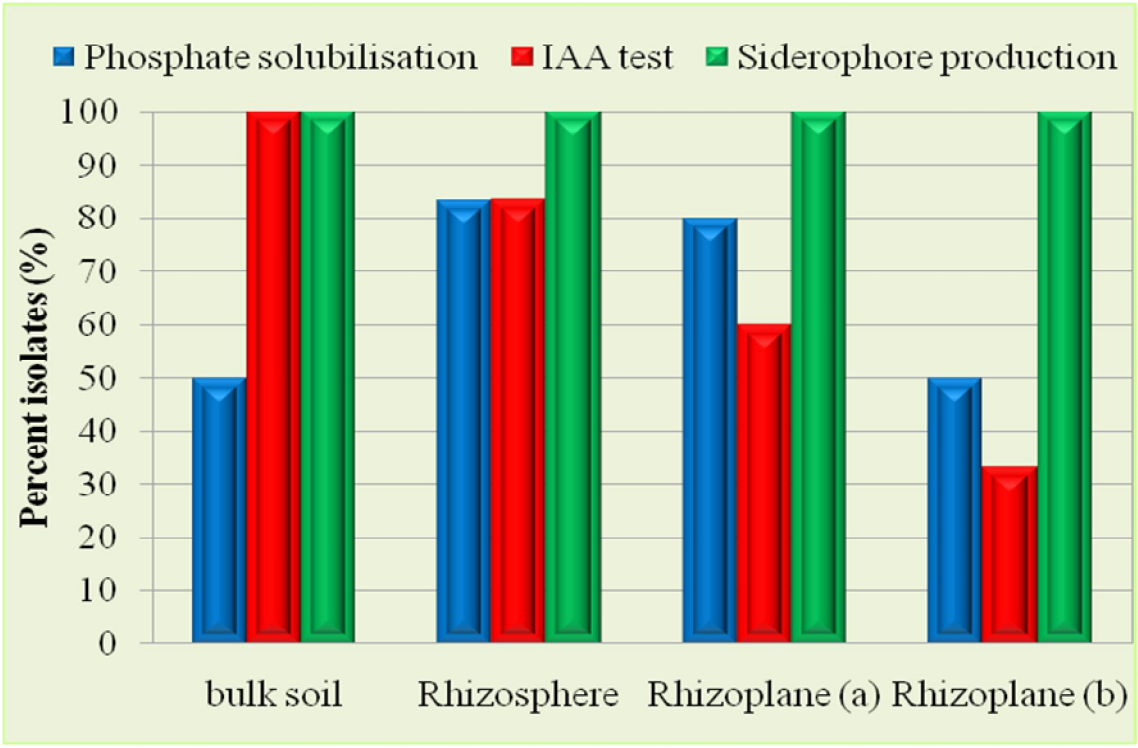

Fig 26. Percent of fluorescent pseudomonad bacteria (FPB) isolated from four different sites of infected field samples showing the plant growth promoting activities: phosphate solubilisation (), IAA test () and siderophore production ()

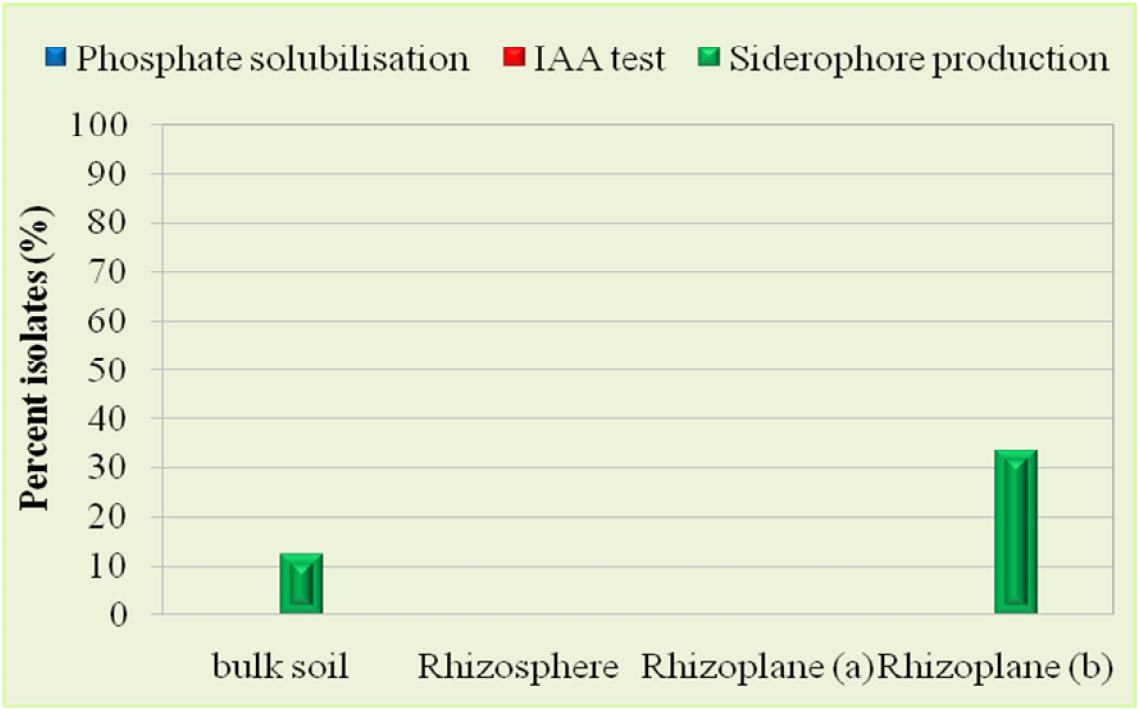

Fig 27. Percent of aerobic spore forming bacteria (SFB) isolated from four different sites of infected field samples showing the plant growth promoting activities: phosphate solubilisation (), IAA test () and siderophore production ()

4.4.2. Correlation among plant growth promoting features and antagonism activities

The plant growth promoting rhizobacteria (PGPR) were able to promote the growth of melon plants by direct mechanisms which produce phytohormones such as CTKs, IAA and Gas, or by indirect mechanisms used to limit the damage of phytopathogens to plants by the production of volatiles as HCN, depletion of iron from the rhizosphere by production of siderophore and solubilisation of minerals such as phosphate. These mechanisms are not mutually exclusive, and the overall biocontrol activity is due to the synergistic effects of different modes of antagonism.

In the previous section (4.3.2.1), some FPB and SFB isolated from ECOS compost, MAIB-ECOS substrate and infected field by FOM were demonstrated for their antagonistic activities towards *F. oxysporum* f. sp. *melonis* (FOM F30) and *Monosporascus cannonbalus* (MC-R), and by studying the PGP activities the mode of action of those antagonistic bacteria can be observable.

The initial screening of PGP activities demonstrated that the strains S.2.1, S.2.2, R.2.1 and R.4.3 produce siderophore and HCN and have the ability to solubilise phosphate (table 20). Moreover, the overall influence and interaction of multiple PGP traits have possible positive effects on the mode of action of antagonist microorganism, therefore those bacterial isolates have shown a higher suppressive effect towards FOM F30 and MC-R isolates. The isolates I.2, I.4, II.1 and II.2 that present only the siderophore production as PGP activity were detected to have a lower suppressive affect against the studied fungal pathogens.

The spore forming bacteria have exposed a single PGP features (siderophores production activity), in addition many authors have demonstrated the importance of siderophore in the inhibition of fungal pathogens, thus the higher antagonism effect of the P22, B1, B2 and C6 can be explained by the production of siderophores (table 21).

Table 20. Detection of Plant Growth Promoting properties and antifungal activities of some fluorescent bacteria isolated from four different sites of MAIB-ECOS substrate

Isolated sites	Pseudomonads fluorescent bacteria	Detection of PGP properties and antifungal activities			
		Phosphate solubilisation	***IAAs***	***Siderophore production***	***HCN production***
Bulk soil	I.2	-	-	+	-
	I.4	-	-	+	-
	II.1	-	-	++	-
	II.2	-	-	++	-
Rhizosphere	S.2.1	++	-	++	+++
	S.2.2	++	-	++	++
Rhizoplane "a"	R.2.1	++	-	++	+++
Rhizoplane "b"	R.4.2	+	-	+	+
	R.4.3	+	-	++	++

Table 21. Detection of Plant Growth Promoting properties and antifungal activities of some spore forming bacteria isolated from three different sites of infected field by FOM and from the water suspension of ECOS compost

Origin of isolates	Isolated sites	Spore forming bacteria	Detection of PGP properties and antifungal activities			
			Phosphate solubilisation	***IAAs***	***Siderophore production***	***HCN production***
Infected field by FOM	Bulk soil	P8	-	-	-	-
	Rhizoplane "a"	P16	-	-	-	-
		P18	-	-	-	-
		P22	-	-	+	-
	Rhizoplane "b"	P24	-	-	-	-
		P25	-	-	-	-
		P27	-	-	-	-
ECOS compost	Water suspension	B1	-	-	+	-
		B2	-	-	+	-
		B3	-	-	-	-
		C6	-	-	+	-
		M2	-	-	-	-
		M3	-	-	-	-

4.5. Standardization of organic growing media for melon crops

4.5.1. Plant growth parameters

4.5.1.1. Germination percentage (G) and mean germination time (MGT)

The maximum germination percentage of melon seeds (70 %), of the first expeiment carried out during June – July 2009, was observed in T3 the substrate based on 30 % of coconut fiber and T5 the substrate based on 40 % of compost (figure 28), while T3 has shown the better germination enhancement (about 8 days) in terms of mean germination time (MGT) than T5 substrate where the melon seeds took the maximum MGT to germinate (about 10 days). The seeds sown in the control substrate (T1) took the minimum MGT to germinate (7 days). However, the presence of ECOS compost residue in T4 substrate composition provided the lowest germination percentage (40 %).

The second experiment carried out during April – May 2010 (figure 29) have shown relatively the same results obtained in the first experiment, that the percentage of germination for T3 substrate based on coconut fiber was 69 %, the lowest germination was for those seeds sown in T4 substrate (25 %) and the seeds sown in T5 substrate took the maximum MGT to germinate (19 days). A 100 % germination was observed for the seeds sown in the control substrate (T1) with a lower MGT about 12 days, while the melon seeds sown in T2 substrate based on 50 % peatmoss took the minimum MGT to germinate (6 days) with a percentage of seed germination (75 %) higher than these (60 %) obtained in first experiment.

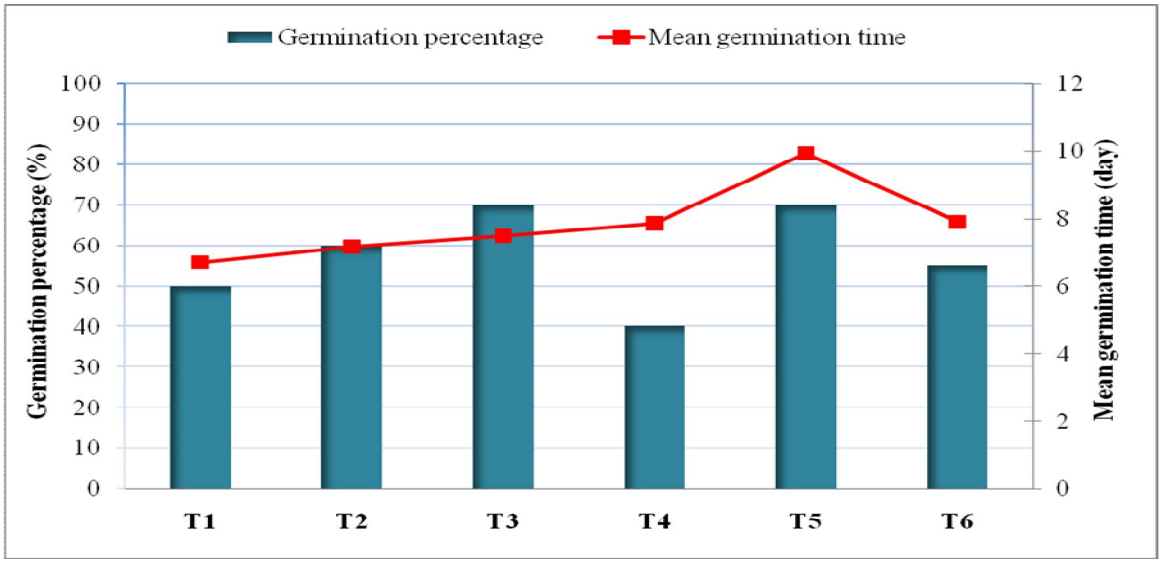

Fig 28. Germination percentage (G) and mean germination time (MGT) of melon plant seedlings grown in the six different organic media (T1 – T2 – T3 – T4 – T5 – T6) during June – July 2009

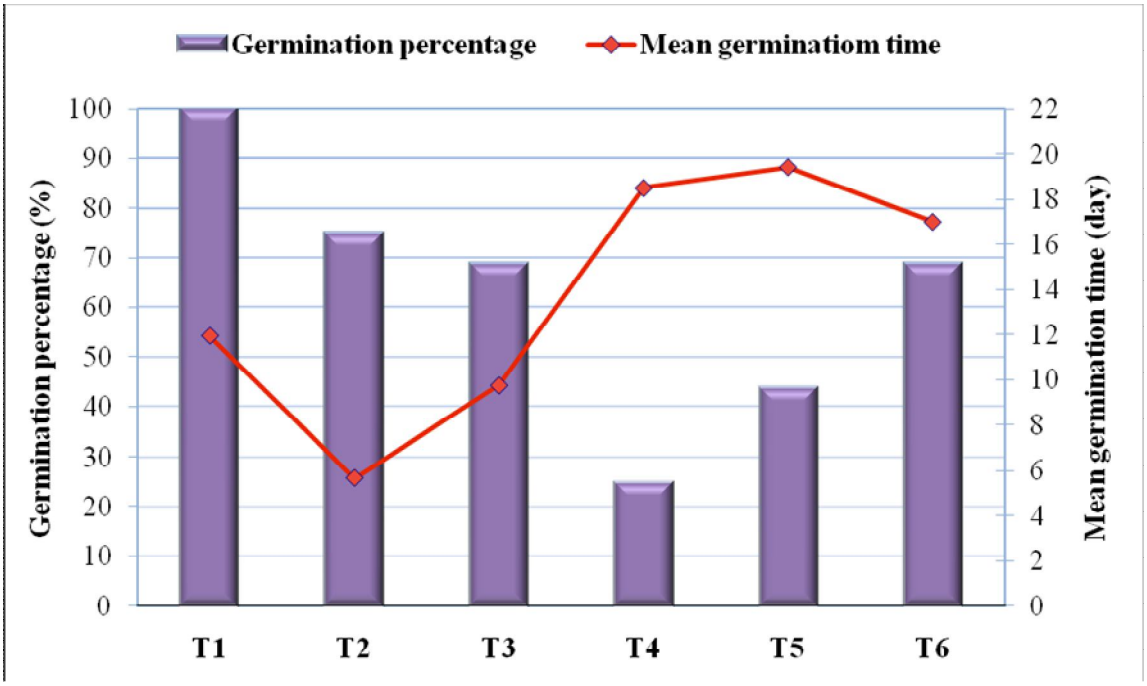

Fig 29. Germination percentage (G) and mean germination time (MGT) of melon plant seedlings grown in the six different organic media (T1 – T2 – T3 – T4 – T5 – T6) during April – May 2010

4.5.1.2. Chlorophyll level

In general, the range for seedling chlorophyll level of the first experiment period (2009) was between 19 and 30 SPAD (figure 30). The first chlorophyll reading (22 days after sowing) discovered that the seedling chlorophyll level in T4 substrate based on 20 % of compost (30 SPAD) had better performance than the other substrates T3 and T5 (28 SPAD), T1 and T6 (26 SPAD), T2 (25 SPAD). The chlorophyll content in the seedlings of all organic substrates decreased at the end of the experiment period and chlorophyll value of T3 seedling substrate (23 SPAD) became better than those of T4 substrate (22 SPAD), followed by the substrates T1 (21 SPAD), T2 and T6 (20 SPAD), T5 (19 SPAD).

The range of SPAD values for seedlings during the second experiment (April- May 2010) were between 27 and 36 SPAD. Significant differences were observed in the effects induced by all the six mixtures, with the highest results in seedling chlorophyll level observed in T3 substrate (36 SPAD), and the best performance of seedling chlorophyll levels overall the experiment period (figure 31). Seedlings in T4 and T5 substrates gave almost the same values (around 35 SPAD), which were near to T3 substrate, no significant difference was observed of the chlorophyll reading from T1, T4 and T5 substrates. The lowest chlorophyll level was detected in the seedlings grown on T1 and T2 substrates. The level of chlorophyll

decrease through the time, the range of the chlorophyll level in the last chlorophyll reading was between 27 (T1 and T2 substrates) and 35 SPAD (T3 substrate).

For both experiment, SPAD values showed that there is significant difference between seedling chlorophyll of the all studied organic substrates, moreover there was a difference of chlorophyll content among the two experiments, the higher chlorophyll seedling level was observed in the second experiment carried out during April – May 2010.

