ANTAGONISTIC EFFECTS OF SOME MICROBIAL INHABITANTS ON PHYLLOPLANE OF SQUASH PLANTS TOWARDS *Sphaerotheca fuliginea*

By

Said Mohamed Hassan Kamel

B.Sc. Agric. Co-operation Sciences, (1995)
Completion Studies, Fac. Agric., Kafr El-Sheikh, Tanta University, 1998

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Approval Sheet

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CONTENTS

1. INTRODUCTION

Cucurbits are one of the most important economic vegetable crops in Egypt as well as in other parts of the world for both local consumption and exportation. It includes approximately, 90 genera most of which are tropical or subtropical plants. The cucurbits cultivated area in Egypt reached about 47216 feddans represent about 11% of the vegetable crops area in which squash cultivated area was 21729 feddan according to the records obtained from the Department of Agricultural Economic Statistical, Ministry of Agriculture and Land Reclamation, Egypt (2001).

Several diseases, specially powdery mildews, are known to attack cucurbit plants all over the world causing serious loss (**Boesewinkel, 1977**). Powdery mildews have been observed on cucurbits at least since 1800. In Egypt the disease was first recorded by **Briton-Jones (1925).** *Sphaerotheca fuliginea* (Schlect. ex: Fr.) Pollacci, is the main causal pathogen infecting leaves, stems and fruits of the cucurbits and causes a serious yield loss. (**El-Kazzaz, 1981**). The pathogen shares the host plant its nutrients as well as interrupts the photosynthesis process.

Chemical control requires high inputs of fungicides though the intensive use of pesticides is regarded undesirable both for environmental reasons and for the risk of the development of resistance by the pathogen (**McGrath, 1991**). In addition, the integrated pest management programs using biological means, which has become common in practice, may be adversely affected by chemical fungicides. So, alternative methods for controlling the powdery mildew are urgently needed to avoid the extensive use of the synthetic chemical fungicides.

This study aimed to search for microbial isolates among the microbial flora inhabiting the phylloplane of squash plants antagonistic to the powdery mildew causal agent *Sphaerotheca fuliginea*. Another aim of this study was to answer the questions: how the probable antagonists affect the studied pathogen? and, in advance, can antagonists effectively act in the open field as in pots?

2. REVIEW OF LITERATURE

2.1. Microbial antagonists and biocontrol of powdery mildew diseases:

Powdery mildew remains one of the most conspicuous diseases on various crops including cucurbits and is still managed by chemical fungicides. However, biological control of various powdery mildew fungi has been quite extensively studied. Regarding to microbial grouping, amongst filamentous fungi, yeasts, actinomycetes and bacteria, several isolates have shown more or less inhibiting effects against the powdery mildew fungi.

2.1.1. Fungal antagonists:

There are two well known filamentous fungi as powdery mildew antagonists.

- ***Ampelomyces quisqualis***

The fungus *Ampelomyces quisqualis* Ces ex Schlecht [= *Cicinnobolus cesatiide* Bary (**Rogers, 1959**)] was first reported as a hyperparasite of powdery mildew on red clover in 1932 (**Yarwood, 1932**). Since that time, it has been shown to antagonize several within the Erysiphaceae including, *Sphaerotheca fuliginea* (Schlect ex: Fr.) Pollacci and *Erysiphe cichoracearum* DC on cucurbits. (**Chona & Munjal, 1956; Jarvis & Slingsby, 1977; Philipp & Crüger, 1979; Rudakov, 1979; Gupta *et al.*, 1985 and Sacham & Sharma, 1988**); *Uncinula necator* (Schwein) Burrill on grapevine (**Gadoury & Pearson, 1988; Puzanova, 1991; Falk *et al.*, 1992 and Falk *et al.*, 1995b**); *Podosphaera leucotricha* (Ellis & Everh.) E.S. Salmon on apple, (**Odintsova, 1975; Bosshard *et al.*, 1987; and Puzanova, 1991**) and *Sphaerotheca pannosae* (Wallr): Fr) Lév. on rose (**Rudakov, 1979 and Puzanova, 1991**).

Kiss (1977) and Falk *et al.* (1995a) reported that more than 65 species of the *Erysiphaceae* representing nine genera are known to be natural hosts of *Ampelomyces*. Experiments have shown that an *Ampeomyces* hyperparasite found in a given species of powdery mildew fungi is not specialized to it and is able to infect many other species of the *Erysiphaceae* (**DeBary, 1870; Philipp & Cruger, 1979; Sztejnberg *et al.*, 1989; Falk *et al.*, 1995a; Kiss & Vajna, 1995**).

Sztejnberg and Mazar (1985) found that effective biocontrol of cucumber and carrot powdery mildew was obtained through hyperparasitism by *Ampelomyces quisqualis* applied as a bio-fungicide suspension of 1-2 million spores/ml. In a trial of greenhouse grown cucumbers heavily naturally infected with powdery mildew (18 plant per treatment), no yield was obtained in the untreated plants. Combined alternate treatments of two sprays with *Ampelomyces quisqualis* and one fungicide spray at a low concentration (Pyrazophos 0.05%) yield 3.6 kg/plant. Plants treated with *Ampleomyces quisqualis* alone yielded 2.5 kg/plant and with the fungicide alone 2.8 kg/plant. Although the combined treatment gave ~27% yield increase over treatment with *Ampelomyces quisqualis* alone or fungicide alone, it was not statistically significant. In carrot field treated weekly with *Ampelomyces quisqualis*, complete parasitism of the powdery mildew was achieved. Plants with hyperparasitized powdery mildew remained more vital than those with unparasitized powdery mildew, although both had the same powdery mildew coverage.

Sztejnberg (1979) isolated *Ampelomyces quisqualis* from parasitized powdery mildew on *Catha edulis*. Successful biocontrol was obtained by repeated *Ampelomyces quisqualis* inoculations every 10 days in the greenhouse on the following mildews; *Sphaerotheca fuliginea* on

cucumber and watermelon, *Erysiphe betae* on sugar beet, *Erysiphe umbelliferarum* on carrot, *Podosphaera leucotricha* on apple, *Phyllactinia suffulta* on mulberry, *Leveillula taurica* on pepper, *Oidium* sp. on zinnia and sow thistle.

Abo-Foul *et al.* (1996) showed that the hyperparasite *Ampelomyces quisqualis* is a potential biological control agent for powdery mildew *Sphaerotheca fuliginea*. Cucumber plants were exposed to powdery mildew with and without subsequent *Ampelomyces quisqualis* treatment. Plants were then examined in comparison with healthy uninfected plants. Disease symptoms, including chlorosis and necrosis were most prominent on the plants exposed only to powdery mildew. Electron micrographs of leaf sections of these diseased plants indicated marked deterioration in the morphological organization of chloroplast membranes. Chloroplasts of *Ampelomyces quisqualis* treated plants seemed undamaged and like those of uninfected plants.

The accumulating evidence of success with *Ampelomyces quisqualis* has promoted commercial interest so that a formulated product (AQ_{10}) was registered in Australia and other countries (**Hofstein & Fridlender, 1994 and Menzies & Belanger, 1996**). Unfortunately, the high humidity requirements of *Ampleomyces quisqualis* have hampered its efficacy (**Verhaar *et al.*, 1999**), in two greenhouse experiments, one in the summer and another in winter/springs, **Dik *et al.* (1998)** failed to achieve disease control by *A. quisqualis*. The advantages of *Ampelomyces quisqualis* were outlined by **Benuzzi & Baldoni (2000)** with particular emphasis to its unique action on overwintering cleistothecia of powdery mildew.

- *Verticillium lecanii*

Another mycoparasite that is attracting attention because of its wide host range is *Verticillium lecanii* (A. Zimmerm) Viégas. This hyphomycete fungus has generated considerable interest because of its potential as a powerful bioinsecticide (**Hall & Burges, 1979; Hall, 1981 and Harper & Huang, 1986**).

Verticillium lecanii also has been speculated as a promising alternative to the use of fungicides since it convincingly has the ability to control several rust fungi (**Spencer, 1980**; **Spencer & Atkey, 1981 and Grabski & Mendgen, 1986**) as well as some powdery mildew pathogens (**Raghavendra Rao & Pavgi, 1977; Hall, 1980; Spencer & Ebben, 1981). Verhaar *et al.* (1996)** reported that *Verticillium lecanii* formulations of which are commercially available in Europe for biocontrol of aphids and whiteflies controlled the powdery mildew on both susceptible and partially resistant cucumber plants. The mycoparasites were much effective against the slaw-growing mildew colonies (on the partially resistant cultivar) than the fast-growing ones (on the susceptible cultivar). On the partially resistant cultivar. Flamingos biweekly treating by *Verticillium lecanii* was able to keep the disease below 15% infected leaf area.