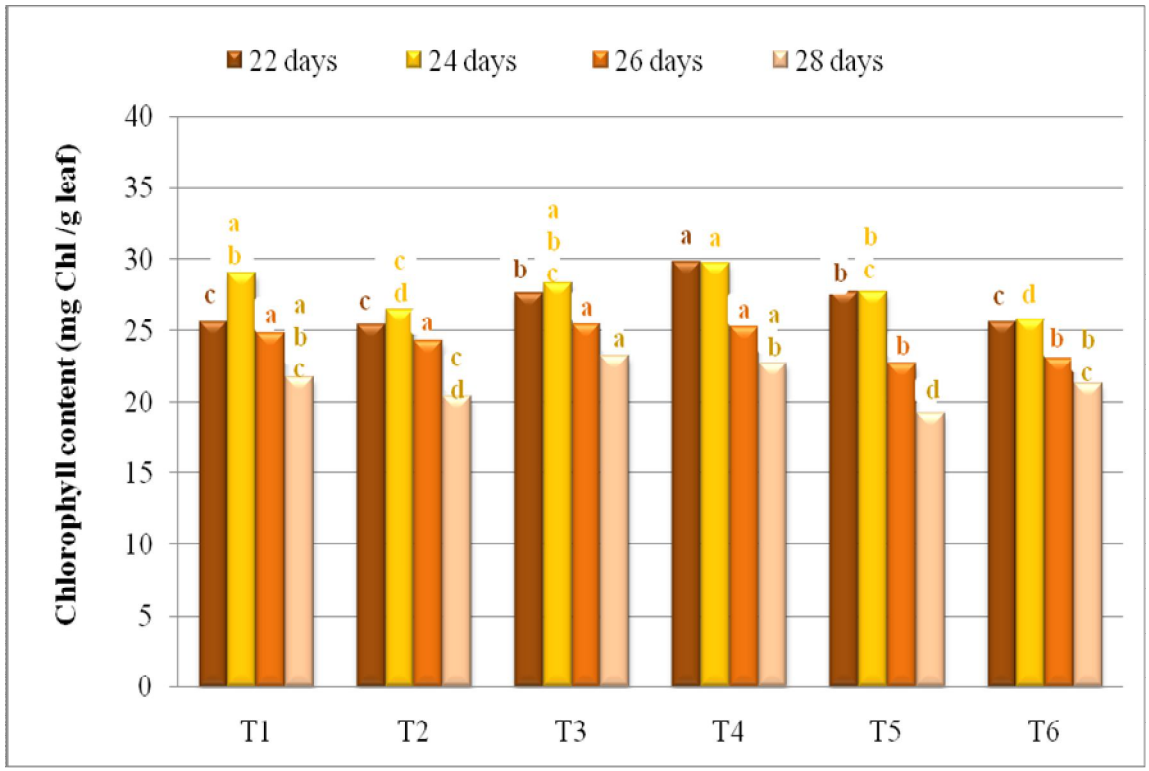

Fig 30. Effect of the six tested organic growing media (T1 – T2 – T3 – T4 – T5 – T6) on seedling chlorophyll level of melon after 22 (■), 24 (■), 26 (■), 28 (■) days of sowing during June – July 2009. Different letters denote significant differences according to Duncan test at 5 % of probability

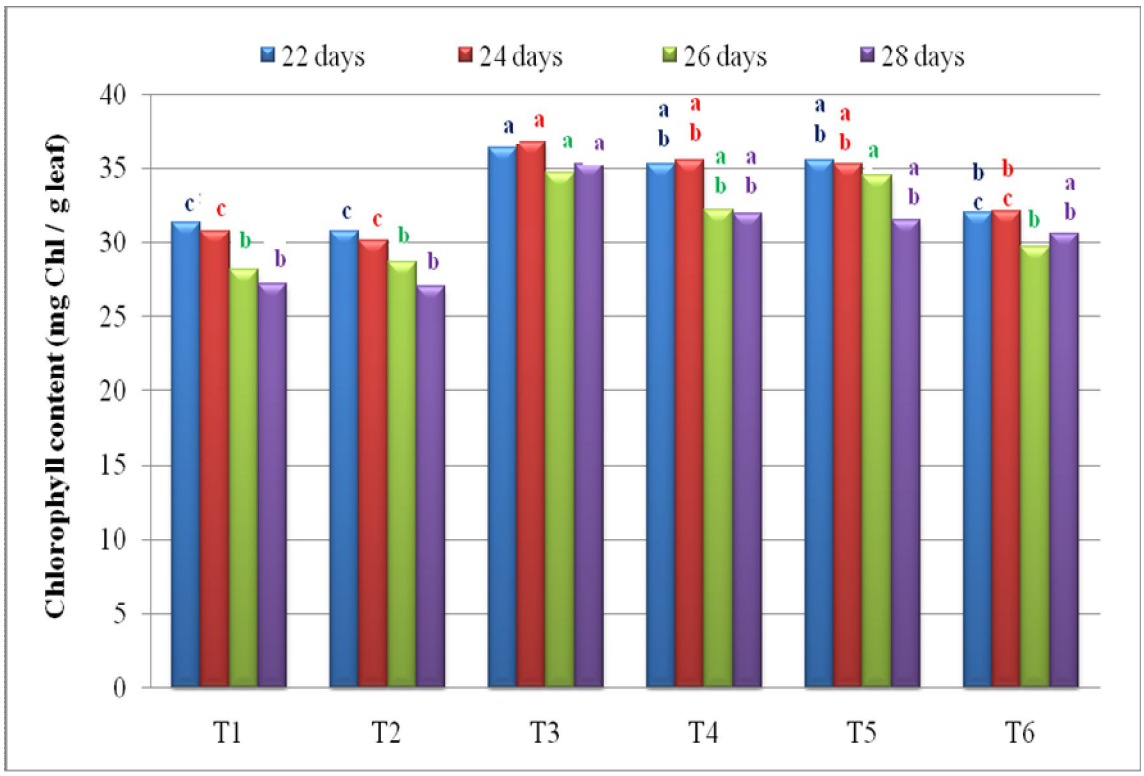

Fig 31. Effect of the six tested organic growing media (T1 – T2 – T3 – T4 – T5 – T6) on seedling chlorophyll level of melon after 22 (■), 24 (■), 26 (■), 28 (■) days of sowing during April – May 2010. Different letters denote significant differences according to Duncan test at 5 % of probability

4.5.1.3. Plant height (H)

Regarding both experiments carried out during 2009 and 2010, there were significant differences between the six studied organic substrates on plant height, besides no difference was shown between the two experiment (figure 32). The highest shoots were obtained for the plants grown in T2 substrate based on 50 % peatmoss during the both growing seasons (49 cm; 2009) and (37 cm; 2010), followed by T3 substrate (46 cm; 2009) and (30 cm; 2010). The lowest plant height was given for those plants grown in T4 and T5 substrates based respectively on 20 % and 40 % of compost; also we noticed that the effect of compost on plant height (24 cm) during the second experiment (2010) was advanced than the first experiment done on June – July 2009 (13 cm). The results further show that the seedlings grown in T1 and T6 substrates during 2009 had a significant difference in plant height while during 2010 no significant difference of plant height were observed between T1 (28 cm) and T6 (16 cm) substrates.

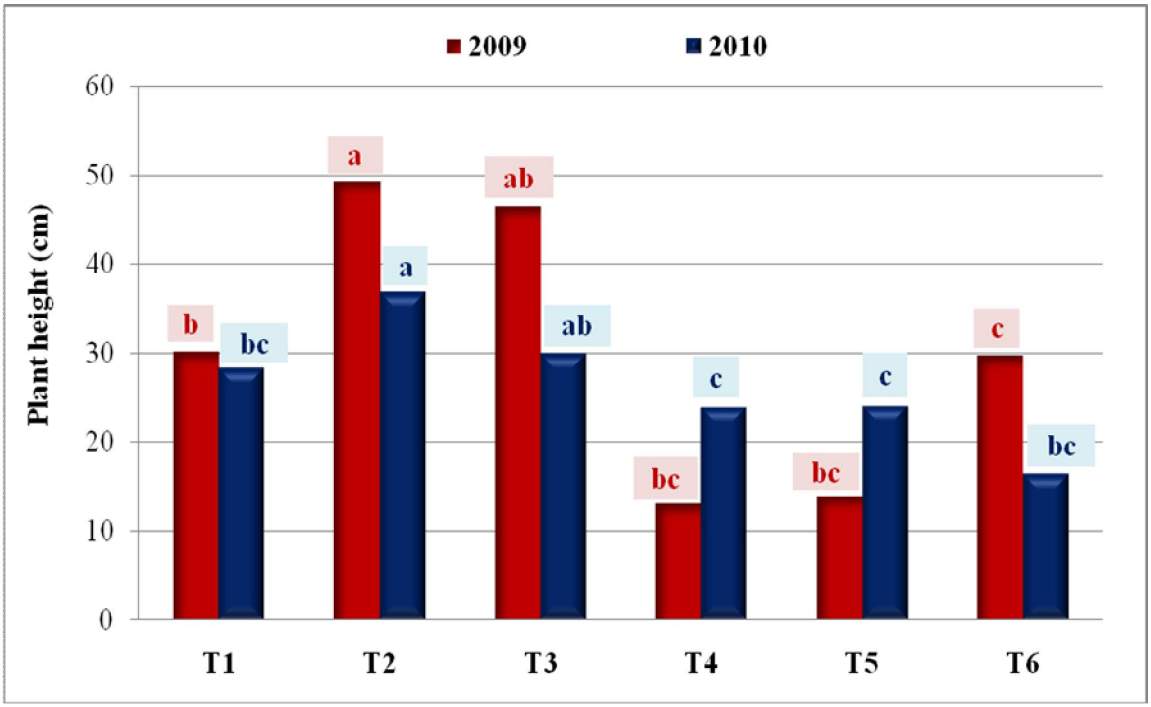

Fig 32. Plant height (H) of melon seedlings grown in the six different based-organic substrates (T1 – T2 – T3 – T4 – T5 – T6) during two different seasons June – July 2009 (■) and April – May 2010 (■). Different letters denote significant differences according to Duncan test at 5 % of probability

4.5.1.4. Root length (L)

In general, we can say that there were no effects of different mixtures on the root length of the melon seedlings (figure 33) during the growing season June – July 2009, whereas there were observed differences between them regarding 2010 growing season (April – May). As well the better root length was obtained for those seedlings grown during June – July 2009. As for the plant height, the T2 substrate shows the longer root (16 cm) of melon seedlings grown on June – July 2009, while for the second experiment that was done on April – May 2010 the longer shoot were obtained for the seedlings grown in T3 substrate (15 cm). The Duncan test identified that the difference of root length seedlings grown in T3 and T2 substrates trough the two experiments was not significant between them, as well the difference between T1 and T6 substrates on the root length was not significant during the both growing season. The lowest root length was obtained for the seedlings grown in T4 substrate (13 cm) during June – July 2009 and T5 substrate (8 cm) during April – May 2010.

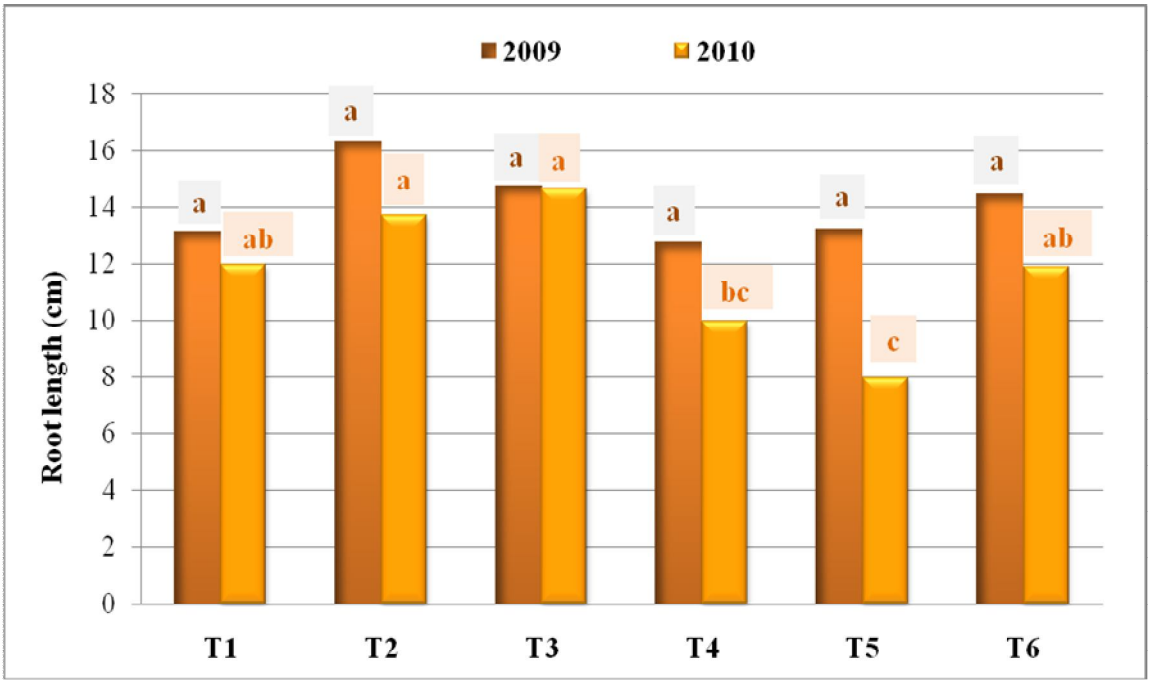

Fig 33. Root length (L) of melon seedlings grown in the six different based-organic substrates (T1 – T2 – T3 – T4 – T5 – T6) during two different seasons June – July 2009 ( ) and April – May 2010 ( ). Different letters denote significant differences according to Duncan test at 5 % of probability

4.5.1.5. Fresh and dry shoot weight

These studied parameters exposed that there were significant differences between the six studied substrates on the fresh and dry weight in both growing seasons (2009 – 2010) (figures 34 and 35). While, it is observed that the effect of different substrates on seedlings fresh weight of the vegetative part was the same on seedlings dry weight. The best result for fresh and dry shoot weight was obtained for the seedling grown during the season June – July 2009, beside in the case of T3 and T6 substrates their effect on fresh shoot weight were elevated in season 2010 more than the season 2009. The higher fresh weight were obtained for the seedlings grown in T3 substrate in both growing season (6 g), moreover the higher dry weight was observed in T3 substrate (0.78 g) only for the growing season 2009, while for the growing season 2010 the higher dry shoot weight were for the seedlings grown in T2 substrate (0.64 g). The substrates based on compost residue (T4, T5 and T6) had a negative effect on fresh and dry shoot weight, that the lowest fresh and dry weight in June – July 2009 was noticed for the plants grown in T5 and T6 substrates, and in April – May 2010 the plants grown in the T4 and T5 substrates had the lowest dry and shoot weight, there were no significant differences between them.

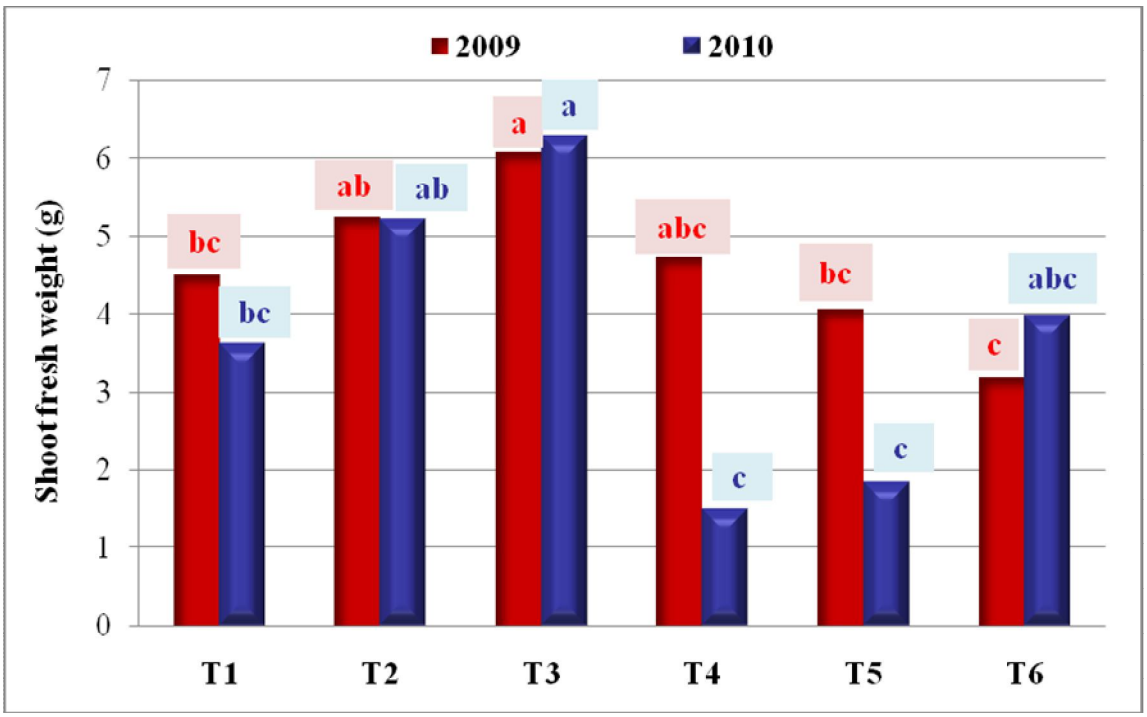

Fig 34. Shoot fresh weight (SFW) of melon seedlings grown in the six different based-organic substrates (T1 – T2 – T3 – T4 – T5 – T6) during two different seasons June – July 2009 (■) and April – May 2010 (■). Different letters denote significant differences according to Duncan test at 5 % of probability

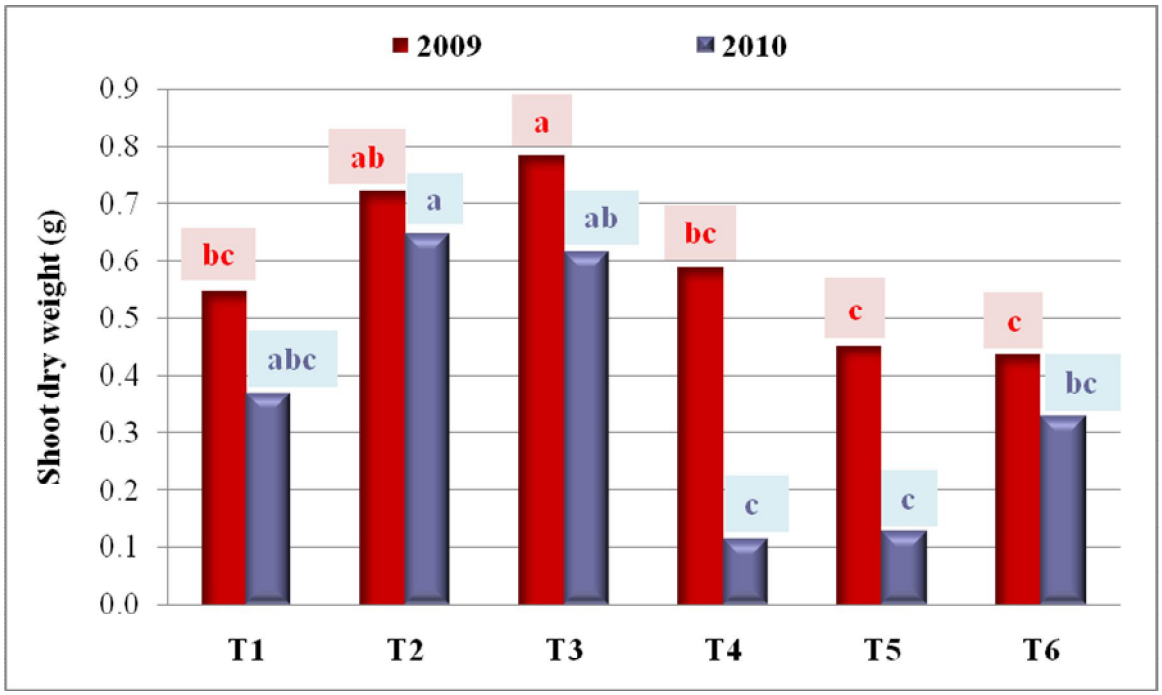

Fig 35. Shoot dry weight (SDW) of melon seedlings grown in the six different based-organic substrates (T1 – T2 – T3 – T4 – T5 – T6) during two different seasons June – July 2009 (■) and April - May 2010 (■). Different letters denote significant differences according to Duncan test at 5 % of probability

4.5.1.6. Fresh and dry root weight

Significant differences among the substrates were observed for the fresh and dry root weight during both studied growing season (figure 36; 37), except the fresh root weight of the first growing season (2009) that no significant difference was shown between the six organic substrates. The fresh and dry root weights of the seedlings grown during June – July 2009 present the best results.

All the studied variables (fresh and dry root weight) of the melon seedlings presented larger values for both growing seasons when grown in T1, T2 and T3 substrates. The smallest values observed for the fresh root weight were 0.20 g obtained from seedlings grown in T5 substrate during the growing season April – May 2010; in addition the lowest dry weight with a value of 0.01 g was revealed for those plants grown in the same substrate (T5) of the same growing season (2010).

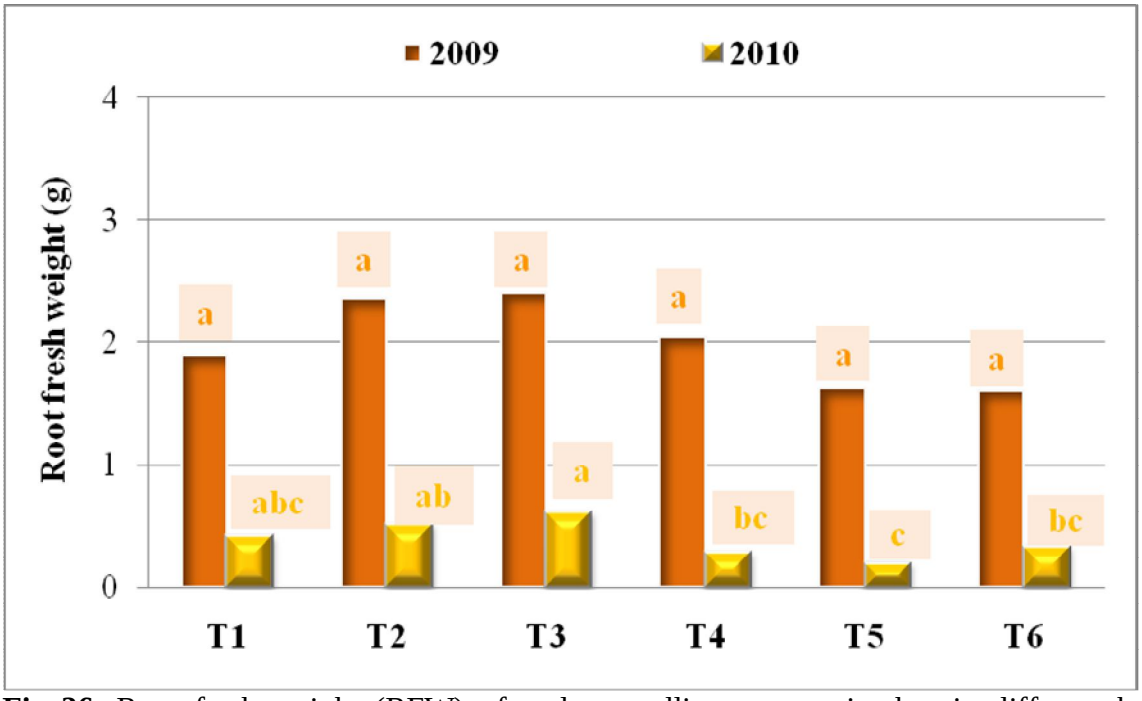

Fig 36. Root fresh weight (RFW) of melon seedlings grown in the six different based-organic substrates (T1 – T2 – T3 – T4 – T5 – T6) during two different seasons June – July 2009 (■) and April - May 2010 (■). Different letters denote significant differences according to Duncan test at 5 % of probability

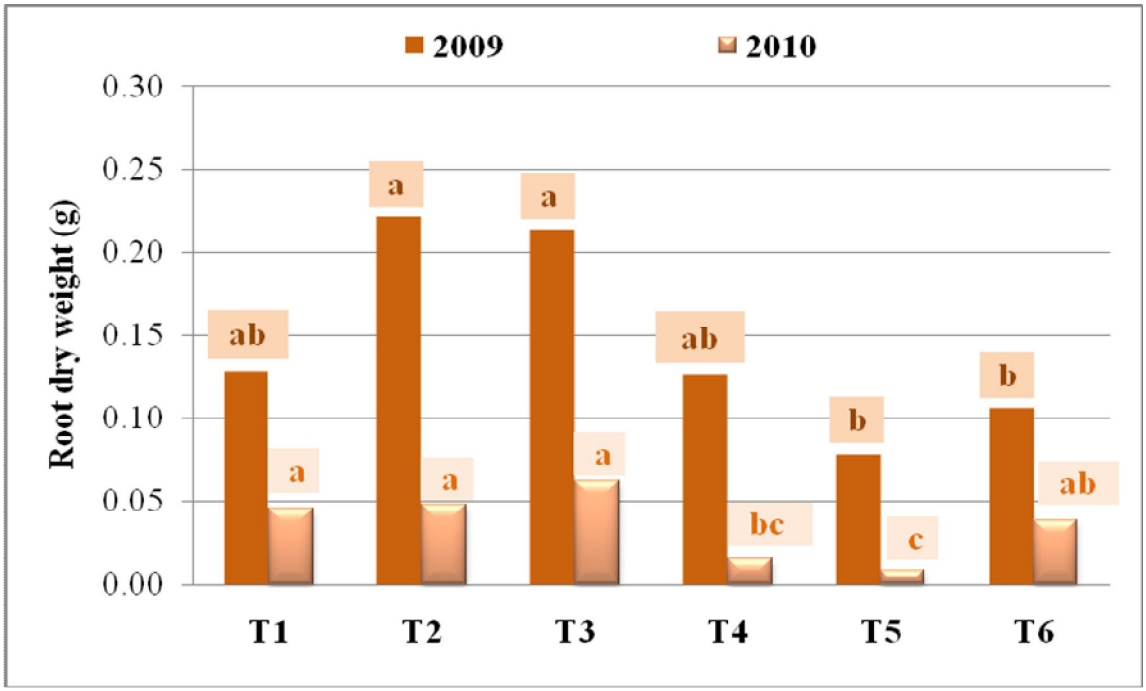

Fig 37. Root dry weight (RDW) of melon seedlings grown in the six different based-organic substrates (T1 – T2 – T3 – T4 – T5 – T6) during two different seasons June – July 2009 ( ) and April - May 2010 ( ). Different letters denote significant differences according to Duncan test at 5% of probability

4.5.2. Colonization of bulk soil, rhizosphere and rhizoplane by bacterial communities

4.5.2.1. Bacterial communities isolated from bulk soil and rhizosphere

Two bacterial groups (fluorescent pseudomonads and spore forming bacteria) and total viable bacteria were isolated from the bulk soil and rhizosphere of all six organic substrates used in this study. The significant differences of bacterial colonies in the case of FPB, SFB and total viable bacteria were observed between the all organic growing substrates. The studied bacterial groups were highly isolated from the rhizosphere of the six organic substrates than from their bulk soil.

The total viable bacteria were largely isolated from the rhizosphere of the control substrate (T1) with a bacterial number of 9.04 x 10^9 cfu/g soil dry weight (SDW), while the viable bacteria extremely colonize the bulk soil of T2 substrate (7.54 x 10^8 cfu/g SDW) (figure 38). The lowest number of total viable bacteria was isolated from the bulk soil (5.06 x 10^7 cfu/g SDW) and rhizosphere (7.08 x 10^8 cfu/g SDW) of T6 substrate.

As for the viable bacteria, the fluorescent pseudomonad bacteria were largely isolated from the bulk soil of T2 substrate (2.05 x 10^6 cfu/g SDW), the rhizosphere of T1 (5.25 x 10^6 cfu/g SDW) (figure 39). The bulk soil of T6 substrate (6.58 x 10^4 cfu/g SDW) and the rhizosphere of T4 substrate (6.63 x 10^5 cfu/g SDW) showed the poorly colonization in number (cfu) of

fluorescent bacteria. The aerobic spore-forming bacteria colonize extremely the substrates based on compost residue, that were mostly isolated from the bulk soil of the T5 (8.76 x 10^7 cfu/g SDW) and T6 substrates (9.88 x 10^7 cfu/g SDW) and from the rhizosphere of T4 substrate (2.67 x 10^9 cfu/g SDW) (figure 40). The small amount of SFB in number of cfu were isolated from the bulk soil (6.05 x 10^7 cfu/g SDW) and the rhizosphere (6.19 x 10^8 cfu/g SDW) of T3 substrate based on 30 % coconut fiber.

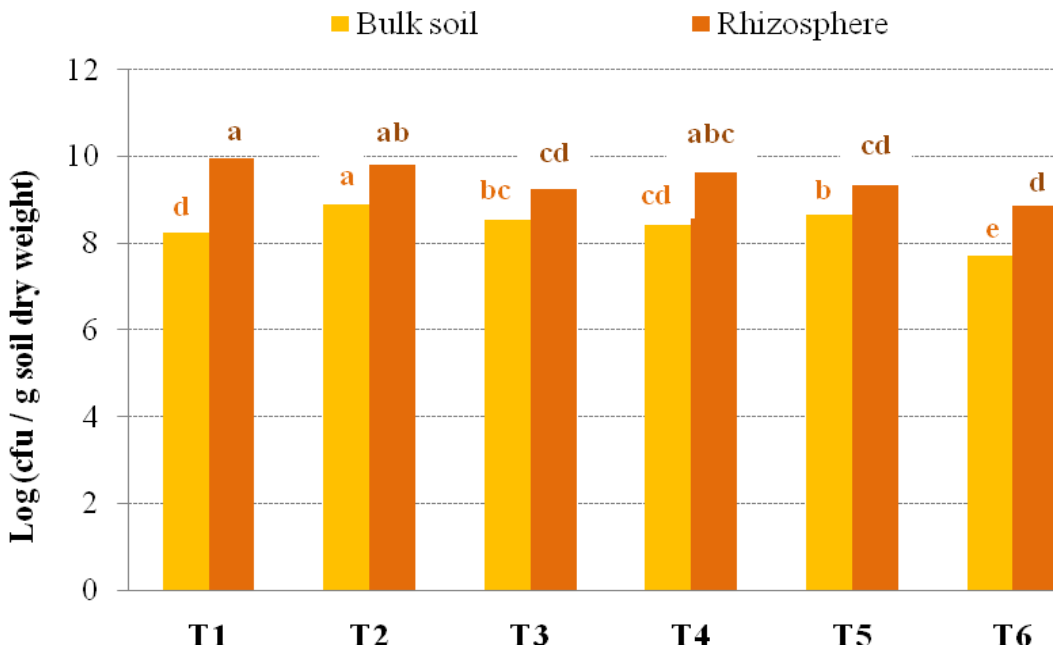

Fig 38. Total numbers of viable bacteria isolated from bulk soil ( ) of the six studied substrates and rhizosphere ( ) of melon seedlings grown in these substrates. Different letters denote significant differences according to Duncan test at $P< 0.05$

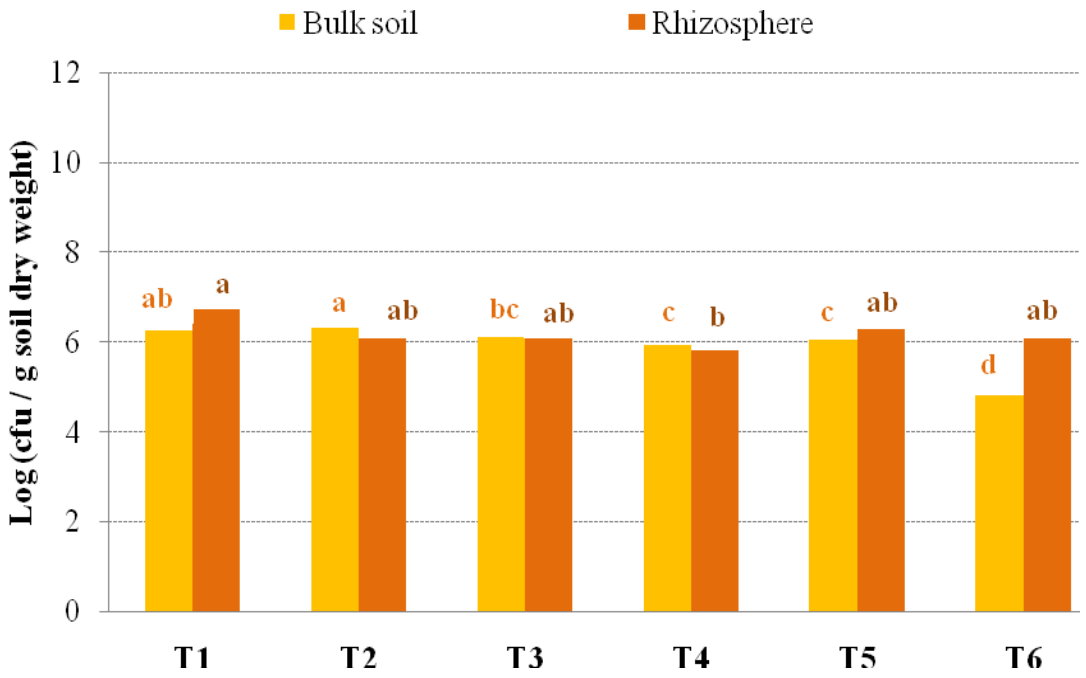

Fig 39. Total numbers of fluorescent pseudomonad bacteria isolated from bulk soil ( ) of the six studied substrates and rhizosphere ( ) of melon seedlings grown in these substrates. Different letters denote significant differences according to Duncan test at $P< 0.05$