Verhaar *et al.* (1997) studied effect of the mycoparasite *Verticillium lecanii* on cucumber powdery mildew *Sphaerotheca fuliginea* in a rooted cucumber leaf bioassay. At near to maximum humidity (> 95% relative humidity), early preventative (9-5 days before mildew inoculation) and early curative (2 days after mildew inoculation) gave considerable reduction in mildewed leaf areas, while late curative treatments resulted in greater mildewed leaf areas.

Using different isolates of *Verticillium lecanii*, **Askary *et al.* (1998)** achieved biocontrol measures to cucumber powdery mildew, and **Verhaar *et al.* (1999)** revealed superior efficiency of a *Verticillium lecanii* isolate than others of *Ampelomyces quisqualis*. Most of the antagonistic isolates lost their effectiveness rapidly at below 100% RH, but one of the tested four isolates of *Verticllium lecanii* achieved > 80% rose mildew control at 90% RH.

On the other hand, **Dik *et al.* (1998)** unfortunately failed to obtain effective control of cucumber powdery mildew using *Verticillium lecanii* in two conducted greenhouse experiments.

- **Other filamentous fungi:**

Beyond the two previously focused powdery mildew fungal antagonists, scattered literatures have been found dealing with other new microorganisms which can antagonize different powdery mildew fungi.

Hijwegen (1989) noticed that treatment of cucumber plants infected with *Sphaerotheca fuliginea* by spraying with culture filtrates of seventeen fungal isolates reduced the number of healthy conidiophores compared with that on untreated control plants. Best results were obtained with *Calcarisporium arbuscula* followed by *Cladobotryum varium*.

Some isolates of *Cladosporium* spp. showed some degrees of powdery mildew control using their spore suspensions against either *Sphaerotheca fuliginea* (**Minuto *et al.*, 1991**) or *Erysiphe cichoracearum* and *Phyllactinia* spp. (**Mathur & Mukerji, 1981**).

Although *Trichoderma harzianum* T39 spray (as TRICHODEX®) reduced *Sphaerotheca fusca* [Syn. *Sphaerotheca fuliginea* (**Elad *et al.*,**

2000)] severity on greenhouse cucumbers by up to 97%, its efficacy declined to 18-55% as the disease progressed. On older leaves the control of *Sphaerotheca fusca* by *Trichoderma harzianum* was poor (**Elad *et al.*, 1998**).

Instead of foliar spraying, the application of *Trichoderma harzianum* T_{39} to soil resulted in a 75-90% reduction in *Sphaerotheca fusca* coverage on the leaves. That led authors to suggest induced resistance as mode of action. Contrarily, incidence of powdery mildew on pea caused by *Erysiphe polygeni* significantly did not differ on either control plants or plants treated with talc-based *Trichoderma viride* formulation (**Rajappan & Yesuraja, 2000**).

In Egypt under greenhouse conditions, disease incidence of powdery mildew on cucumber plants reached about 40% and 43% using a local isolate of *Trichoderma harzianum* and the commercial biofungicide based on *Trichoderma harzianum* "Plant Gard®", respectively (**Abdel-Sayed, 2000**). This was significantly lower than disease incidence (about 55%) on untreated plants.

Vozenilkova *et al.* (1992) stated that accompanied treatment using mixture of *Trichoderma* sp. and fungicides previcuvn (propamocarb hydrochloride, fundazol 50 WP (benomyl) and Topsin M70 WP (thiophanate-methyl) gave the best control of *Sphaerotheca fuliginea* in greenhouse-grown cucumber Sandra.

Other filamentous fungi such as *Ulocladium atrum* and *Clonostachys rosea* also showed promises as tools in foliar disease management including powdery mildews (**Yohalem, 2000**). Isolates of *Phoma glomerata* could colonize and suppress development of powdery mildew (*Phyllactinia guttata*) and may be used as a mycoparasitic agent

(**Sullivan & White, 2000**). One isolate of the fungus *Aphanocladium album* was included in a bioassay conducted by **Verhaar *et al.* (1999)** to study its effectiveness as a mycoparasite to control rose powdery mildew (*Sphaerotheca pannosa* var. *rosa*) under selected environmental conditions. **Jarvis (1992)** reported that *Aphanocladium album* previously has been considered as an antagonist of the powdery mildew. He also made studies on the antagonism exhibited by the fungus *Paeciliomyces farinosus* towards powdery mildew pathogens.The pycnidium-forming hyperparasite *Acremonium alternatum* Linc. Fr. was recorded as an antagonistic fungus against the cucurbits powdery mildew pathogen, (**Malathrakis, 1985**).

2.1.2. Yeast and yeast-like fungi:

Although the yeast or yeast-like isolates of *Aureobasidium pullulans*, *Cryptococcus* spp., *Rhodotorula* spp. and *Sporobolomyces* spp. showed promises as tools in foliar disease management including powdery mildew (**Yohalem, 2000**), the main yeast-like fungi antagonistic to powdery mildews are belonging to *Sporothrix* and *Tilletiopsis* spp.

- ***Sporothrix* spp.:**

Sporothrix flocculosa Traqunir, Shaw & Jarivs and *Sporothrix rugulosa* Traquair, Shaw & Jarvis [Anamorph: *Stephanoascus flocculosus* and *S. rugulosus*] are yeast-like, epiphytic fungi that were isolated and identified by **Traquair *et al.* (1988)**. Subsequently, it was demonstrated that both fungi were powerful antagonists of cucumber powdery mildew *Sphaerotheca fuliginea* (**Jarvis *et al.*, 1989**). When was tested against other powdery mildew fungi, *Sporothrix flocculosa* demonstrated the same antagonistic activity on wheat powdery mildew *Erysiphe graminis* var. *tritici* (**Hajlaoui & Belanger, 1993**) and on rose

powdery mildew *Sphaerotheca pannosa* var. *rose* (**Hajlaoui *et al.*, 1992; Hajlaoui & Belanger, 1991** and **Belanger *et al.*, 1994**).

Under strict commercial conditions *Sporothrix flocculosa* offered a good control of rose powdery mildew comparable to that obtained with the common used fungicides. Moreover, biological control treatments improved flowers quality than those of chemical control treatments (**Belanger *et al.*, 1994**).

In comparative experiments under controlled conditions, *Sporothrix flocculosa* showed more rapid colonization of powdery mildew colonies than *S. rugulosa* and was less affected by unfavourable climatic conditions (**Hajlaoui & Belanger, 1991**). The superior efficacy of *Sporothrix flacculosa* than other known antagonists was again confirmed by **Dik *et al.* (1998)**.

Pseudozyma flacculosa is the synonym of *Sporothrix flocculosa* (**Boekhout, 1995** and **Begerow *et al.*, 2000**). **Belanger and Avis (1998)** reported that *Pseudozyma flocculosa, Ampelomyces quisqualis*, *Tilletiopsis* sp. and *Verticillium lecanii* were used as bioagent to control numbers of *Erysiphales*. In comparative studies *P. flocculosa* achieved the highest rate of powdery mildew control over the other known biocontrol agents under both small and commercial scale experiments. More attention was payed to *Pseudozyma flocculosa* as a biocontrol agent of powdery mildew in terms of taxonomy (**Urquhart *et al.*, 1997 and Avis *et al.*, 2001**).

- ***Tilletiopsis* spp.:**

Several species belonging to the genus *Tilletiopsis* have been reported to have antagonistic properties against powdery mildew of several plants. **Hoch & Provvidenti (1979)** were the first to substantiate

the antagonism between *Tilletiopsis* sp. and the cucumber powdery mildew *Sphaeratheca fuliginea*. Subsequently **Hijwegen & Buchenauer (1984)** and **Menzies & Belanger (1996)** confirmed the common occurrence of *Tilletiopsis* spp. with *Erysiphaceae*.

Interestingly *Tilletiopsis* spp. may come in advance as biocontrol agents compared with *Ampelomyces* sp. A protectant applications of *Ampelomyces* sp. (one isolate) and *Tilletiopsis* spp. (eight isolates) as biocontrol agents against powdery mildew on cucumber cotyledons and leaves resulted in suppression of infection by 90-97% in case of all *Tilletiopsis* spp., while *Ampelomyces* did not significantly control the pathogen (**Hartmann *et al.*, 1984**).

The genus *Tilletiopsis* seems having numerous species antagonistic not only to cucumber powdery mildew but also to other powdery mildews. **Sundheim and Tronsmo (1988)** found a suppression effect of an isolate of *Tilletiopsis* on the cucurbit powdery mildew *Sphaerotheca fuliginea* as well as the apple powdery mildew *Podosphaera leucotricha* and the powdery mildew of grape *Uncinula necator*. Also, **Klecan *et al.* (1990)** demonstrated an antagonistic relationship between *Erysiphe graminis. sp. hordei* and *Tilletiopsis pallescens* on the surface of barley leaf segments, *Tilletiopsis pallescens* caused marked reduction in mycelial expansion and in spore production. On the other hand, another species known as *Tilletiopsis minor* was antagonistic to a lesser extent on the cucumber powdery mildew under greenhouse conditions (**Hijwegen, 1992a**).

Kundsen & Skou (1993) also reported about another effective species of *Tilletiopsis*, *T. albescens* as a biocontrol agent against the powdery mildew pathogens either on barley (*Erysiphe graminis* f. sp.

hordei) or on cucumber (mixed population of *Sphaerotheca fuliginea* and *Erysiphe cichoracearum*). Application of ballistospores or cut mycelium was equally effective. The efficacy increased exponentially with concentration, and two applications were more effective than one.

Urquhart *et al.* (1994) recovered 143 isolates of *Tilletiopsis* spp. from powdery mildew infected leaves of 22 plant species. They found two species, *Tilletiopsis washingtonensis* Nyland, and *T. pallescens* Gokhale, and when applied at a rate of 1 x 10^8/ml, significantly reduced the incidence of cucumber powdery mildew under greenhouse conditions.

To control rose powdery mildew [*Sphaerotheca pannosa* (Wallr. Fr.) Lev. var. *Rosae* Waronichin], **Ng *et al.* (1997)** conducted two trials on potted rose plants under greenhouse growing conditions. On plants treated with one application of *Tilletiopsis pallescens* either as spore suspension or as culture filtrate, sporulation of the pathogen was significantly reduce by 78%-94% with no significant difference between the two tested treatments (spore suspension and culture filtrate).

2.1.3. Bacterial antagonists:

Tajika *et al.* (1997) isolated a novel amino acid metabolite produced by a *Streptomyces* strain which exhibited a weak preventive effect on cucumber powdery mildew disease caused by *Sphaerotheca fuliginea* in pot tests.

Bettiol *et al.* (1997) reported that a wettable formulations based on cells or concentrated culture filtrates of two *Bacillus subtilis* isolates sprayed on cucumber and zucchini squash plants totally controlled powdery mildew disease and improved plant growth. **Schmitt *et al.* (1999)** studied the antifungal activity of gramicidin S and possible use of

Bacillus brevies to control *Sphaerotheca fuliginea*. *In vitro* studies with conidia of *Sphaerotheca fuliginea* revealed that the antifungal metabolite gramicidin S inhibited conidial germination by about 80%. In *in vivo* studies on cucumber plants, *Bacillus brevis* cultures significantly reduced the disease intensity.

Vogt and Buchenauer (1997) used the florescent *Pseudomonas* strains as soil or seed treatment in order to reduce damping off in cucumber, variable reductions of powdery mildew were obtained.

Singh *et al.* (2000) found that, seed bacterization by *Pseudomonas fluorescens* and *Ps. aeruginosa* alone and/or in combination with aerial spray of their cell suspension or Neemazal (a product of neem) at different concentrations controlled powdery mildew of pea. Combinations of seed bacterization with either aerial sprays of bacterial cells suspensions or nemazol were more effective in controlling the disease than seed bacterization alone. Bacterization by both bacteria and aerial spray of Neemazal increased the dry weight of aerial parts, number of nodes and pods as well as seed weight of pea plants.

Abd El-Sayed (2000) found that among five tested antagonistic microorganisms *Bacillus subtilis* (isolated from cucumber plants) gave the highest protection against the powdery mildew on cucumber plants when applied either 3 days before or 5 days after infection (disease intensity averaged 31.04 and 32.99% respectively), followed by *Pseudomonas fluorescence* (isolated from potato plants) where the disease intensity averaged 39.16 and 39.66% (before and after infection, respectively).

Abd El-Moneim (2001) evaluated some non-chemical methods to control some foliage diseases of cucumber. Results indicated that

Pseudomonas fluorscens and mixture of *Trichoderma* isolates were the most effective biocontrol agents in controlling powdery mildew in primo variety.

2.2. Mode of action of antagonism:

Biological control agents of fungal diseases are known to exert their activity generally by substrate competition, antibiosis, lytic enzymes and/or direct parasitism (**Deacon, 1991; Whipps, 1992 and Belanger *et al.*, 1995**). Strict biotrophs including powdery mildews pathogens are out of nutrient-competition with the epiphytic necrotic antagonists (**Cook & Baker, 1989**). Direct parasitism, antibiosis and lytic enzymes are considered the evident events in the relationships between powdery mildews and their, up to now, registered antagonists.

Ampelomyces acts as a hyperparasite on the powdery mildew fungi. It penetrates and feeds on the hyphae of the powdery mildew fungus (**Hashioka & Nakai, 1980**). The mycoparasite is specific to powdery mildews (Erysiphales) but has an extremely broad host range within this diverse group of important plant pathogens. It has been recorded on more than 64 species in the genera, *Brasilomyces, Erysiphe Leveillula, Microsphaera, Phyllactinia, Podosphaera, Sphaerotheca* and *Uncinula* as well as the an amorphic genera *Oidium* and *Oidiopsis* representing powdery mildews on 256 plant species within 172 genera in 59 families and which occur in 28 countries around the world (**Falk *et al.*, 1995a**). According to **Falk *et al.* (1995b)**, *Ampelomyces quisqualis* directly penetrates the walls of hyphae, conidiophores, and immature cleistothecia, but may be unable to infect mature cleistothecia. For approximately 7-10 days, the mycoparasite spreads within the hyphae of the mildew colony without killing it. Thereafter, the process of pycnidial formation begins and is then completed within 2-4 days. Infected cells

generally die soon after pycnidial formation begins. Any evidence of toxin production by *Ampelomyces quisqualis* have not been detected (**Beuther *et al.*, 1981**).

The effect of *Verticillium lecanii* appears to be mediated by the production of antifungal substances prior to physical contact between the fungi, followed by cell-surface interaction between the two fungi, and finally pathogen cell invasion. **Askary *et al.* (1997)** studied the chronological events of the interaction between *Verticillium lecanii* and cucumber powdery mildew *Sphaerotheca fuliginea* at different time after inoculation. Events involved attachment, mechanical pressure and production of cell well degrading enzymes. The interaction between *Verticillium lecanii* and *Sphaerotheca fuliginea* also affected the morphological and structural features of the haustorial lobes. The well documented ability of *V. lecanii* to secrete toxins with insecticidal properties (**Clydon & Grove 1982 and Gindin *et al.*, 1994**) reinforce the implication of toxic metabolites as an early step in the antagonistic process between *V. lecanii* and *S. fuliginea*.

Trichoderma harzianum Rifai, the antagonist against several soil borne plant pathogens (**Dennis & Webster, 1971**) produced extracellular lytic enzymes B-1,3-glucanase and chitinase (**Hadar *et al.*, 1979**) and **Chet *et al.*, 1979**). *T. harzianum* attacks the pathogen's mycelium first by dissolving its cell wall in certain locations, followed by hyphal penetration (**Chet *et al.*, 1981**), it then uses other extracellular enzymes e.g. lipase and protease (**Elad *et al.*, 1982**). On the other hand, **Martinez *et al.* (1999)** prepared a cellulase produced by *T. harzianum* to be studied for its effect on the natural defences of plants. The mode of action was determined as on eliciting effect, triggering peroxides and chitinase activity to produce systemic acquired resistance with the production of

ethylene and salicylic acid leading to reduction in powdery mildew infection on greenhouse melon plants. **Elad *et al.* (1998 and 1999)** also suggested induced resistance since the application of *T. harzianum* T_{39} conidia to root zone of plants resulted in the reduction of foliar diseases including powdery mildew. **Ahmed (1995)** examined the influence of fungal *Trichoderma* on spore germination of *Sphaerotheca fuliginea*. He found that culture filtrates of *Trichoderma harzianum* and *T. viride* inhibited spore germination on dry slides incubated in damping chamber at 25°C by 75.9% and 77.56%, respectively compared with control. In the same time, this percentage reached only 5.28% in the case of *Alternaria* sp.

The effect of the powdery mildew antagonist *Sporothrix flocculosa* is not based on direct hyperparasitism or chitinolytic activity. *Sporothrix floculosa* is believed to act exclusively by antibiosis (**Hajlaoui *et al.*, 1992; Hajlaoui & Belanger, 1993,** and **Hajlaoui *et al.*, 1994**). **Belanger and Deacon (1996)** conducted a vidoemicroscopy study upon the interaction specificity of the biocontrol agent *Sporothrix flocculosa*. They revealed that individual conidia of *S. flocculosa* produced several germ tubes, but did not attack live, necrotic, or dead hyphae of the pathogen. The typical reactions included plasmalemma aggregation and vacuolation of the cytoplasm with 8 h and finally cell death without any apparent cell wall alteration. Fatty acids with antibiotic activity have been isolated and characterized from liquid cultures of *S. flocculosa* (**Benyagoub *et al.*, 1996 and Choudhury *et al.*, 1994**). Bioassay of these fatty acids confirmed that they induce the same toxic effect in fungal cells as *S. flocculosa* it self (**Avis & Belanger 2000; Hajlaoui *et al.*, 1994, and Avis *et al.*, 2001**). Cytochemical observations revealed that the antagonist *S. flocculosa* induced rapid collapse of powdery mildew

conidial chains and cytoplasmic disintegration of fungal cells (**Hajlaoui *et al.*, 1992**).