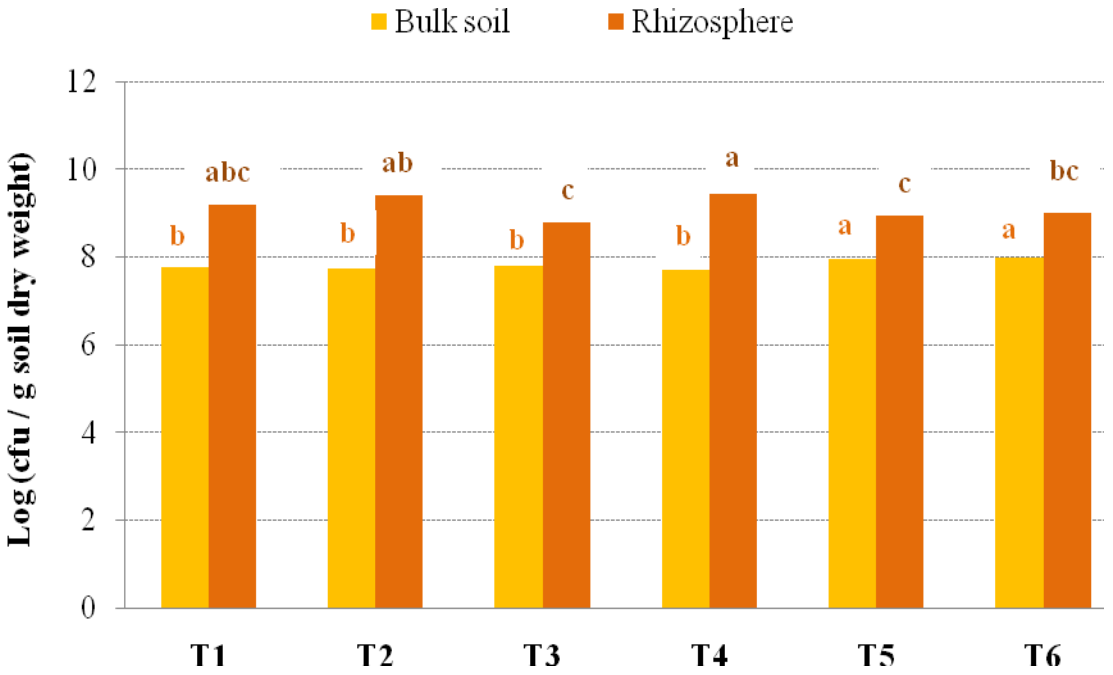

Fig 40. Total numbers of aerobic spore-forming bacteria isolated from bulk soil ( ) of the six studied substrates and rhizosphere ( ) of melon seedlings grown in these substrates. Different letters denote significant differences according to Duncan test at $P< 0.05$

4.5.2.2. Bacterial communities isolated from rhizoplane “a” and “b”

The analysis of bacterial populations was carried out on the rhizoplane “a” and “b” of melon seedlings grown in the six different substrates. From the rhizoplane “a” we isolate the bacteria adhering to root surface and the endophyte bacteria were isolated from the rhizoplane “b”.

Significant differences in number of the bacterial groups were observed among the studied substrates. The both isolated sites (rhizoplane “a” and “b”) of all the studied organic growing substrates were mostly colonized by the spore forming bacteria.

The total viable bacteria appear to colonize better the rhizoplane “b” than the rhizoplane “a” (figure 41), the higher number of viable bacterial colonies were isolated from the rhizoplane “a” of T1 substrate (2.87×10^8 cfu/g RFW) and from the rhizoplane “b” of T1 (3.5×10^8 cfu/g RFW) and T2 (2.34×10^8 cfu/g RFW) substrates. While, the values of total bacteria were found slightly lower in those samples taken from the rhizoplane “a” (3.26×10^6 cfu/g RFW) and “b” (5.31×10^6 cfu/g RFW) of the T6 substrate based on compost and coconut fiber residue.

The larger amount of Fluorescent pseudomonad bacteria was isolated from the rhizoplane “a” of T1 (6.23×10^4 cfu/g RFW) and T3 (4.64×10^4 cfu/g RFW) substrates and from the rhizoplane “b” of the same substrates T1 (4.42×10^4 cfu/g RFW) and T3 (4.64×10^4 cfu/g RFW) (figure 42). These results shown that the FPB colonize deficiently the substrates based on compost residue that the rhizoplane “a” of T6 substrate (9.19×10^3 cfu/g RFW) and rhizoplane “b” of T5 substrate (8.14×10^3 cfu/g RFW) have the lesser FPB populations.

The aerobic spore-forming bacteria show a variation in the number of cfu/g among the six organic substrates (figure 43), that were mostly isolated from the rhizoplane “a” (7.17×10^6 cfu/g RFW) and rhizoplane “b” (6.75×10^6 cfu/g RFW) of T4 substrate based on compost residue. In addition the SFB were slightly present in those samples taken from the rhizoplane “a” (6.14×10^5 cfu/g RFW) and rhizoplane “b” (6.65×10^5 cfu/g RFW) of T3 substrate.

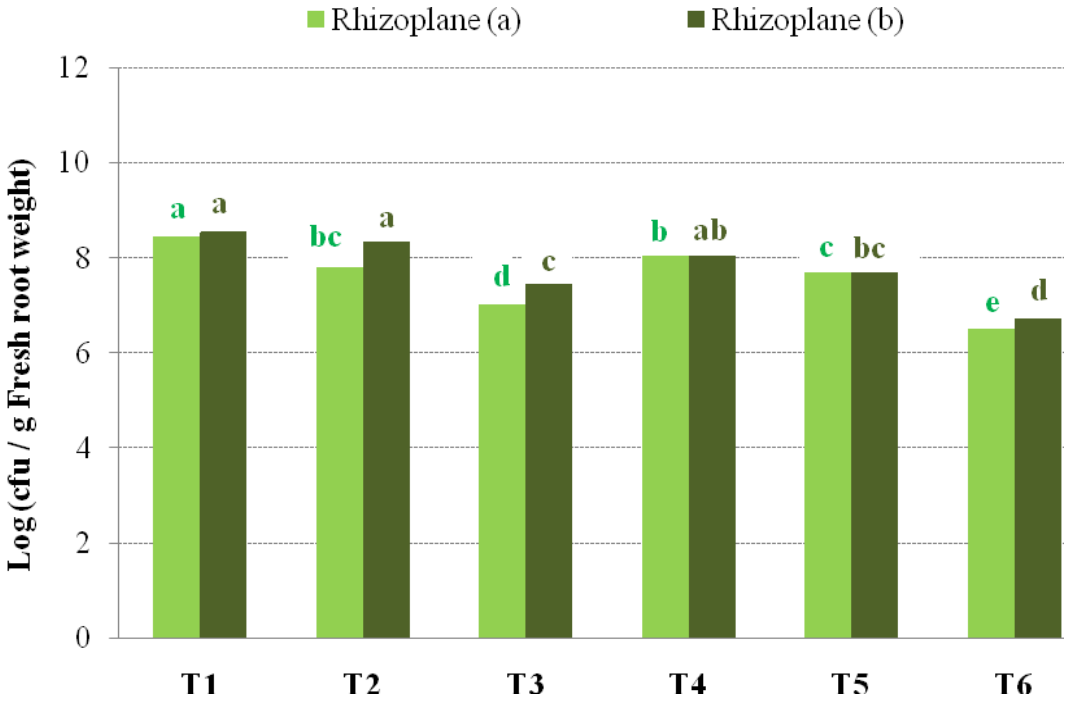

Fig 41. Total numbers of viable bacteria isolated from rhizoplane "a" ( ) and rhizoplane "b" ( ) of melon seedlings grown in the six studied substrates. Different letters denote significant differences according to Duncan test at $P< 0.05$

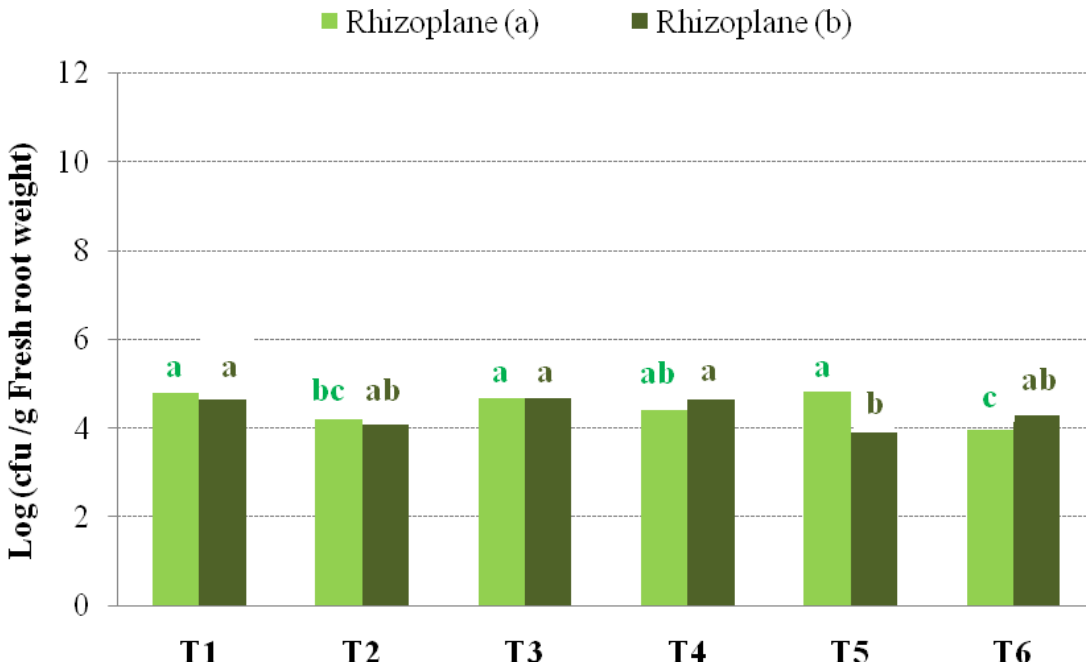

Fig 42. Total numbers of fluorescent pseudomonad bacteria isolated from rhizoplane "a" ( ) and rhizoplane "b" ( ) of melon seedlings grown in the six studied substrates. Different letters denote significant differences according to Duncan test at $P< 0.05$

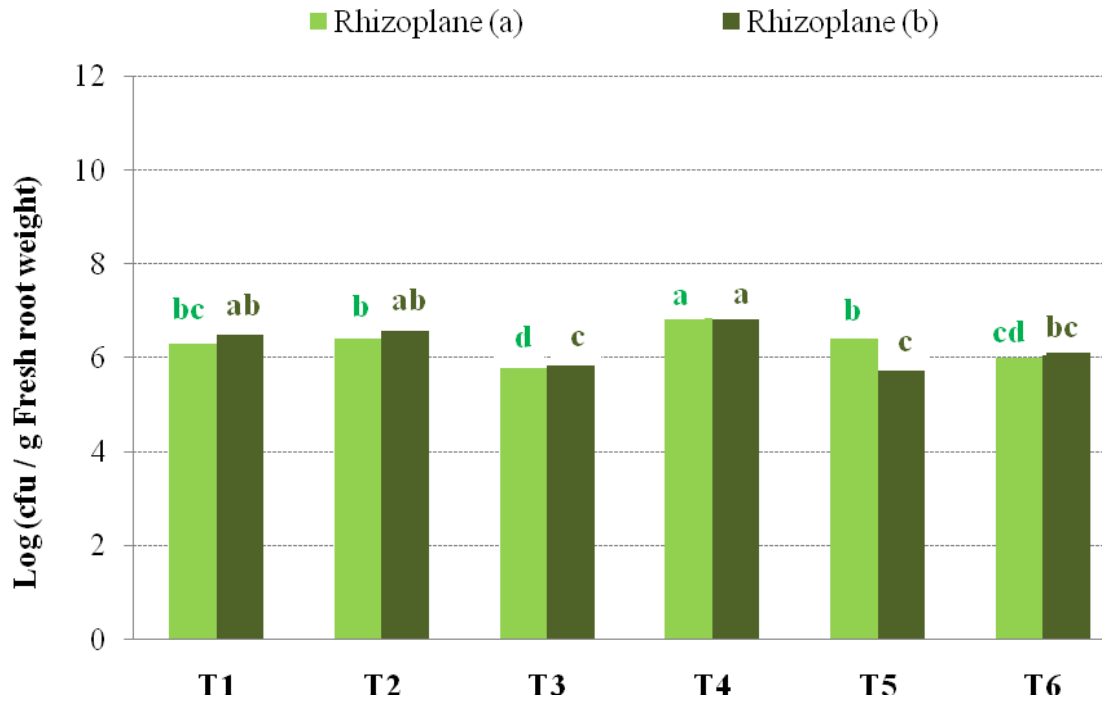

Fig 43. Total numbers of aerobic spore-forming bacteria isolated from rhizoplane "a" ( ) and rhizoplane "b" ( ) of melon seedlings grown in the six studied substrates. Different letters denote significant differences according to Duncan test at $P< 0.05$

4.6. *In vivo* antagonism assay against *Fusarium oxysporum* f. sp. *melonis*

4.6.1. Disease severity (DS) and area under the disease progress curve (AUDPC)

Distributions on the first experiment of the fungal pathogen (FOM F30) in classes of disease severity at different dates and in classes of AUDPC are shown in figure 44 and table 22, respectively.

No symptom was observed in the control melon plants trial (A), in contrast the untreated plants inoculated with FOM F30 (trial "B") showed typical wilting symptoms: initial marginal yellowing followed by general yellowing of the older leaves, and then wilting. A good number of untreated melon plants died 14 days after inoculation (AUDPC = 151.13) (table 22). The AUDPC of infected melon plants trials (H, I, J, K), that were treated by drenching the soil respectively with bacterial suspension of B1 (AUDPC = 154.17), B2 (AUDPC = 73), B3 (AUDPC = 89.77) and C6 (AUDPC = 94.45) isolates, was relatively elevated than those trials (D, E, F, G) in which seeds were coated respectively by those bacterial isolates B1 (AUDPC = 92.5), B2 (AUDPC = 70), B3 (AUDPC = 73.86) and C6

(AUDPC = 81.52). Moreover, the B2 bacterial isolate treated by applying the two different techniques was the most effective for biological control of FOM.

The first symptoms in the inoculated melon plants were observed 10 days after inoculation (figure 44), the highest disease severity percentage was shown by the untreated trial and the trials treated by B1 isolate. The disease severity of the untreated and all treated trials increase exponentially with time. No difference of disease severity, 14 days after pathogen inoculation, was observed among the treated melon plant trials, while the disease severity of those treated plants differs from that of the untreated plants. Application of the tested antagonistic bacteria reduced the severity of melon Fusarium wilt, beside the disease severity of the treated plants by drenching the soil with bacterial suspension of B1 isolate (DS= 78.13) did not differ from that of the untreated melon plants (DS= 78.41).

Table 22. Effect of four bacterial isolates (B1, B2, B3, C6) collected from water suspension of ECOS compost on the area under the disease progress curve (AUDPC) of Fusarium wilt caused by *F. oxysporum* f. sp. *melonis* (FOM F30) in melon plants in greenhouse experiment.

Trials code[1]	**Techniques of bacterial treatment**	**Bacterial isolates**	**FOM**	**AUDPC**
A	-	-	-	**0**
B	-	-	+	**151.13**
C	Drenching suspension	Sublic*	+	**82.95**
D	Seed bacterization	B1	+	**92.5**
E		B2	+	**70**
F		B3	+	**73.86**
G		C6	+	**81.52**
H	Drenching suspension	B1	+	**154.17**
I		B2	+	**73**
J		B3	+	**89.77**
K		C6	+	**94.45**

‘‘ + ” and ‘‘_” represent the presence and absence of the treatment, respectively.

* Commercial bio-product formulation used as positive control and based on *B. licheniformis* and *B. subtilis*

(1): trials code were mentioned in the table 11.