Hoch & Provvidenti (1979) revealed the interaction between *Sphaerotheca fuliginea* and *Tilletiopsis* sp. They observed that hyphae of *Tilletiopsis* sp. were entwined around *Sphaerotheca fuliginea* hypha as well as leaf trichomes. However cells of *Sphaerotheca fuliginea* in contact with *Tilletiopsis* usually were necrotic, while penetration of *Sphaerotheca fuliginea* structure by hyphae of *Tilletiopsis* was rarely observed and probably represented postnecrotic invasion. This leads to the assumption that *Tilletiopsis* sp. *Sphaerotheca fuliginea* interaction apparently do not involve penetration of the alive host fungus. Some other factors presumably substances secreted by *Tilletiopsis* sp. are involved. *A Sporobolomyces* sp. (Sporobolomycetaceae) relative to *Tilletiopsis* sp. produces fungistatic antibiotics in culture (**Yamasaki *et al.*, 1951**). In addition, no degradation of hyphal walls of *Erysiphe graminis hordei* at sites of contract with *Tilletiopsis* was detected (**Klecan *et al.*, 1990**), although collapse of hyphae, vacuolation of the hyphal cytoplasm and aggregation of damaged cytoplasmic components were observed. **Ng *et al.* (1997)** succeeded to suppress sporulations of the powdery mildew causal agent *Sphaerotheca pannosa* (Wallr: Fr.) Lev. var. *rosae* Woronichin on rose using one application of culture filtrate (without spores) of *Tilletiopsis pallescens*. They mentioned that the mode of action of *T. pallescens* appears to be associated with enzymes or metabolites produced in the culture filtrate. **Urquhart *et al.* (1994)** reported that chitin and its components sugar, N-acetyl glucosamine were not utilized by *Tilletiopsis* which agrees with results obtained by **Hajlaoui *et al.* (1992) and Hijwegen (1992b)** suggesting no chitinas activity. On the other hand, **Urquhart *et al.* (1994)** found that

laminarin (B-1, 3 glucan polymer) was utilized by *Tilletiopsis* providing indirect evidence of B-1, 3 glucanase activity that was subsequently confirmed in their enzymatic assays. **Urquhart *et al.* (1994)** observed that the hyphae of *Sphaerotheca fuliginea* appeared collapsed when viewed under scanning electron microscopy after *Tilletiopsis* treatment without ruptures in the mycelium. Sine any broad-spectrum activity against other fungi was not observed, **Urquhart *et al.* (1994)** did not expected antibiotic activity in contrast with that previously suggested by **Hajlaoui *et al.* (1992)**.

Belanger *et al.* (1993) studied the mode of action of *Stephanoascus flocculosa*, *St. rugulosus* and *Tilletiopsis washingtonsis* on *Sphaerotheca pannosa* var. *rosae*. The absence of wall dissolution associated with a rapid collapse of host hyphae leads to speculation on the importance of antibiosis in the antagonistic activity of the tested organisms.

Numerous members of actinomycetes antagonize other microorganisms by means of the produced antibiotics. *Streptomyces* strain 185, isolated by **Tajika *et al.* (1997)**, produced an antibiotic which was identified as N delta-(5-methyl-4-oxo-2-imidazolin-2-yl)-L-ornithine, this substance exhibited a preventive effect on cucumber powdery mildew disease (caused by *Sphaerotheca fuliginea*) in pot tests.

It is well-known that the fungal antagonistic isolates of *Pseudomonas fluorescens* acts through more than one mode of action, *Ps. fluorescens* produces siderophores which cleat iron and prevent other microorganisms to utilize this element, and as a result to iron starvation, the pathogen can not grow, penetrate and cause disease (**Paulitz and Loper, 1991**). In addition, *Ps. fluorescens* isolates produce some

antifungal substances i.e. pyrrolinitrin, pyroluteroin and 2, 4 diacetyl ploroglucinol (**Sarnigue *et al.*, 1995; Duffy and Defago, 1997 and Sharifi *et al.*, 1998**).

The success achieved by **Bettiol *et al.* (1997)** in controlling *Sphaerotheca fuliginea* on cucumber and zucchini squash using metabolites of *Bacillus subtilis* affirmed the mode of action of *Bacillus* sp. as antibiosis. Throughout such mechanisms of action, *Bacillus subtilis* inhibits many phytopathogenic fungi (**Baker *et al.*, 1983; McKeen *et al.*, 1986; Pusey, 1989; Thirumalachar & O'Brien, 1979; Belal *et al.*, 1996 and Abd El-Wahab *et al.*, 1997**). Inhibition of *Sphaerotheca fuliginea* conidial germination by the antifungal metabolite gramicidin S, produced with *Bacillus brevis* (**Schmitt *et al.*, 1999**) again assured the mode of *Bacillus* spp. against the causal agents of powdery mildew diseases.

3. MATERIALS AND METHODS

3.1. Sampling of microbial flora:

From different localities of squash commercial plantation in Kafr El-Sheikh and El-Gharbia governorates, leaves of squash plants at different stages of maturity were used. Whole blades of healthy as well as powdery mildew infected leaves were cut and then were separately washed using 20 ml of sterilized tap water (containing 0.2% tween 20) under aseptic conditions. Individual aliquots of washing water or its dilutes (0.2 ml) were then spread over the surface of potato dextrose agar (PDA) as well as of nutrient agar (NA) plates media (**Dhingra & Sinclairy, 1995**). Inoculated plates were incubated at room temperature (28 $\pm$ 2°C) until arising maximum numbers of microbial colonies separated enough to be picked up. The picked colonies were repurified before maintenance on slants of potato dextrose agar medium (for fungi or yeasts) and of nutrient agar medium (for bacteria and actinomycetes). The purification techniques used were based on single hyphal-tip for fungi and single colonies for bacteria, yeast and actinomycetes obtained by steeking on agar media.

3.2. Screening for antagonism against squash powdery mildew:

3.2.1. Preparation of the experimental plants:

In glasshouse, seeds of squash (c.v. Escandrani) were planted in 15 cm plastic pots filled with 2 kg/each of unsterilized loamy-clay soil. The apical growing parts of 15 days old seedlings were completely removed and the cotyledons were allowed to grow until the end of the experiment. The pruned plants were irrigated whenever needed and pots were fertilized with calculated doses of the mineral elements as recommended (50 kg ammonium sulphate, 25 kg urea and 60 kg potassium sulphate/feddan).

3.2.2. Preparation of microbial inocula potent for antagonisms:

The microorganisms previously isolated from leaves of squash plants were recultured on suitable liquid media [potato dextrose broth (PDB) for fungi and yeasts or nutrient broth (NB) for bacteria and actinomycetes] in flasks of 500 ml capacity containing 200 ml medium. Cultures were incubated at 28°C for 10 days in shaking incubator (140 r/min.). Cultures were paper filtered and hand homogenized, and counts of cells (in cases of yeast and bacteria) or spores (in case of fungi) were adjusted to 10^7/ml.

3.2.3. Preparation of the pathogenic inoculum:

Potted mature plants of squash (c.v. Escandrani) naturally infected with the powdery mildew disease *Sphaerotheca fuliginea* was used as an inoculum-source of the pathogen. Pots containing plants with heavy infection were symmetrically distributed among the potted experimental plants as a natural source of infection.

3.2.4. Procedure of application:

The prepared microbial cultures were introduced to 21 days old experimental plants. Cultures were amended with calculated aliquots of an adhesive surfactant (New-Film, 1265 registered by ministry of Agric., Egypt) as recommended (30 ml/100 L) and hand homogenized before fine spraying onto the upper and the lower leaf surfaces of plant cotyledons until runoff. Plants, sprayed with sterilized tap water (likely amended with the adhesive surfactant), served as check treatment (control). Spraying was repeated weekly for 3 times and the lesions of powdery mildew were calculated.

The efficacy of the various treatments were determined by counting the number of powdery mildew colonies on both surfaces of

cotyledons before and after application, and disease severity was assessed as mentioned by **McGrath and Staniszewska (1996)**.

Each experiment was repeated three times using the completely randomized design with three pots/treatment, each containing two plants/pot (i.e. 12 cotyledons/treatment)

3.3. Identification of antagonists:

All the microorganisms, isolated as previously mentioned in the 1st step, were grouped as fungi, yeasts, bacteria and actinomycetes with preliminary description was generally done and advanced identification to the probable genus and species was conducted specially for the effective isolates. The identification tests were achieved in the guidance, of **Barnett and Hunter (1972), Domsch *et al.* (1980); Barnett and Pankhurst (1974), Parry *et al.* (1983) and Bergey's Manual of Systematic Bacteriology (1984)**.