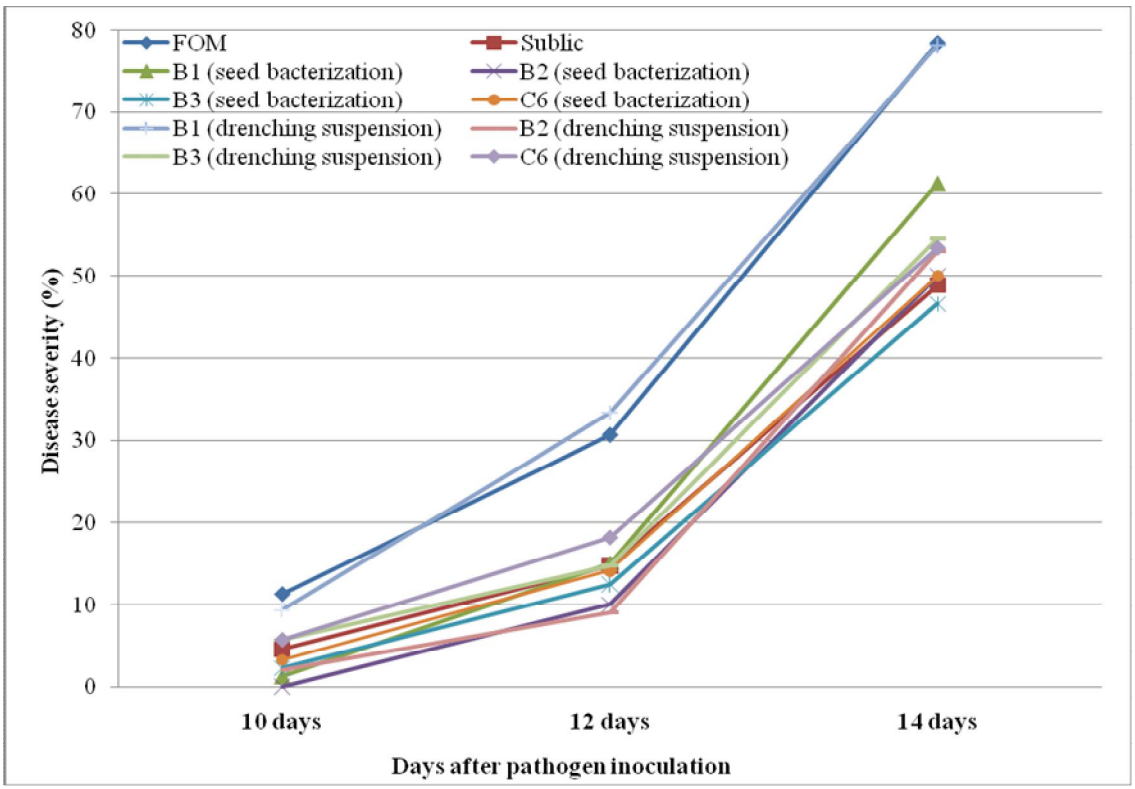

Fig 44. Severity of Fusarium wilt caused by FOM on melon plants treated with four bacterial isolates (B1, B2, B3, C6) collected from ECOS compost by using two techniques of treatment

The AUDPC and disease severity values, reported respectively in table 23 and figure 45, pointed out that the tested fluorescent (S.2.2 and R.2.1) and spore forming bacteria (P8 and P18), isolated respectively from MAIB-ECOS substrate and field infected by FOM, showed a highest suppressive effect to FOM F30.

Symptoms of Fusarium wilt appeared earlier in the untreated melon plants (trial II) than in treated melon plants. Furthermore, untreated melon plants showed symptoms of Fusarium wilt (22.83 % disease severity) 8 days after transplanting (AUDPC = 415.22). The AUDPC of the infected melon plants that were treated with the antagonistic bacteria was highly reduced in case of treating the plants 7 days before pathogen inoculation by drenching the soil with S.2.2 (AUDPC = 281.55) and P18 (AUDPC = 330.95) bacterial suspension. However, the treated melon plants by R.2.1 (AUDPC = 353) and P8 (AUDPC = 369) bacterial isolates showed few symptoms of Fusarium wilt when applying the seed bacterization technique. No disease was observed in the control plant trial (I).

Untreated melon plants showed 85.87 % Fusarium wilt at day 16 after pathogen inoculation (figure 45). The disease severity of the untreated melon plants 16 days after inoculation did not differ from that trials “IX” and “X” of the melon plants treated respectively by R.2.1 (DS

= 83.33 %) and P8 (DS = 82.29 %). Application of S.2.2 by drenching the soil with the bacterial suspension reduced the severity of melon Fusarium wilt by 63.54 %, but not the severity of melon Fusarium wilt when applying the seed bacterization technique (DS = 76.04 %).

We noticed that the bacterial isolates B1, B2, B3 and C6 of the first experiment were more efficient to control fusarium wilt of melon plants than the bacterial isolates S.2.2, R.2.1, P8 and P18 of the second experiment. In fact, the first disease symptom of the first experiment appeared after 10 days of pathogen inoculation with a lowest percentage of disease severity equal to 0 %, observed in the trial "E" based on coating the seeds by B2 bacterial isolate, while the first symptom of the second experiment appeared 8 days after pathogen inoculation with the lowest disease severity value 9.52 % for trial "IX" based on drenching the soil by P18 bacterial suspension. Simultaneously, the pathogen was vigorous in the second experiment (DS 8 days after the pathogen inoculation of the untreated trial "II" = 22.83 %) more than the first one (DS 10 days after the pathogen inoculation of the untreated trial "B" = 11.36 %).

Table 23. Effect of two FPB isolates (S.2.2, R.2.1) collected from MAIB-ECOS substrate and of two SFB isolates (P8, P18) collected from infected field by FOM on the area under the disease progress curve (AUDPC) of Fusarium wilt caused by *F. oxysporum* f. sp. *melonis* (FOM F30) in melon plants in greenhouse experiment.

Trials code[a]	**Techniques of bacterial treatment**	**Bacterial isolates**	**FOM**	**AUDPC**
I	-	-	-	**0**
II	-	-	+	**415.22**
III	Drenching suspension	Sublic*	+	**201.1**
IV	Seed bacterization	S.2.2	+	**378.14**
V		R.2.1	+	**353**
VI		P8	+	**369**
VII		P18	+	**340**
VIII	Drenching suspension	S.2.2	+	**281.55**
IX		R.2.1	+	**388.55**
X		P8	+	**384.37**
XI		P18	+	**330.95**

'' + " and ''_" represent the presence and absence of the treatment, respectively.

* Commercial bio-product formulation used as positive control and based on *B. licheniformis* and *B. subtilis*

[(a)]: trials code were mentioned in the table 12.

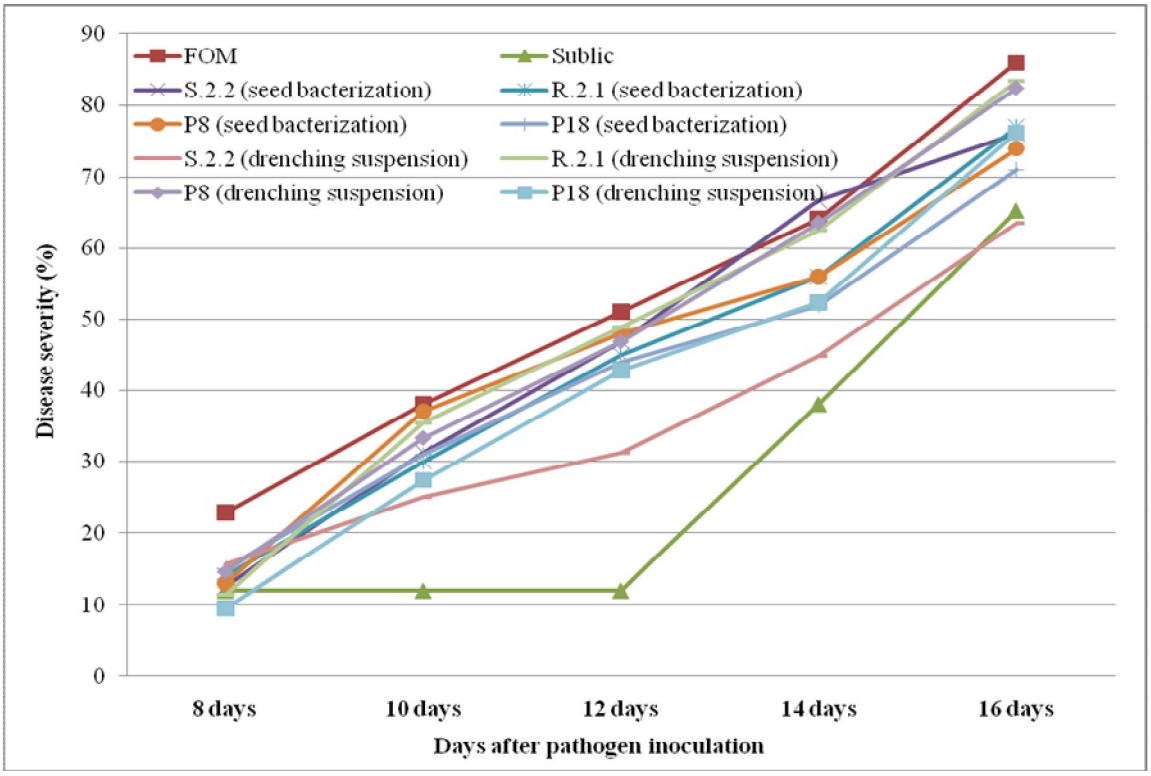

Fig 45. Severity of Fusarium wilt caused by FOM on melon plants treated with two bacterial isolates (S.2.2, R.2.1) collected from MAIB-ECOS substrate and two bacterial isolates (P8, P18) collected from infected field by FOM by using two techniques of treatment

4.6.2. Growth measures

4.6.2.1.Plant height

Experiment with the S.2.2, R.2.1, P8 and P18 antagonistic bacteria gave the most satisfactory plant height results. An analysis of variance for the plant height parameter, considering the trials of the both experiments, revealed the influence of the antagonistic bacteria in the expression of the plant height in all trials. Duncan's test results indicated that there were significant differences in the average value of the plant height among trials in both *in vivo* antagonism assays. There were significant differences in results between untreated and treated melon plants of both experiments. In the two *in vivo* assays there were significant differences between the two treatment techniques, in which the trials treated by drenching bacterial suspension showed the better plant height than those treaded by coating the seeds by the bacterial isolate. The plant height of the treated trials, "H" (8.29 cm) (figure 46) and "VI" (30.96 cm) (figure 47), showing a high disease severity was not significantly different from the untreated trial "B" (9.85 cm) and "II" (35.13 cm).

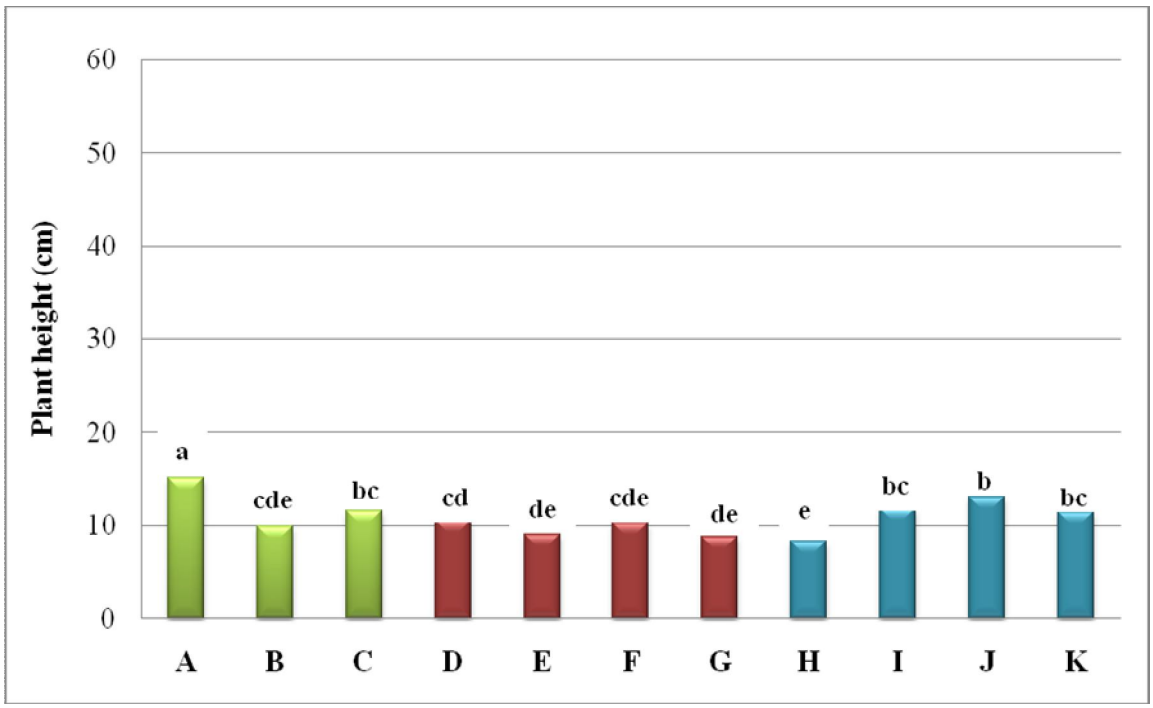

Fig 46. Plant height of melon seedlings treated with four spore forming bacterial isolates "B1" (D, H), "B2" (E, I), "B3" (F, J) and "C6" (G, K) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

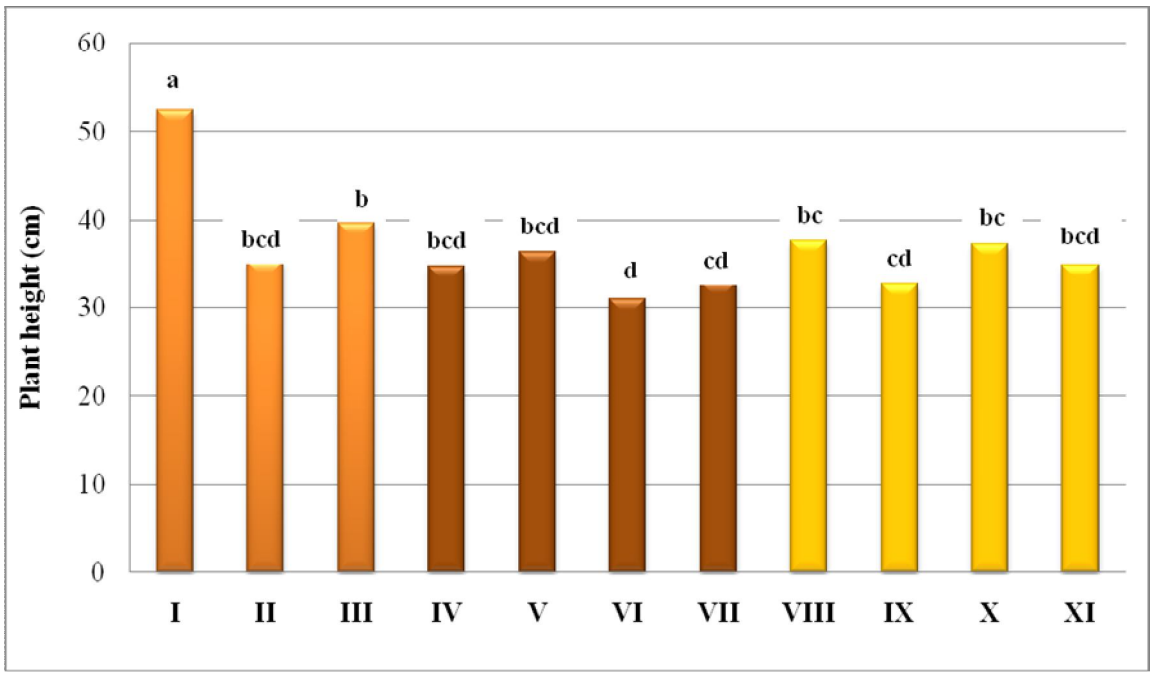

Fig 47. Plant height of melon seedlings treated with two pseudomonad bacterial isolates "S.2.2" (IV, VIII) "R.2.1" (V, IX) and two spore forming bacterial isolates "P8" (VI, X) "P18" (VII, XI) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

4.6.2.2.Root length

The effect of the antagonistic bacteria on plant growth under pathogen pressure demonstrated that these bacteria produced significantly higher influence on the root length of melon plant. Root length was significantly elevated on trials treated with B1, B2, B3 and C6 antagonistic bacterial isolates of the first experiment (figure 48) and with S.2.2, R.2.1, P8 and P18 bacterial isolates of the second experiment (figure 49). The second experiment showed considerably higher root length than the first experiment. As for the plant height, the root length was considerably different among the treated and untreated plant trials in both experiments. The treated trials by drenching bacterial suspension showed the better plant height than those treaded by coating the seeds by the bacterial isolate. The lowest root length was observed on those trials showing a high disease severity as the trial "H" of the first experiment (5.33 cm) and the trial "V" (16.26 cm) of the second experiment.