3.4. Definition the mode of the antagonistic action:

To reveal how the antagonistic microorganisms act to inhibit the powdery mildew, the effects of the separated washed cells and the filtered metabolites (in comparison with the whole liquid cultures) were determined on the disease incidence. The cell-free filtrates and cells of the tested microorganisms (grown up to 10 days old) were separated by centrifugation at 10000 r/min. for 15 minutes. The supernatants were filter-sterilized using 0.2 µ filter membrane (Sartorius Minisart Nmlpf SM 16596 HY) and the sedimentary cell-biomasses were rewashed three times using physiological saline solution 0.8% NaCl. Cells were resuspended in this solution and the concentrations were adjusted to be 10^7 cfu/ml before spraying onto the tested plants. Spore germination of the tested pathogen was determined in the presence of metabolites of the

tested antagonists. Light and electron microscopy examinations were also carried out to observe any of direct or indirect effects harmful to the pathogen.

3.4.1. Spore germination of *Sphaerotheca fuliginea* as affected by culture filtrates:

Conidial spores of squash powdery mildew were obtained from young sporulating lesions. To avoid the old unviable conidia, lesions were gently shaken by a glass rod, and 24 hrs later (as recommended by **Godwin *et al.*, 1987**), new conidia were deposited on glass slides according to **Nair *et al.* (1962)**. Slides were previously cleamed with ethyl alcohol and air dried before covering with a thin smear of 2% water agar amended with filter-sterilized culture filtrate of the tested antagonist. Slides were placed on V-shaped glass rods in sterilized petri-dishes containing several layers of water moistened filter papers. Slides with conidia were incubated at 25°C for 24 hours under continuous light (**Reifschneider *et al.*, 1985**) before microscopical examination at x100 magnification to determine the spore-germination. Conidia were considered to have germinated if a germ tube, at least as long as the width, was produced (**Menzies *et al.*, 1991**). Percentages of germination were calculated for 100 conidia on a slide. Three slides were examined for each treatment. Slides with water agar free from culture filtrate were used as control treatment.

3.4.2. Microscopy examination of the affected pathogen:

For microscopy examination, specimens of cotyledons bearing powdery mildew lesions treated with either a whole liquid culture, suspension of washed cells or cell-free culture filtrate of either of the tested antagonist were prepared at the 7th day after treating. Untreated powdery mildew lesions and others treated with recommended systemic

fungicide Topas 100 EC a.e.: (Penconazole 10% w/v 1-[2(2,4 dichlorophyenyl)-pentyl]-1H-1,2,4 triazole, Syngenta, Swiss) at the concentration 25 ml/L were used for comparison. Each treatment was represented by 10 replicates.

3.4.2.1. Light microscopy examination:

According to methods conducted by **Falk *et al.*, (1995b)**, stereomicroscope was used to examine mildew lesions at 50 x magnification power and the percentages of the conidiophores that had collapsed were estimated by recording the number of erect conidiophores present in ten fields of view. Sporulation was also assessed as the number of conidial spores on ten erect conidiophores and the percentages of inhibiting the sporulation were calculated. Each treatment was represented with ten tested lesions.

3.4.2.2. Scanning electron microscopy (SEM):

Squash cotyledons bearing lesions of powdery mildew (with and without treatment) were collected and processed for (SEM) work according to **Urquhart *et al.* (1994)**. Tissue pieces of ~ 5 mm^2 were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 1 h, followed by exposure to osmium tetroxide vapor for 24 h and then rinsed three times in distilled water. The samples were dehydrated through a graded ethanol series (from 10 to 100% for 30 min. in each) and critical point-dried with liquid CO_2. The specimens were mounted on stubs with double-sided adhesive tape, coated with gold, and viewed in a scanning electron microscopy (JEOL-1005). Photographs were taken with FORTEPAN 200, ISO 200/24° film. All the work of specimens processing, SEM observations and photographia was carried out at Electron Microscope Unite, Fac. of Sciences, Cairo Univ., Egypt.

3.5. Biological control of the powdery mildew disease on squash and cucumber crops in the open field:

At the Experimental Farms, Faculty of Agriculture at Kafr El-Sheikh, Tanta University, this work was carried out using randomized complete blocks design with three replications. Seeds of squash (cv. Escandrani) as well as of cucumber (cv. Bremo) were sown in rows of 1 m. width at 1 m distance apart. Each plot contained 5 plants. At the time of ~10% natural occurrence of disease severity, application of treatments was started as spraying of plants with liquid cultures 10^7 cfu/ml prepared as previously mentioned in 3.2.2. In addition, plants sprayed with the fungicide 4 Topas 100 were also involved with the recommended concentration (25 ml/L). Plants were treated 1, 2 or 3 times with 10 days apart. Cultural practices, irrigation and fertilization were carried out as recommended in the program of production improvement of cucurbitaceious crops (**Ministry of Agric. & Land Reclamation, Egypt, 1994**). To avoid the use of chemical insecticides, plant protection against insects (mainly white fly and mites) was carried out using the yellow traps. The experiments were conduced under natural filed infection.

3.5.1. Disease measurements:

Disease severity was assessed immediately before treatment application (initial disease severity) and 30 days later, i.e. 10 days after the third time of application (final disease severity). According to **McGrath and Staniszewska (1996),** the powdery mildew disease severity was estimated by counting visible sporulating mildew colonies on both adaxial and abaxial surfaces per leaf. Percent leaf area covered by mildew were converted to severity value by using a conversion factor of 1% = 10 colonies. Five old leaves per plant were examined for five

plants in each plot (i.e. 25 leaves/treatment). Assessments were also made on fully expanded leaves from the middle and upper thirds of a plant and data from all three age-classes of leaves were averaged together.

As the initial disease severity widely ranged under field conditions, it was worthy to calculate corrected percentages of disease inhibition (CDI%) to evaluate the real comparable efficacy of treatments as follow:

$$CDI\% = \frac{A - B}{A} x100$$

Where

A = The corrected final disease severity of treatment.

B = The occurred final disease severity of treatment.

The corrected final disease severity (A) was calculated as the product of L/M x N where L is the initial disease severity of a treatment, M is the initial disease severity of the check (control) and N is the final disease severity of the check (control).

3.5.2. Yield measurement:

The potential yield was estimated through counting the fruit initials as a yield parameter (**McGrath, 1996**). Fruit initial was considered when it reached the size of ~ 10 cm long and ~ 3 cm diameter. When majority of fruits were at marketable size, fruits were harvested. Harvesting was repeated every 5 days and extended for 45 days and the accumulated yield was expressed as number of fruit/plant.

3.5.3. Influence of antagonists on some botanical measurements:

To determine whether the selected antagonistic microorganisms under test have any positive or negative effects on squash plants, some of

botanical vegetative measurements were determined in the plants subjected to the three times of application as following:

3.5.3.1. Total chlorophyll contents:

The SPAD-501 portable leaf chlorophyll meter (Minolta Corp) was used for greenness measurements using the 5th apical fully expanded leaf according to **Marquard & Timpton (1987); Tenga *et al.* (1989) and Yadava (1986)**.

3.5.3.2. Number of leaves per plant:

The fully expanded leaves were considered for each plant.

3.5.3.3. Leaf area:

Leaf area was determined using the dry weight method. The leaves of a plant were cleaned from dust and then representative 50 disks were taken using a cork borer known for its area (78.5 mm^2). The leaf area was calculated using the following formula:

$$\text{Leaf area} = \frac{\text{Weight of leaves}}{\text{Weight of 50 disks}} \times 50 \times n$$

Where:

n: the area of one disk

3.5.3.4. Plant height

Plant height was measured from the base of stem up to the apex for each plant (cm).

3.5.3.5. Fresh and dry weight of plant:

Cleaned uprooted plants free from lateral root-hairs were weighted freshly and then after drying at 105°C until constant weight.

3.6. Statistical analysis:

The completely randomized design was used for the laboratory and glasshouse experiments, while the randomized complete blocks design

was used for the open field experiment. Each experimental design has its previously mentioned replication. Data were transformed before subjection to analysis of variance using IRRI Stat Computer Program. Means were compared using LSD method **(Stell and Torrie, 1980)** and multiple range test **(Duncan, 1954)**.

3.7. The used media:

According to **Dhingra & Sinclair (1995)**, the following media were used for isolation and preparing the obtained antagonists.

- Nutrient agar:

Beef extract	3 g
Peptone	5 g
Glucose	2.5 g
Water	1 L
Agar	15 g

- Potato-dextrose agar:

Potatoes	200 g (peeled, sliced and boiled until softing before cheesecloth filtered)
Water	1 L (as a final volume)
Dextrose	10 g
Agar	15 g

Mixtures of a medium were autoclaved at 121°C for 20 min and agar is avoided if required to obtain the liquid medium.