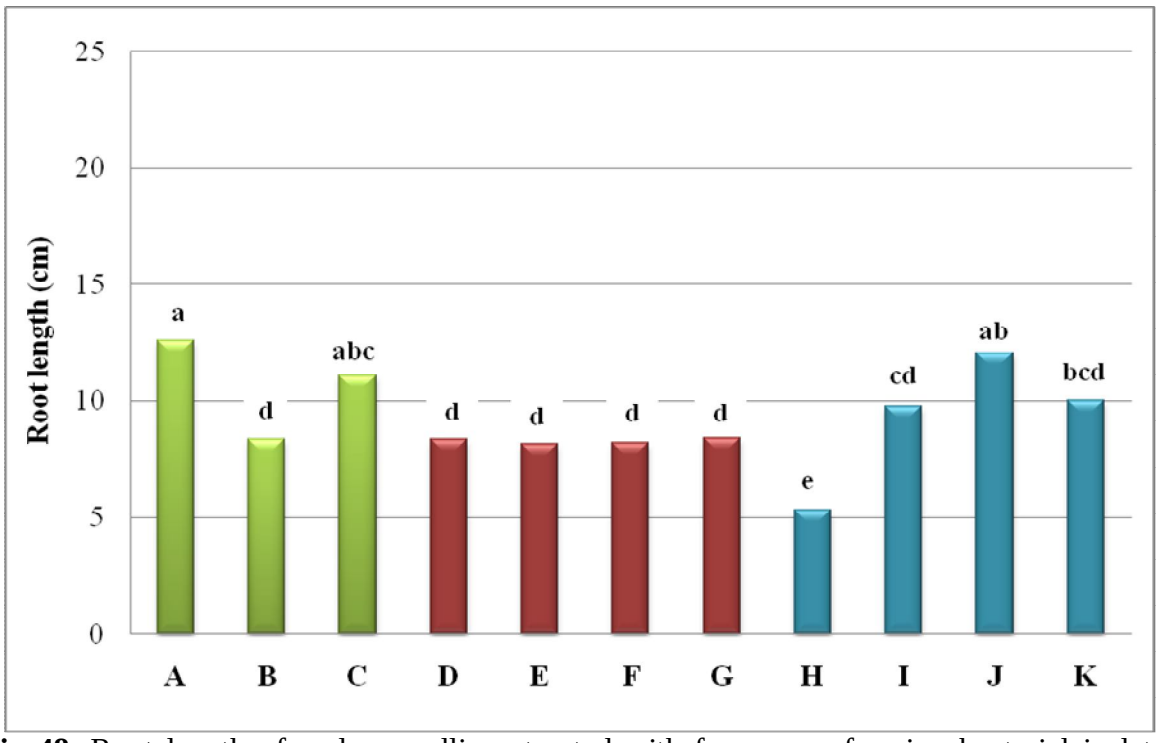

Fig 48. Root length of melon seedlings treated with four spore forming bacterial isolates "B1" (D, H), "B2" (E, I), "B3" (F, J) and "C6" (G, K) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

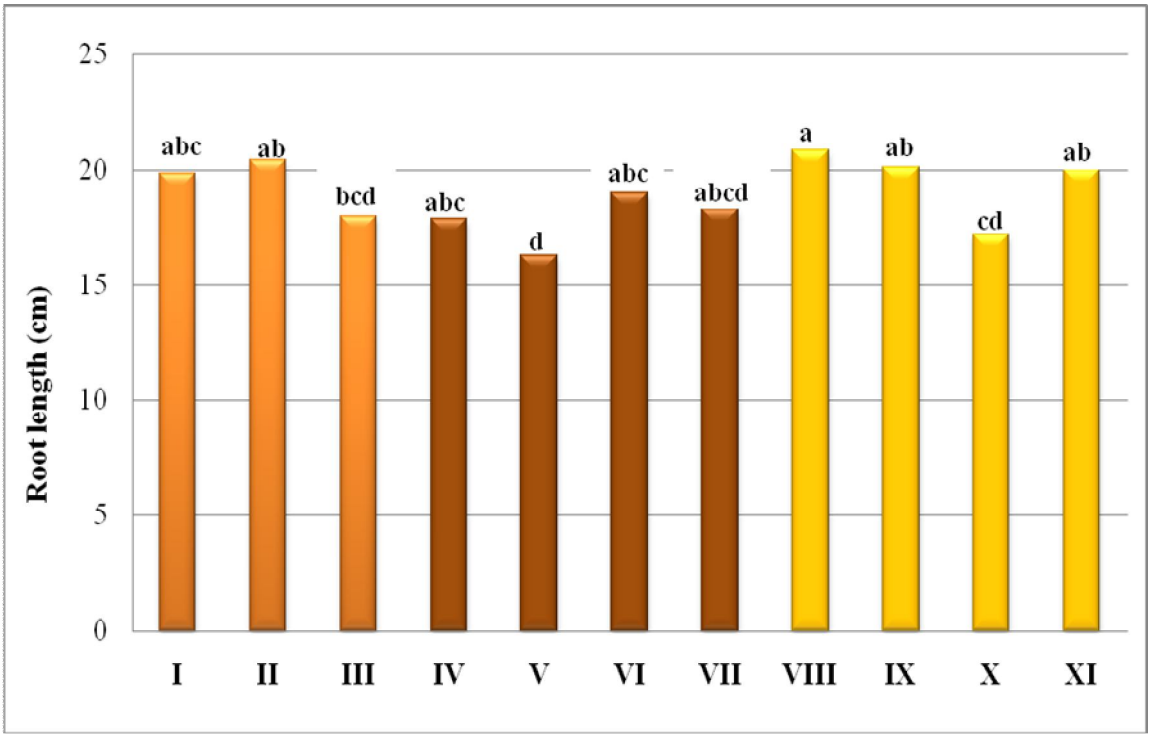

Fig 49. Root length of melon seedlings treated with two pseudomonad bacterial isolates "S.2.2" (IV, VIII) "R.2.1" (V, IX) and two spore forming bacterial isolates "P8" (VI, X) "P18" (VII, XI) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

4.6.2.3.Fresh and dry shoot weight

The present study showed a significant difference in the values of the fresh and dry shoot weight among trials of both *in vivo* antagonism assays. Results of the fresh shoot weight trials (figures 50 and 52) of the two experiments showed relatively the same significant differences obtained on the dry shoot weight trials (figures 51 and 53), that the lesser fresh and dry shoot weight were observed for the trial "H" of the first experiment and trials "II", "IX" and "X" of the second experiment. Melon plants treated by drenching the bacterial suspension show a significantly high dry and shoot weight values as compared to those plants treated by coating the melon seed by the bacterial isolates. The effect of the antagonistic bacteria in increasing shoot weight (fresh and dry) was more evident in the control trial ("C" and "III"), the treated "I", "J" and "K" trials of the first experiment and the treated "IV", "VII", "VIII" and "XI" trials of the second experiment with values significantly different from untreated trial "B" of the first experiment and "II" of the second experiment.

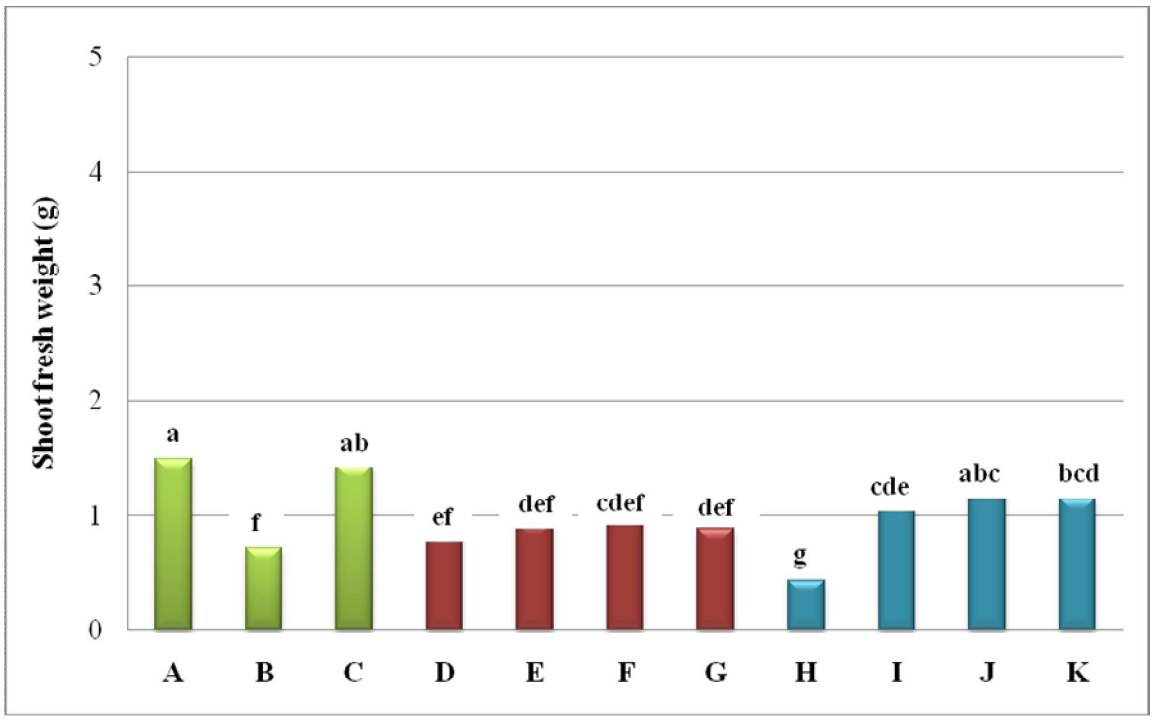

Fig 50. Shoot fresh weight of melon seedlings treated with four spore forming bacterial isolates "B1" (D, H), "B2" (E, I), "B3" (F, J) and "C6" (G, K) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

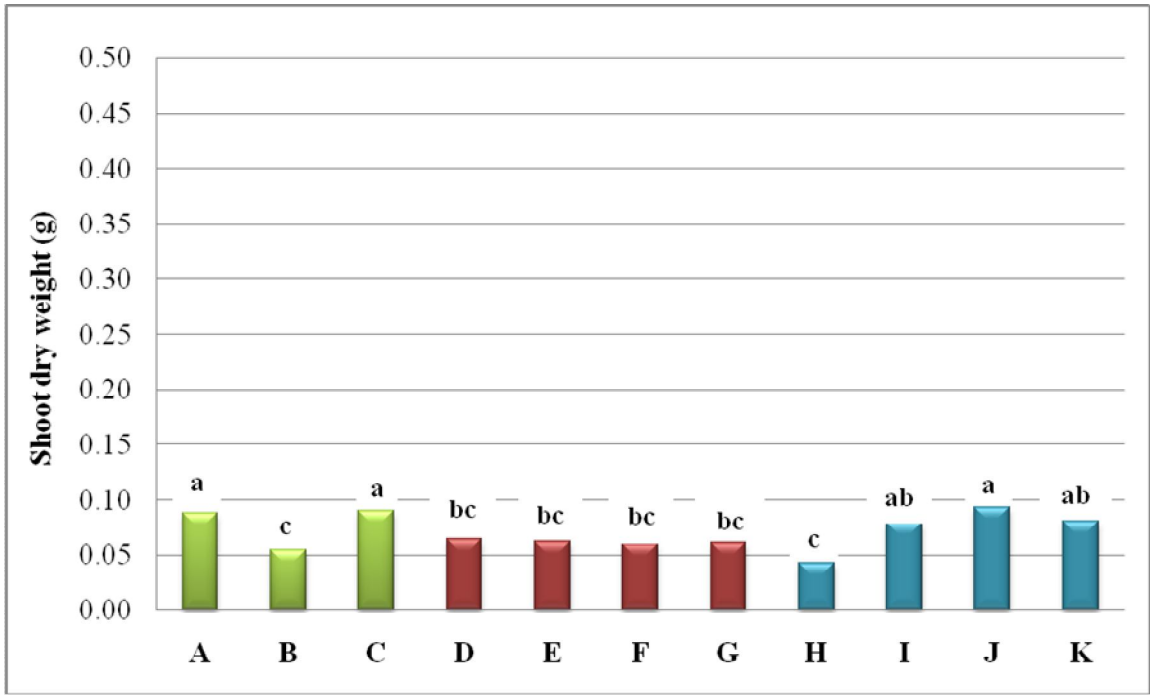

Fig 51. Shoot dry weight of melon seedlings treated with four spore forming bacterial isolates "B1" (D, H), "B2" (E, I), "B3" (F, J) and "C6" (G, K) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

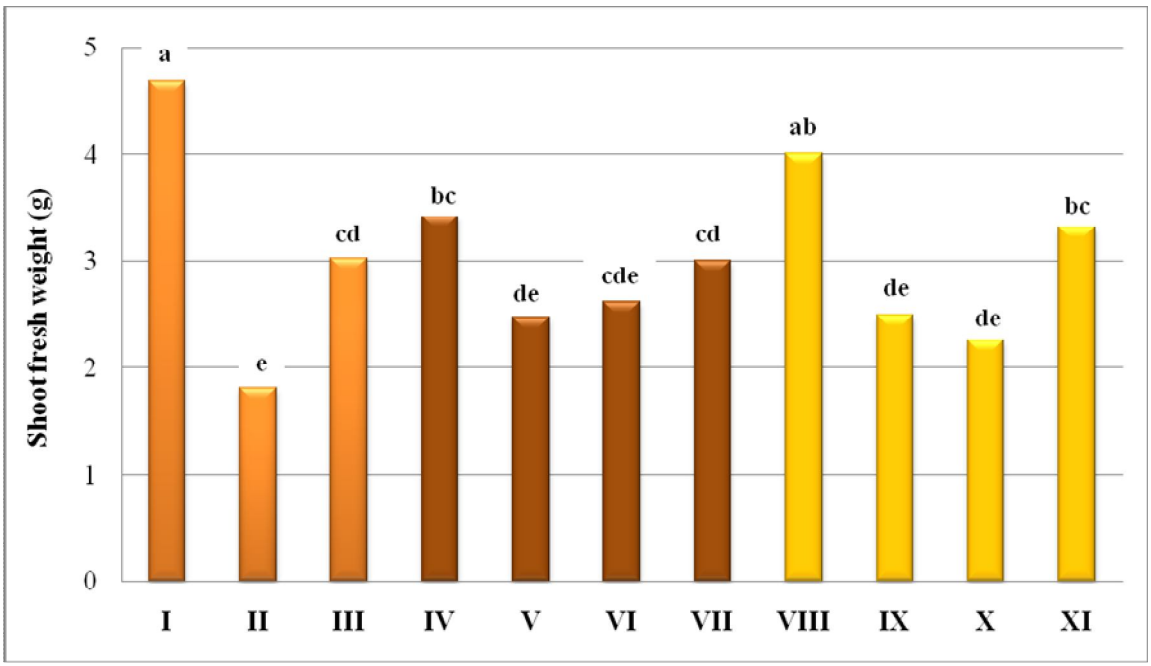

Fig 52. Shoot fresh weight of melon seedlings treated with two pseudomonad bacterial isolates "S.2.2" (IV, VIII) "R.2.1" (V, IX) and two spore forming bacterial isolates "P8" (VI, X) "P18" (VII, XI) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

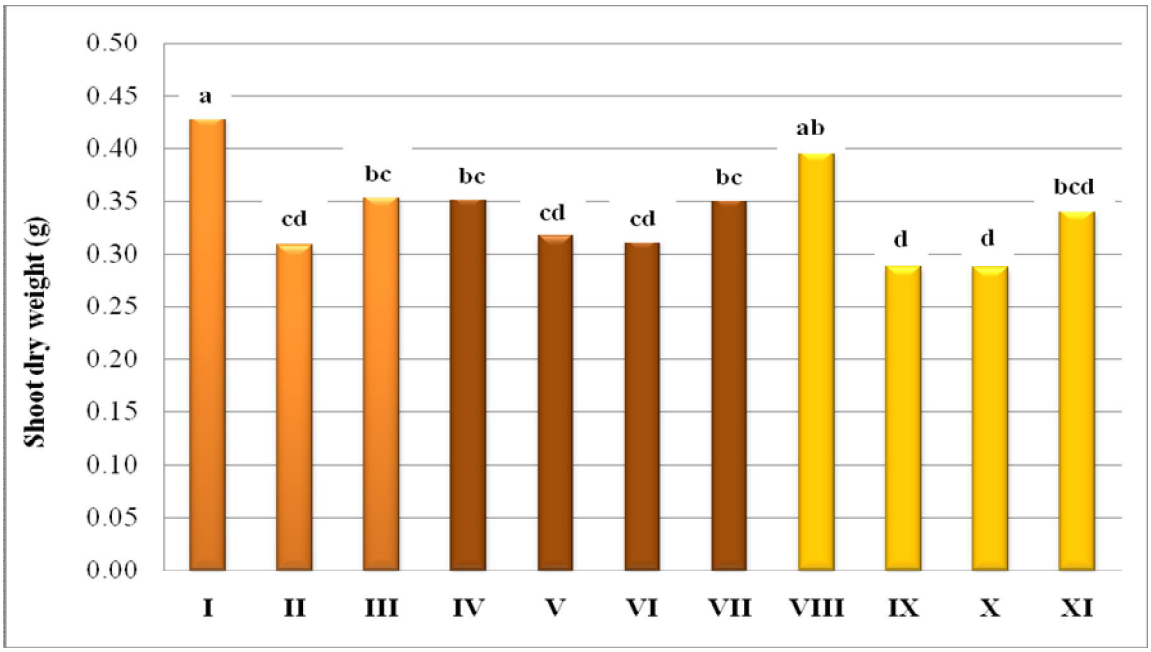

Fig 53. Shoot dry weight of melon seedlings treated with two pseudomonad bacterial isolates "S.2.2" (IV, VIII) "R.2.1" (V, IX) and two spore forming bacterial isolates "P8" (VI, X) "P18" (VII, XI) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

4.6.2.4.Fresh and dry root weight

The analysis of variance of treated and untreated trials in both assays revealed the influence of the inoculated antagonist on the fresh and dry root weight. The root weight of trials (fresh and dry) treated with S.2.2, R.2.1, P8 and P18 bacterial isolates (figures 56 and 57), either treated by drenching the soil by bacterial suspension or by seed bacterization, was higher than that trials treated with B1, B2, B3 and C6 bacterial isolates (figures 54 and 55). The untreated and the all treated trials of both experiments were significantly different between them for the root weight values. As for the all other parameters, the lower expression of the fresh and dry root weight was obtained for trial "H" of the first experiment based on drenching the soil by B1 bacterial suspension, and we noticed that the bacterial strain S.2.2 of the second experiment represented the higher value of fresh and dry root weight.