4- RESULTS AND DISCUSSION

4.1. Isolation and identification of effective antagonists inhibiting the squash powdery mildew:

This study was carried out to search for microbial agents inhabiting the leaf surfaces of squash plants which having any of counteracting ability against the incidence of the powdery mildew disease. Isolation of microorganisms from squash leaves, collected from different fields of squash crop in Kafr El-Sheikh and El-Gharbia Governorates, resulted in various microbial isolates comprising fungi, yeasts, eubacteria and actinomycetes. The predominant groups were gram positive bacteria (58.54%), followed by fungi (30.48%); where the gram negative bacteria had lower occurrence (6.1%) and the lowest percentages of the isolated microorganisms have been occurred by yeast and actinomycetes (2.44% for each).

The preliminary screening of the obtained microbial isolates to inhibit the powdery mildew disease on cotyledons of squash potted plants showed variable efficiencies for this purpose (Table 1). The most effective fungal isolate was the isolate No. 84 which was identified as an isolate of *Epicoccum* sp. Link (Fig. 2 and Table 2). It inhibited the disease incidence by 100% after the third application onto the infected cotyledons (Fig. 1).The other isolated fungi, identified (according to the morphological structures) as *Aspergillus* sp., *Penicillium* sp., *Mucor* sp., *Alternaria* sp., *Ulocladiu* sp., *Cladosporium* sp., *Botrytis* sp. and *Stachybotrys* sp. widely ranged in their lower effects (Table 1).

Among the isolated microorganisms belonging to yeasts, the isolate No. 69 identified as *Rhodotorula* sp. Grinbergs et Yarrow (Fig. 4 and Table 3) showed 98.38% disease inhibition (Fig. 3) at the end of the experiment compared to the check treatment (treated with sterilized water).

Concerning to the bacterial isolates, numerous antagonistic agents lay amongst this microbial group. The isolates bearing the code numbers 27, 40, 58, 59, 83 and 32 identified as *Bacillus subtilis* (Chrenberg) Cohn, *Bacillus thuringiensis* Berliner, *Bacillus coagulans* Hammer, *Bacillus chitinosporus* Gordon, Haynes and Pang, *Bacillus pumilus* Meyer and Gottheil and *Derxia* sp. Jensen, Peterson, De and Bhattacharya, respectively (Tables 4-9 and Figs., 6, 8, 10, 12, 14 and 16) exhibited obvious inhibition effects against the development of powdery mildew lesions. Four of them (*B. subtilis*, *B. thuringiensis*, *B. coagulans* and *B. chitinospours*) prevented the establishment of any powdery mildew colonies on squash cotyledons (Figures 5, 7, 9 and 11), while the other two bacterial isolates (*Bacillus pumilus* and *Derxia* sp.) inhibited the disease incidence at the end of the experiment (three applications) by 98.9% and 99.3%, respectively as compared to the check treatment (Figs. 13 and 15). On the other hand, no antagonistic activities were detected by the isolated actinomycetes against the powdery mildew disease even after three times of application with a week intervals.

Table (1): Preliminary screening of the isolated microbial flora for inhibition effect against the powdery mildew infection on squash cotyledons in glasshouse.

No.	Isolated microorganisms		Disease severity*			
	Grouping and from genus	(code No.	At the application start	A week ** after the 1st application	A week after the 2nd application	A week after the 3rd application
	Fungi					
1	*Aspergillus* sp.	25	0.6	4.83	12.00	63.33 yz
2	*Aspergillus* sp.	26	0.33	5.00	10.83	60.00 x
3	*Aspergillus* sp.	31	3.67	8.17	72.32	106.67 h
4	*Aspergillus* sp.	34	5.67	8.83	15.67	31.33 lm
5	*Aspergillus* sp.	38	0.83	7.50	101.67	140.00 j
6	*Aspergillus* sp.	42	1.17	6.17	10.50	26.67 hij
7	*Aspergillus* sp.	43	1.50	7.83	10.85	43.33 pq
8	*Aspergillus* sp.	51	1.67	3.67	11.83	40.00 o
9	*Penicillium* sp.	52	2.83	10.83	18.17	73.67 c
10	*Penicillium* sp.	68	1.50	6.83	15.00	45.67 qr
11	*Mucor* sp.	39	0.00	18.83	45.33	60.00 x
12	*Mucor* sp.	41	2.83	6.33	36.70	52.67 uv
13	*Mucor* sp.	45	1.83	8.17	13.00	42.33 op
14	*Alternaria* sp.	18	2.33	6.33	37.83	65.00 zA
15	*Ulocladium* sp.	72	1.50	7.67	12.00	43.00 pq
16	*Cladosporium* sp.	29	1.50	5.30	18.00	63.33 yz
17	*Botrytis* sp.	71	1.83	6.50	11.67	50.00 stu
18	*Stachybotrytis* sp.	17	1.67	5.33	15.00	70.67 B
19	*Stachybotrytis* sp.	19	1.33	3.67	21.70	50.00 stu
20	*Epicoccum sp.*	84	2.00	1.67	1.17	0.00 a
21	Unknown	8	0.00	8.83	16.67	53.00 v
22	Unknown	9	2.00	7.50	14.67	80.00 D
23	Unknown	50	2.00	8.17	12.33	46.67 r
24	Unknown	74	2.83	5.33	7.12	50.00 stu
25	Unknown	80	0.83	8.50	12.17	49.67 st
	Yeasts					
26		64	1.17	4.83	8.83	36.00 n
27		69	6.00	6.33	5.50	5.20 cd

Table (1): Cont.

No.	Isolated microorganisms		Disease severity*			
	Grouping and from genus	(code No.	At the application start	A week ** after the 1st application	A week after the 2nd application	A week after the 3rd application
	Bacteria (Gram +)					
28		1	4.83	17.50	51.33	80.00 D
29		2	4.00	9.17	19.17	80.00 D
30		3	2.67	3.83	4.83	5.00 cd
31		5	3.17	6.61	7.83	20.00 g
32		6	2.17	2.67	2.83	6.00 cd
33		7	6.50	15.50	68.67	86.00 E
34		11	13.17	18.67	93.00	160.00 K
35		12	1.17	3.83	10.33	36.67 n
36		13	0.17	7.83	10.83	30.67 klm
37		14	0.50	10.11	24.67	61.33 xy
38		15	0.00	1.00	3.16	5.00 cd
39		16	2.00	5.00	9.17	35.00 n
40		20	3.00	5.00	7.00	14.00 f
41		21	0.00	3.17	6.17	17.67 g
42		22	12.67	15.17	92.00	124.67 I
43		24	0.66	2.33	2.50	14.00 f
44		27	1.17	1.00	0.67	0.00 a
45		28	0.83	3.17	9.50	28.67 jkl
46		30	2.00	5.83	10.83	36.67 n
47		33	8.17	13.50	25.50	51.00 tuv
48		35	2.17	20.00	41.67	56.67 w
49		36	9.00	33.33	66.67	106.67 H
50		37	4.17	7.50	11.83	43.00 pq
51		40	0.30	0.20	0.00	0.00 a
52		47	2.83	6.33	86.70	106.67 H
53		44	2.17	2.33	13.00	47.67 rs
54		46	10.33	14.00	36.67	66.67 A
55		48	2.67	9.17	44.17	63.33 yz
56		49	5.00	13.83	83.33	100.00 G
57		53	8.67	11.67	12.00	31.67 m
58		54	7.33	10.33	15.00	31.00 m
59		55	1.83	3.33	5.83	14.00 f
60		56	1.50	7.33	16.50	63.33 yz
61		57	1.50	3.00	4.00	5.00 cd
62		58	0.33	0.33	0.00	0.00 a
63		59	1.67	0.33	0.17	0.00 a
64		60	0.67	1.00	5.50	14.00 f
65		61	0.17	7.50	13.33	41.00 op
66		62	0.67	10.33	15.33	35.00 n
67		63	10.60	13.67	133.33	180.00 L
68		67	0.50	15.50	28.33	90.00 F
69		70	2.50	9.00	14.17	63.33 yz
70		73	0.00	10.33	19.17	50.00 stu
71		75	3.00	5.33	6.33	31.00 klm
72		76	1.67	4.17	4.85	10.33 e
73		77	5.33	11.33	23.33	66.67 A
74		78	2.83	6.33	8.50	25.67 hi
75		83	2.00	2.83	3.83	5.00 cd

Tale (1): Cont.

No.	Isolated microorganisms		Disease severity*			
	Grouping and from genus	(code No.	At the application start	A week ** after the 1st application	A week after the 2nd application	A week after the 3rd application
	Bacteria (Gram -)					
76		4	1.50	4.00	7.67	25.33 h
77		32	0.17	1.17	2.50	5.00 cd
78		79	1.17	18.33	58.00	78.67 D
79		81	0.00	3.67	5.33	20.00 g
80		82	2.33	5.17	9.33	40.00o
	Actinomycetes					
81		10	7.33	13.50	66.67	107.00 H
82		23	1.17	3.50	15.30	28.33 ijk
	Control* **		1.5	18.33	383.33	486.3 M

* Exact lesion counts were made when there were less than approximately 50 lesions per cotyledon. Thereafter, severity was estimated using a conversion factor of 1% 10 lesions per cotyledon.

** Application was repeated three times with a week intervals.

*** Treated with sterilized water

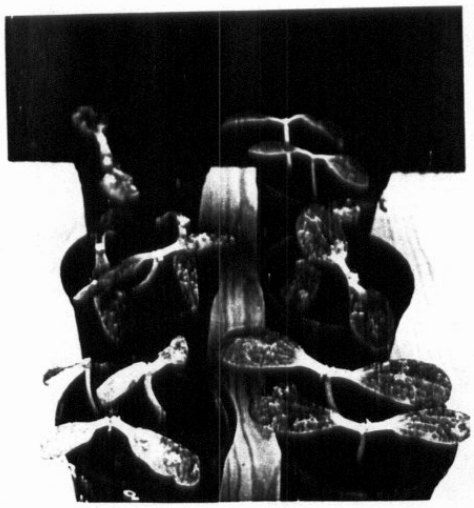

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Fig. (1): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 84 (*Epicoccum* spp. Link).

1. Untreated plants (control) 2. Treated plants.

Table (2): Characters of the effective antagonistic isolate No. 84 *Epicoccum sp.* Link.

The tested characters	Description
Growth on agar medium	Abundantly developed mycelium on PDA.
Pigment	Mycellium is characterized by production of orange to brown pigments soluble in media.
Sprodochia	Spores are formed in dark sporodochia
Conidial spores	Conidiospores are olivaceous in color and globose shaped. Cross-walls are developed to cut up the spore into a compact ball of thick-walled cells.

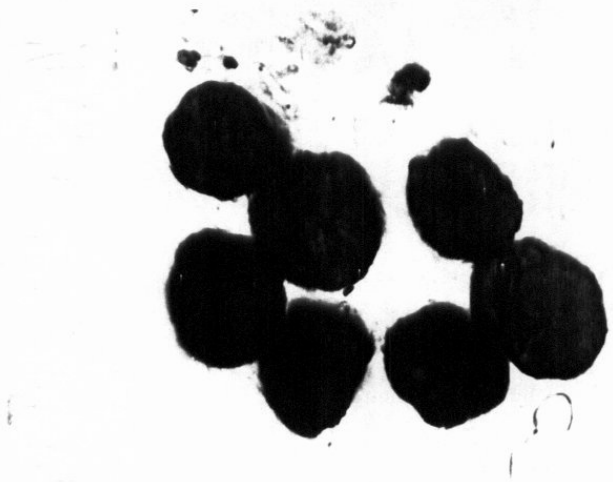
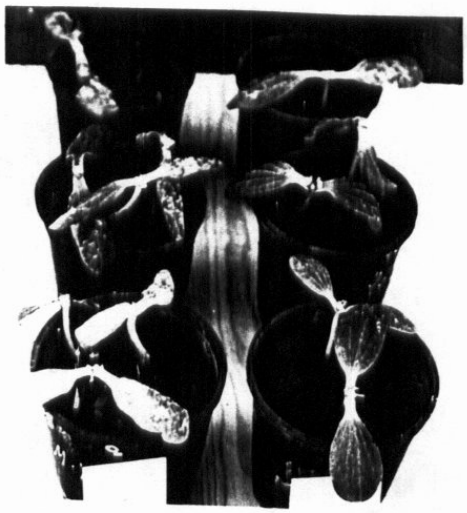


Fig. (2): Lactophenole stained cells of the isolate No. 84 (*Epicoccum* sp.) (X1600).



1 2

Fig. (3): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 69 (*Rhodotorula* spp.)

1. Untreated plants (control) 2. Treated plants.

Table (3): Characters of the effective antagonistic isolate No. 69 (*Rhodotorula sp.* Grinbergs et Yarrow).

The tested characters	Results
Vegetative cells	Spherical, oval or elongated
	No pseudomycelium
Growth on:	
Nitrate	-
Inositol	-
Pigmentation	red pigment

+ = Positive, - = Negative

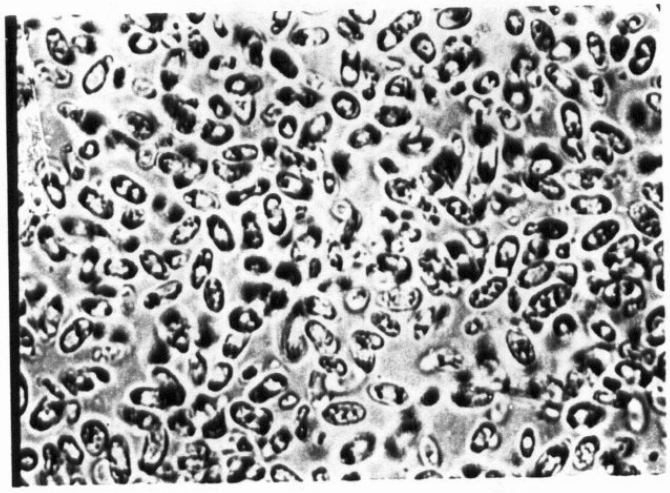
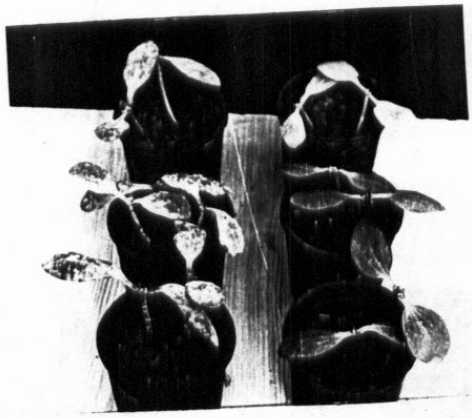


Fig. (4): Lactophenole stained cells of the isolate No. 69 (*Rhodotorula sp.*) (X1600)



1 2

Fig. (5): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 27 (*B. subtilis*)

1. Untreated plants (control) 2. Treated plants.

Table (4): Characters of the effective antagonistic isolate No. 27 [*Bacillus subtilis* (Ehrenberg) Cohn.]

The tested characters	Results
Formation of endospores	+
L.V. reaction	-
Citrate utilization	+
Anaerobic growth	-
V.P. reaction	+
Nitrate reduction	+
Indole production	-
Growth on 7% NaCl	+
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Urease activity	V
Motility	+
Shape of cell	Rods
Gram reaction	+
Catalase reaction	+

+ = Positive - = Negative V = Variable

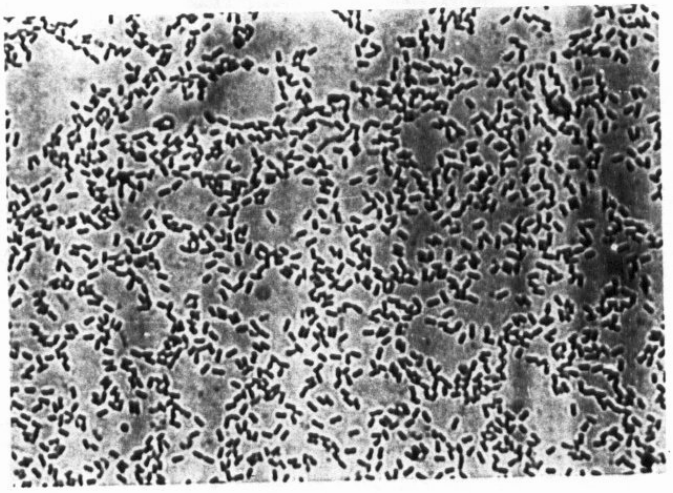
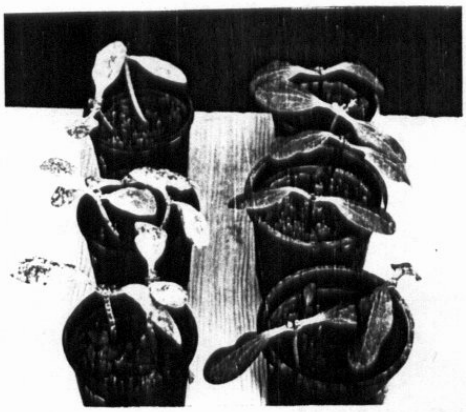


Fig. (6): Gram stained cells of the isolate No. 27 (*B. subtilis*) (x 1600)



1 2

Fig. (7): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 40 (*B. thuringiensis*)

1. Untreated plants (control) 2. Treated plants.

Table (5): Characters of the effective antagonistic isolate No. 40 (*Bacillus thuringiensis* Berliner).