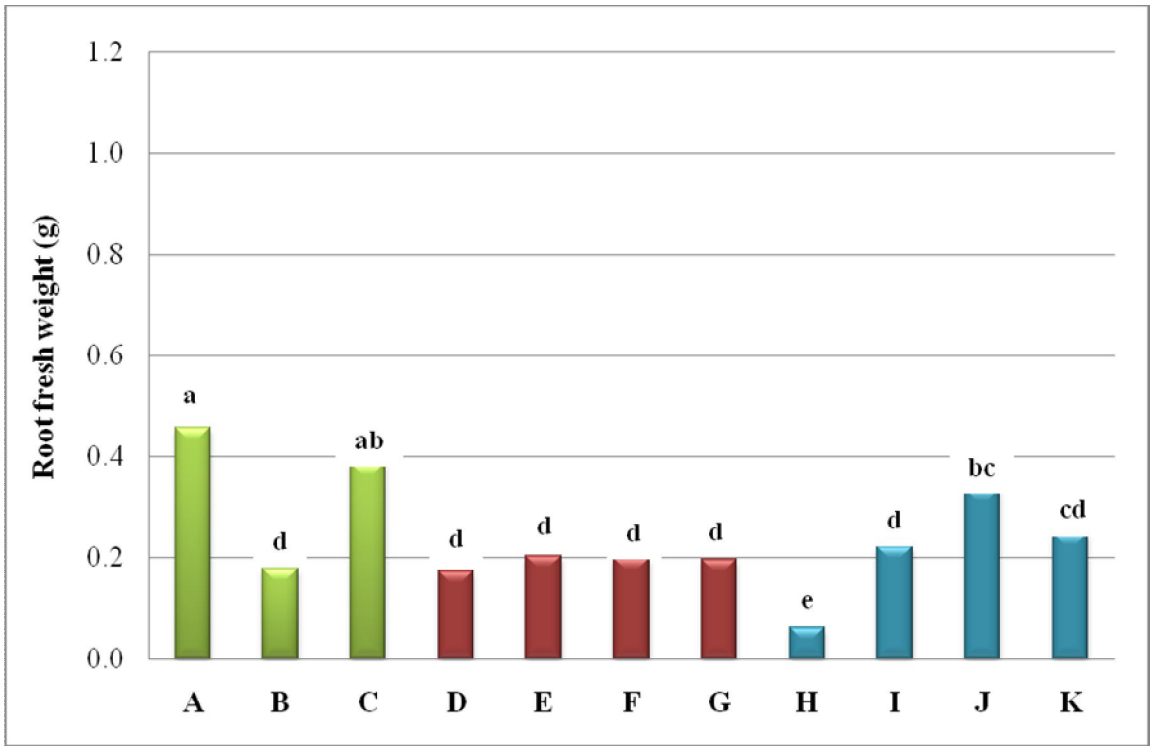

Fig 54. Root fresh weight of melon seedlings treated with four spore forming bacterial isolates "B1" (D, H), "B2" (E, I), "B3" (F, J) and "C6" (G, K) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

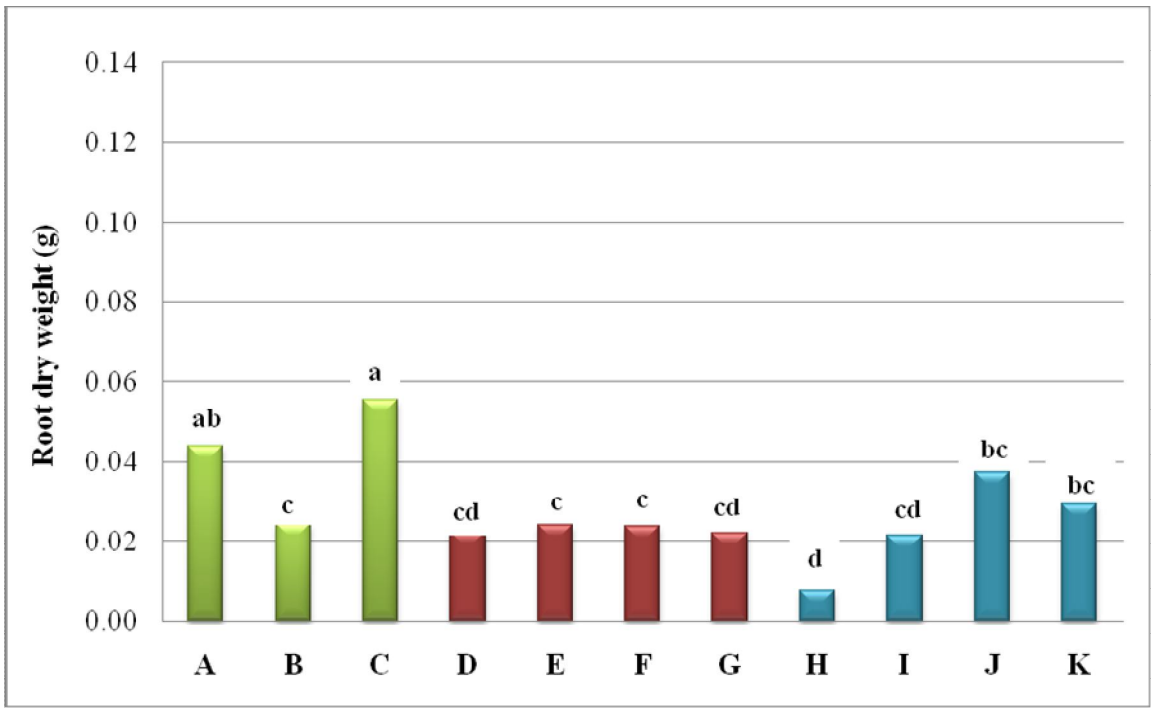

Fig 55. Root dry weight of melon seedlings treated with four spore forming bacterial isolates "B1" (D, H), "B2" (E, I), "B3" (F, J) and "C6" (G, K) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

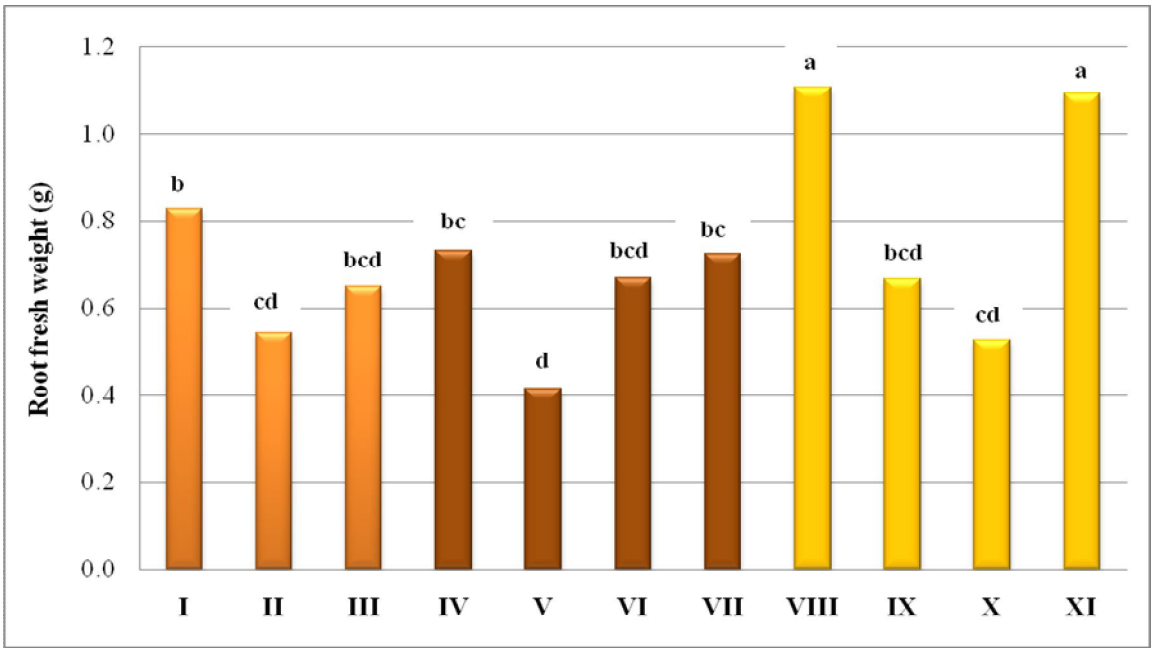

Fig 56. Root fresh weight of melon seedlings treated with two pseudomonad bacterial isolates "S.2.2" (IV, VIII) "R.2.1" (V, IX) and two spore forming bacterial isolates "P8" (VI, X) "P18" (VII, XI) by using ( ) control and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

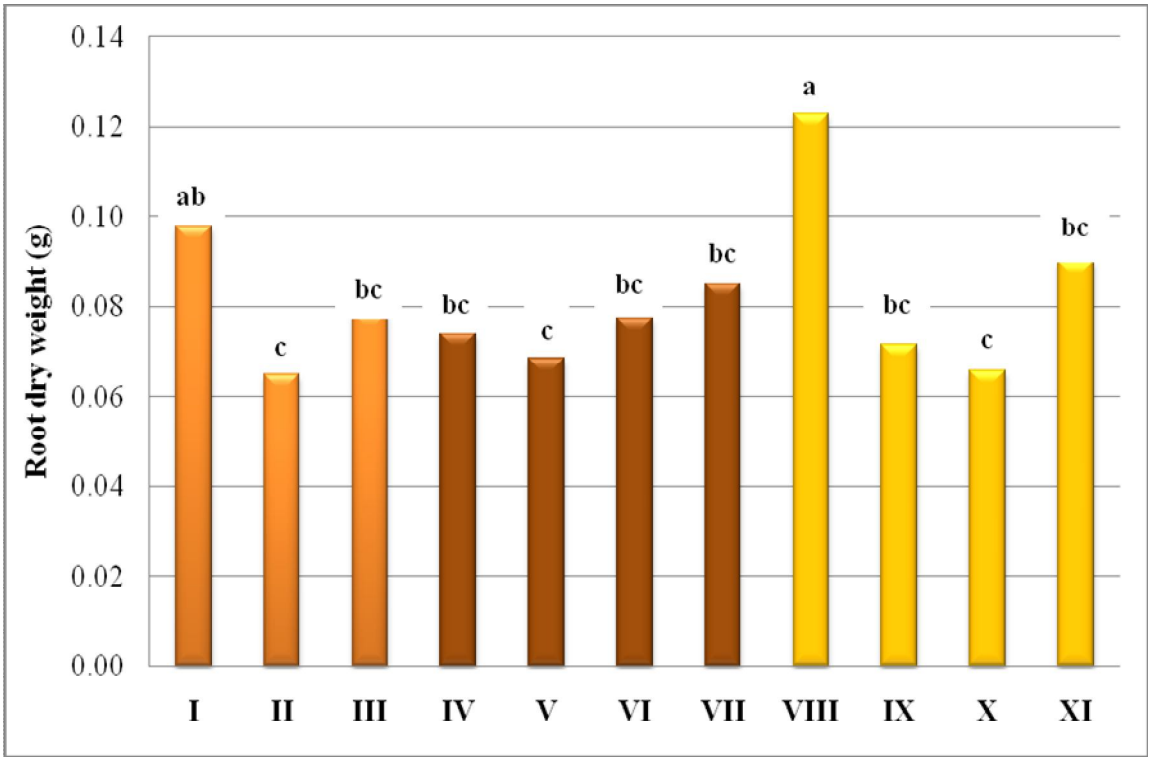

Fig 57. Root dry weight of melon seedlings treated with two pseudomonad bacterial isolates "S.2.2" (IV, VIII) "R.2.1" (V, IX) and two spore forming bacterial isolates "P8" (VI, X) "P18" (VII, XI) by using ( ) controls and two techniques of treatment: ( ) seed bacterization and ( ) drenching bacterial suspension, in the presence of FOM F30. Different letters denote significant differences according to Duncan test at 5 % of probability

4.7. Molecular identification of antagonistic bacteria

Molecular methods were used to identify the bacteria most effective as biocontrol agents of Fusarium wilt of melon. These bacteria belonged to different species of the genus *Bacillus* or related groups. Our molecular analysis showed that the resulting sequences of the two bacterial strains were closely identical to *B. subtilis*. More specifically, the identified species is an essential component of the compost microbiota and it is the most frequently found into the thermophyllic bacteria, it is able to grow at 55 °C and can survive temperatures of up to 80 °C.

5) Discussion

To reduce the deleterious effects of pesticide applications in agriculture, request of alternative control methods are required. To achieve this goal, many strategies are needed to promote IPM at global, regional and national levels. One of the most promising mean is the development of economic biological control procedures for feasible use in agricultural applications. Also, it is well known that some soils and compost proved recently to have the potential to provide biological control against some soil borne plant pathogens such as *Fusarium oxysporum*, *Pythium* and *Phytophthora* species and this suppression relates to both physiochemical and microbiological features of the substrate (McKellar and Nelson *et al*., 2003; Mazzola M., 2002).

Microorganisms present in the tested commercial compost that may have a suppressive effect were isolated. Numerous bacteria were recovered from ECOS compost that appears to be highly colonized by the aerobic spore forming bacterial (SFB) group (the predominant bacterial taxa isolated were *Bacillus* spp.), this can be due to the interior temperature of the compost during composting that rose to 50 – 55 °C. A few species are able to grow at high temperatures (Pitt, 1979). The elevated temperature is suitable for thermophiles and thermotolerant bacteria. One of the most frequent thermophilic and thermotolerant species present in the compost was *Bacillus* spores. These bacteria have a worldwide distribution and they can resist adverse environmental conditions such as growing at high temperature.

Isolation results from the four different isolate sites (bulk soil, rhizosphere and rhizoplane "a" and "b") of the compost based substrate (MAIB-ECOS) and infected field by FOM, revealed a variation in bacterial populations as well as in the number of cfu g^{-1}. The microbiological complexity and richness of the bulk soil of organic substrate and infected field make them useful for melon seedling growth, in order to produce plants with the root system colonized by beneficial bacteria. The positive impact of roots and root activity on rhizosphere and rhizoplane microbial diversity, especially the fluorescent pseudomonads, was confirmed. In fact, the roots can protect and maintain bacterial communities against strong physicochemical perturbation by nutrients secreted by the roots. Moreover, the results of this study made it possible to assess the major effect of roots on their microbial environment reproducibly (Antonelli *et al.*, 2009; Sebaaly *et al.*, 2009).

There were conspicuous differences in number of species compositions between the MAIB-ECOS substrate and infected field by FOM. From the results obtained, it can be concluded

that the higher various bacteria genera were isolated from the bulk soil, rhizosphere and rhizoplane of melon seedling grown in the infected field by FOM. The infected field is an environment that is high in microbial species diversity, for the reason that it is colonized by a greater variety of biocontrol agents to suppress the fungal pathogen of melon *F. oxysporum* f. sp. *melonis*. Moreover, we characterized the biodiversity of the bacterial microflora and the elevated microbial community of the infected field in response to physicochemical stabilization. Indeed, differences in published results may be due to differences in the amount of soil adhering to the roots, which is affected by soil type, soil moisture and root morphology, having a profound effect on the bacterial rhizosphere community (Marschner *et al.*, 2001).

In this study, the results of the direct contact condition (*in situ*) between the bacterial content of ECOS compost and the phytopathogenic fungus *Fusarium oxysporum* f. sp. *melonis* (FOM F30 isolate) and *Monosporascus cannonballus* (MC-R isolate), indicated that these bacteria are the main effective agent in the antagonistic process against FOM F30 and MC-R isolates. The compost contains biocontrol agents that appear to play an important role in fungal suppression, although the mechanisms by which these agents provide control remain unidentified (Boulter *et al.*, 2000). However, it has been postulated that the protective effects of compost could be due to one or a combination of factors as microbial richness of compost, high concentration of chemical components, or presence of a low molecular weight compound that lyses fungal parts. The mechanisms of suppressive action of compost against phytopathogenic fungi require further work to fully elucidate these mechanisms.

In the present study, the bacterial microorganisms isolated from the water suspension of ECOS compost revealed their potential biocontrol effect against FOM F30 and MC-R isolates more than those isolated from bulk soil of compost. The *in vitro* antagonism assay for bacterial extraction from water suspension of ECOS compost was extremely efficient to find the bacterial strains having an important role in suppressing the hyphal growth of FOM F30 and MC-R isolates, where six selected bacterial isolates (B1, B2, B3, C6, M2 and M3) belonging to *Bacillus* spp. shown a clear inhibition zone for MC-R isolate, while only four of these isolates (B1, B2, B3 and C6) had an antagonistic effect against FOM F30 isolate. The variability of suppressiveness reaction of these microorganisms toward FOM F30 and MC-R isolates has been attributed to their diverse antagonist mechanisms that depend on the production of antibiotics and antifungal compounds and the production of variable metabolites, such as the siderophore and the volatile compounds.

The findings reported in "*in vitro*" antagonism assays demonstrate that the use of microbial communities to induce a disease suppressiveness of soil could be a potential tool for the management of soil borne pathogens. The bacterial microorganisms isolated from the MAIB-ECOS substrate, especially those belonging to fluorescent pseudomonad group, demonstrated their potential biocontrol effect against fusarium wilt and monosporascus root rot in melon, meaning that the organic substrate may be considered for use in controlling soil borne diseases. Although, the microorganisms collected from the infected field samples shown an antagonist effects against the studied pathogens, while only the spore forming bacteria present a greater degree of biological control specially those isolated from the rhizoplane "a" and "b".

The isolated bacteria looked show a mechanism of action by the production of antibiotic-type secondary metabolites, which spread through the medium, leaving a clear band (inhibition zone) that separates the antagonist from the pathogen (Hoitink and Boehm, 1999). It was shown that only few bacterial isolates expressed direct antagonistic activity against the assayed soil borne fungi by the production of antimicrobial diffusible and volatile compounds such as hydrocyanic acid, while a considerable number of bacterial strains were able to produce Plant Growth Promoting traits such as phosphate solubilization and siderophore production, although a low percentage of bacterial IAA producers have been isolated.

In this review, the same bacterial isolates studied for their antagonistic effect were evaluated *in vitro* for their plant growth promoting traits. The PGP assays revealed that the *Pseudomonas* spp. isolated from the four different sites of MIAB-ECOS substrate and infected field samples have shown all the studied PGP traits, though the spore forming bacteria showed only the siderophore production activity, so as the cell walls of gram-positive bacteria have strong metal-binding properties (Beveridge *et al.*, 1982), also some bacteria produce extracellular polysaccharide sheaths that bind metals (Matthews *et al.*, 1979). Moreover, *Bacillus* spp. have been known for their siderophore production for many years and therefore many reports on the isolation and characterization of these siderophores have been published (Xicheng Hu and Boyer, 1996), as well the uptake of iron by the spore forming bacteria under limiting conditions is particularly well studied where iron are absorbed through specific uptake receptors (Archibald *et al.*, 1984; Tabillion *et al.*, 1977; Winkelmann, 1991).

The studied bacterial isolates can exert a positive effect on plant growth through direct and indirect mechanisms. Direct growth promotion can be through the solubilization of inorganic phosphate and mineralization of organic phosphate, which makes phosphorous available to the plants (Lifshitz *et al.*, 1987), stimulation of root growth by production of phytohormones (Bothe *et al.*, 1992; Kloepper *et al.*, 1980). Indirect growth promotion was completed by decreasing or preventing the deleterious effect of pathogenic microorganisms. The mechanism(s) by which bacteria can act as biocontrol agents to limit the damage to plants by phytopathogens were mostly attributable to: (i) the ability to produce siderophore that chelate iron, making it unavailable to pathogens; (ii) the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens (Bowen and Rovira, 1999; Glick and Bashan, 1990); (iii) the ability to successfully compete with pathogens for nutrients or specific niches on the root; and (iv) the ability to induce systemic resistance (Bloemberg *et al.*, 2001; Glick, 1995; Persello-Cartieaux *et al.*, 2003). These mechanisms are not mutually exclusive, and the overall biocontrol activity is due to the synergistic effects of different modes of antagonism (O'Sullivan and O'Gara, 1992). Different microorganisms, including associative bacteria such as *Azospirillum*, *Bacillus*, *Pseudomonas* and *Enterobacter* group, have being used for their beneficial effects on plant growth (Höflich *et al.*, 1994; Kloepper *et al.*, 1992) and for their antagonism against soil borne pathogens (Hoflich *et al.*, 1994).

The antifungal activities of the bacterial antagonists used in our study were previously mentioned, but these activities were correlated with the secretion of diffusible compounds or production of siderophores. Here we present an additional mode of action production of volatile compounds such as the HCN against the two soil borne pathogens of melon. Volatiles of the *Pseudomonas* spp. and *Bacillus* spp. notably inhibited the growth of both studied fungal diseases. To our knowledge this is the first documentation that VOCs of these bacterial antagonists inhibit the mycelium growth of *Fusarium oxysporum* f. sp. *melonis* and *Monosporascus cannonballus*.

Previously, it was demonstrated that VOCs of randomly selected soil bacteria isolates stimulate or inhibit the growth rate of *Trichoderma viridae, Phaenaerochaete magnoliae, Phytophthora cryptogea, Gaeumannomyces graminis* and *Microdochium nivale* (Wheatley, 2002). Alstrom (2001) reported that 21 strains of soil bacteria (*e.g.*, *Serratia proteamaculans*, *Pseudomonas putida*, *P. acidovorans*, *P. chlororaphis*, *Stenotrophomonas* spp. and *Alcaligenes* spp.) isolated from oil seed rape roots suppressed the pathogen *V. dahliae*, and

Fernando *et al.* (2005) demonstrated inhibitory effects of *Pseudomanas* spp. volatiles on *Sclerotinia sclerotiorum*. Also, bacillus species including *Bacillus subtilis* are known for their antifungal activity as the volatile compounds, hence their importance in the biological control of a number of plant diseases (Broadbent *et al*., 1977; Fravel, 1988; Milner *et al*., 1996; Pandey *et al*., 1997; Ryder *et al*., 1999; Weller, 1988; Whipps, 2001).

Together, these investigations clearly demonstrate that VOC-mediated interactions between bacteria and fungi occur. Such interactions can be species specific, but it also appears that VOCs of many microorganisms can have effects on multiple members of the ecological community. *In vitro*, these interactions range from almost complete mycelium growth inhibition to small growth reduction as well as mycelial and conidial morphological abnormalities (Chaurasia *et al.*, 2005).

The intensity of volatile activity produced by the selected bacteria showed strong, moderate, slight or no mycelium growth inhibition, moreover the same bacterial isolate exhibit a broad difference of volatile fungistasis action against each of the tested fungi (FOM F30 and MC-R isolates). Furthermore, these discrepancies may be the result of the use of different growth conditions or the growth phase of the bacterial population (Fernando *et al.*, 2005; Fiddaman and Rossall, 1994; Ryu *et al.*, 2003, 2004, Sebaaly *et al.*, 2010) or the effect of temperature on volatile. So as the level of control of fungal growth by the volatiles was correlated well with temperature that the highest temperature (30 °C) produce the best reduction in growth of fungi, while the volatile metabolite(s) failed to cause complete inhibition of fungal growth at temperatures closer to optimum for the fungus (< 25 °C) (Fiddaman *et al.*, 1992).

The results obtained from the two experiments, carried out in June – July 2009 and April – May 2010, for the standardization of different organic growing media for melon crops leaded us to the following discussion. Soilless culture based on raw, residual, mineral or organic materials for melon seedling production may be an alternative to traditional melon production that permits the development of root system, playing, therefore, a support role for the plant. This study suggests that the use of different percentage of compost (20 % or 40 %) in growing substrate is not highly beneficial, points out that the negative effect of compost in the substrate on the plant characteristics can be related to the possible presence of phytotoxic compounds which inhibit root growth and affect the growth of melon plants. In addition, mixing the compost with the coconut fiber also provide a negative influence on the growth of the plants. The proportion of the organic materials is recommended to be better evaluated in the substrate composition for the production of melon seedlings.

Among the several tested materials with potential for melon substrate production, the organic growing substrates based on 30% of coconut fiber and 50 % of peatmoss were demonstrated having the better activity on melon seedling growth in terms of germination rate, in correspondence with time and of root and plant biomass. According to Carrijo *et al*. (2002), the good physical properties of the coconut fiber, its non-reaction with the nutrients from fertilization, its long durability without alteration of physical characteristics, the sterilization possibility, the abundance of the raw material that is renewable and the low cost for the producer makes coconut fiber a substrate difficult to be replaced with other material types, as well the Sphagnum peat is characterized by a high physical and chemical stability and low degradation rate (Garcia-Gomez *et al.,* 2002).

The melon seedlings grown in the six growing substrates during June – July 2009 presented a larger value, for all the studied variables, than those grown during April – May 2010. That many factors can affect the behavior of melon seedlings such as temperature since melons are warm-season fruits which thrive in temperatures of 25 °C to 30 °C, and variation in moisture content of the substrate thus the substrate should be prepared to provide ample soil moisture.

Furthermore, the addition of simple substrate, such as compost, coconut fiber or peatmoss, has the ability to increase the microbial communities present in the soil. Indeed, the organic media are characterized by their great variety of substrates, permitting a large variety of microorganisms to become established. The values of bacterial populations present in the substrates were high. Those belonging to fluorescent pseudomonad group were mostly found in T1 and T3, whereas, the large amount of aerobic spore-forming bacteria was isolated from the bulk soil, the rhizosphere and the rhizoplane “a” and “b” of melon plants grown in 20 % (T4) and 40 % (T5) compost based substrates. As well, some soil bacteria are able to associate with the roots of crop plants and can exert beneficial effects such as increasing plant growth and root growth, speed up seed germination, improve seedling emergence, responses to external stress factors, protect plants from disease (Lugtenberg *et al*., 2002).

In vivo experiments were also carried out to establish any relation between the antagonist bacteria and the growth and development of FOM F30 isolate inoculated in a conducive medium based on peatmoss. Results from our pathogen inoculations into the conductive peatmoss substrate corroborate statements by Borrero *et al.* (2004) and by Hoitink and Grebus (1997). Furthermore, this assay confirmed that the use of antagonist microorganisms had a high potential for the management of fusarium wilt of melon. The biocontrol agents

isolated from ECOS compost, MAIB-ECOS substrate and infected field by FOM, appear to play an important role in fungal suppression. B2 and S.2.2 bacterial isolates were the most effective for biological control of FOM F30 isolate.

In addition, the obtained results *in vivo* indicated that seed treatment appears to represent the most effective delivery method for *Bacillus* spp. in controlling Fusarium vascular wilt of melon and it is perhaps more available and acceptable to growers to use the seed treatment with natural biocontrol agents. Seed treatments set the stage for early colonization of germinating seedlings by the antagonistic bacterium and it has been shown to be an important determinant for rhizosphere and rhizoplane colonization, thereby, protects the plant from pathogen attack for a longer time. While, the drenching bacterial suspension technique was more efficient for the fluorescent bacterial isolates that significantly decreased the disease incidence and increased the plant growth on melon seedlings. This could be due to the faster proliferation, at the seedlings application site (around the crown of the plant), of the antagonist propagules provided by drenching bacterial suspension, consequently they colonize the rhizosphere of the developing seedlings. Both techniques demonstrated that the microbial communities established early on the roots were robust and resistant to the effects of pathogen introduction or a switch from a recirculating to a run-to-waste nutrient supply.

Moreover, there are several factors, other than the use of the proper techniques that affect activities of soil microorganisms such as soil moisture, temperature, soil organic matter and agronomic practices such as irrigation (Hiltunen and White, 2002). As well, the degree of disease suppression probably depends on the antagonistic microorganism and the host, as well as the behavior of the pathogen inoculum (Landa *et al.*, 1997). The microorganisms capable of initiating this type of specific suppression include the non-pathogenic strains of *Pseudomonas* sp. (Kannangara *et al.*, 2000; Scher and Baker, 1982) and *Bacillus* spp. (Phae *et al.*, 1990).

The conflicting results among the bacterial isolates of the two *in vivo* experiments may be due to the fact that the application of this type of bacterial groups to a soil may situate the biocontrol activity against pathogens at a particular level of the rhizosphere; in other words, it is the root–bacteria interaction that inhibits pathogen access to the plant by creating a film of microorganisms, which acts by a system of mycoparasitism, competition and the production of antibiotics to prevent the pathogen from entering the plant (Duijiff *et al.*, 1999; Eparvier and Alabouvette, 1994; Olivain and Alabouvette, 1997). It is also possible that some microorganisms might install themselves in the root tissues, thus potentiating the

plant's defense mechanism (Kleifield and Chet, 1992). It is also postulated that incorporating such biocontrol agents may produce a systemic resistance as a consequence of an improvement in the nutritional state of the plant (Kale *et al.*, 1992; Kalembasa, 1996; Nowak *et al.*, 1995), thereby improving its quality and thus contributing directly to the defense of the plant against pathogen infection (Duijiff *et al.*, 1998; Fuchs *et al.*, 1997). Inducing a plant's own defense mechanism by prior application of a biological agent is a novel strategy in plant disease management.

The growth of the melon plants in the soil infected by FOM F30 isolate and amended by antagonistic bacteria were significantly higher than that obtained in the non-amended trial. Therefore, the use of biocontrol agents, besides controlling the action of pathogens *F. oxysporum* f. sp. *melonis* which frequently attack melon crops, may also improve crop yield.

Furthermore, the protective effects of the antagonistic microorganisms could be due to one or a combination of these factors as competition for nutrients, antibiosis, volatiles compounds and siderophore production, although the mechanisms by which some of these antagonistic bacteria provide control remain unidentified, such as for B3 isolate. The antagonistic mechanism of B1, B2 and C6 bacterial isolates depends on the production of metabolites, such as the siderophore and the volatile compounds, additionally the HCN production for S.2.2 and R.2.1 bacterial isolates.

In this way, the incorporation of antagonistic microorganisms into the soil may be used to improve plant growth and to control soil borne diseases like Fusarium wilt and Monosporascus root rot of melon plants.

6) Conclusion

The two main fungal pathogens of melon (*Cucumis melo* L.) causing a critical problem and economic losses in Central Italy are *Monosporascus cannonballus* (MC) and *Fusarium oxysporum* f. sp. *melonis* (FOM). These pathogens are agents of collapse and vascular wilts of the melon plants, respectively. The use of compost to suppress soil borne plant pathogens has been extensively reviewed in recent decades. As well, the use of antagonist microorganisms for increasing the yield and crop protection is an attractive approach in the modern system of sustainable agriculture, in which several fluorescent and spore forming bacteria have been identified as biocontrol agents.

Fluorescent pseudomonads and aerobic spore-forming bacteria were isolated and quantified from bulk soil of commercial compost (ECOS), organic growing substrate (MAIB-ECOS) and infected field by FOM used for melon cultivation. The same bacterial groups were also isolated from the rhizosphere and the rhizoplane of the melon plants grown in MAIB-ECOS substrate and in infected field. The bacteria isolated and identified from the compost were generally found belonging to the aerobic spore forming bacterial group (*Bacillus* spp). A large number of both bacterial groups were obtained from bulk soil, rhizosphere and rhizoplane of the tested substrate and collected field samples. In addition, the positive effect of roots on bacterial multiplication was confirmed.

Representative strains of these bacteria were characterized, and then were *in vitro* tested to investigate their antagonism toward MC-R or FOM F30 isolates and for their Plant Growth Promoting ability. As well as, it was shown that few bacterial isolates expressed an antagonistic feature against the assayed fungi; whereas a higher number of bacteria were able to promote plant growth, in terms of phosphorous solubilization and production of siderophore and phytohormone (IAA).

Moreover, the suppressive effect of the compost against the tested fungi was examined using *in situ* and *in vitro* assays. *In situ* assay was designed to detect the suppression effect of the matured compost, in which autoclaved and non-autoclaved compost was placed onto the center of the fungi seeded plate's surface, and *in vivo* assay to find the bacterial strains present in the compost that have an important role in suppressing the growth of tested fungi by producing clear inhibition zones. *In situ* results indicated considerable decrease in fungal growth in plates containing non-autoclaved compost compared to the autoclaved one. The *in vitro* results have shown that the bacterial content of ECOS compost is the main effective

agents in the antagonistic process against phytopathogenic fungi. In addition, the most probable antagonistic mechanism of these bacteria was the production of diffusible and volatile antifungal compounds.

Based on the results obtained in the *in vitro* assays, eight bacterial isolates were selected to be tested for their suppressive effect in further bioassays *in vivo*. Studies of *in vivo* assay on suppression of disease caused by fusarium wilt have revealed the importance of the microbial community on pathogen suppression as well as on disease development. The suppressive effect can come from the joint action of the microbial community than from the individual action of an actual microorganism, further work should be done to prove the suppressive effect by applying a combination of the studied microbial agents.

Although the treatment techniques influence on the action of bacterial isolates that the seed bacterization technique was more efficient for *Bacillus* spp., while fluorescent bacteria involved a higher suppressive expression by applying drenching suspension technique. This study, in general, confirms previous studies made with *Bacillus* spp. and Fluorescent *Pseudomonas* spp., either as a soil drench or seed treatment, in which control of different soil borne plant pathogens on melon crop has been demonstrated.

The antagonist microorganisms isolated from suppressive compost or from organic suppressive substrate or from infected field samples did not induce the same degree of FOM F30 suppression when inoculated into a conductive peatmoss substrate. Nevertheless, the study for standardization of organic growing media for melon crops have shown that substrates based on coconut fiber and on peatmoss represent the best organic amendment substrates for the production of horticultural melon seedlings.

In summary, the results of these studies suggest several considerations for cover melon crop, where *F. oxysporum* f. sp. *melonis* and *Monosporascus cannonballus* are the most common soil borne melon pathogens, such as, the use of compost that ECOS compost obtained from ornamental residues showed important suppressive effects (*in situ*) against the studied diseases or the application of antagonist microorganisms that reduce the incidence of the fungal pathogens. According to this research work, several antagonist microorganisms appeared to inhibit FOM and MC growth and could be used as biocontrol agents in future researches, in addition the evidence from this study would suggest that the fungal pathogen treatment *via* these biocontrol agents can be scaled-up for field use to reduce the adverse environmental effects of hazardous pesticides.

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