The tested characters	Results
Formation of endospores	+
L.V. reaction	+
Citrate utilization	+
Anaerobic growth	+
V.P. reaction	+
Nitrate reduction	+
Indole production	-
Growth on 7% NaCl	+
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Urease activity	V
Motility	+
Parasporal bodies	+
Shape of cell	Rods
Gram reaction	+
Catalase reaction	+

+ = Positive - = Negative V = Variable

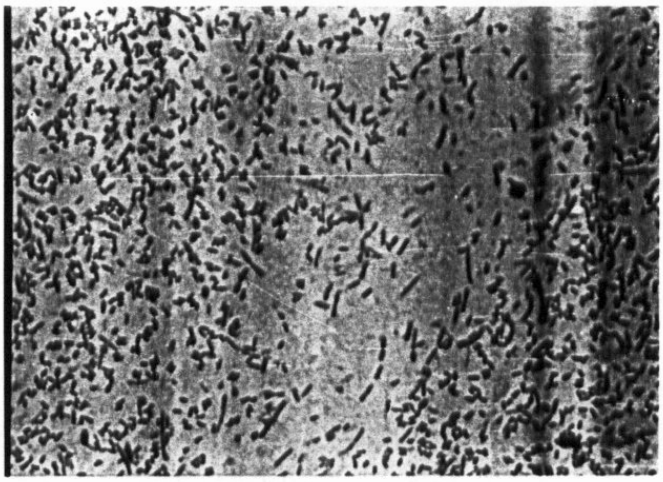
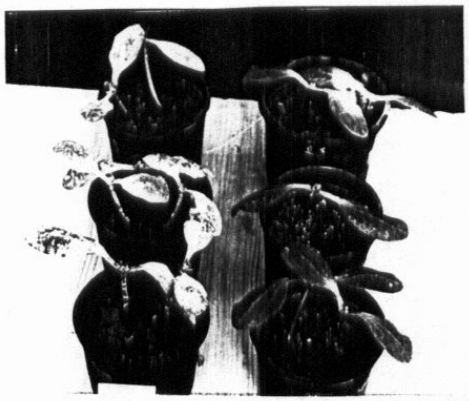


Fig. (8): Gram stained cells of the isolate No. 40 (*B. thuringiensis)* (x 1600).



1 2

Fig. (9): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 58 (*B. coagulans*)

1. Untreated plants (control) 2. Treated plants.

Table (6): Characters of the effective antagonistic isolate No. 58 (*Bacillus coagulans* Hammer).

The tested characters	Results
Formation of endospores	+
L.V. reaction	-
Citrate utilization	v
Anaerobic growth	+
V.P. reaction	v
Nitrate reduction	v
Indole production	-
Growth on 7% NaCl	-
Starch hydrolysis	+
Casein hydrolysis	v
Gelatin hydrolysis	-
Urease activity	-
Motility	+
Shape of cell	Rods
Gram reaction	+
Catalase reaction	-

+ = Positive - = Negative V = Variable

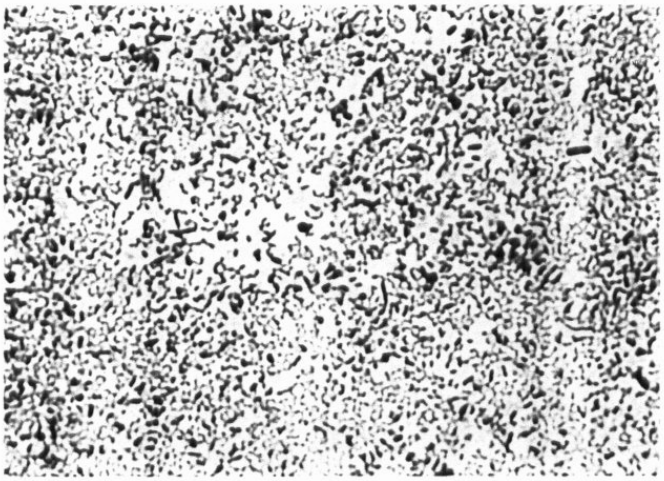
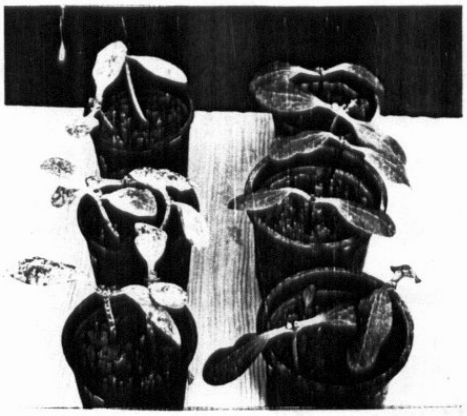


Fig. (10): Gram stained cells of the isolate No. 58 (*B. coagulans*) (x1600).



1 2

Fig. (11): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 59 (*B. chitinosporus*)

1. Untreated plants (control) 2. Treated plants.

Table (7): Characters of the effective antagonistic isolate No. 59 (*Bacillus chitinosporus* Gordon, Haynes and Pang).

The tested characters	Results
Formation of endospores	+
L.V. reaction	-
Citrate utilization	-
Anaerobic growth	-
V.P. reaction	-
Nitrate reduction	-
Indole production	-
Growth on 7% NaCl	-
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Urease activity	-
Motility	+
Shape of cell	Rod-shaped cells usually in chains
Gram reaction	+
Catalase reaction	+

+ = Positive, - = Negative

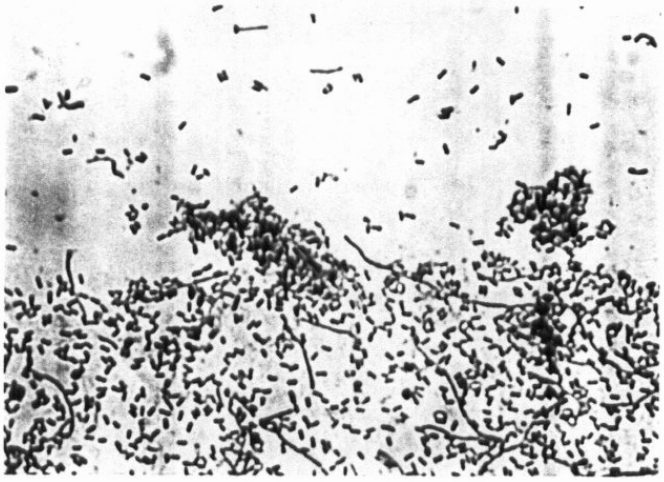
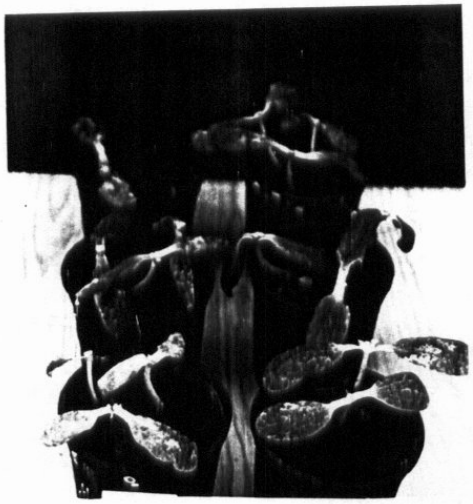


Fig. (12): Gram stained cells of the isolate No. 59 (*B. chitinospours*) (x 1600)



1 2

Fig. (13): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 83 (*B. pumilus*)

1. Untreated plants (control) 2. Treated plants.

Table (8): Characters of the effective antagonistic isolate No. 83 (*Bacillus pumilus* Meyer and Gottheil).

The tested characters	Results
Formation of endospores	+
L.V. reaction	-
Citrate utilization	+
Anaerobic growth	-
V.P. reaction	+
Nitrate reduction	-
Indole production	-
Growth on 7% NaCl	+
Starch hydrolysis	-
Casein hydrolysis	+
Gelatin hydrolysis	+
Urease activity	-
Motility	+
Shape of cell	Rods
Gram reaction	+
Catalase reaction	+

+ = Positive, - = Negative

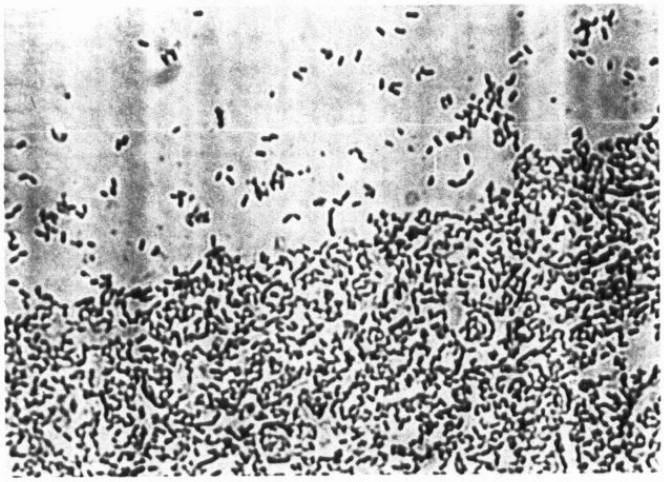
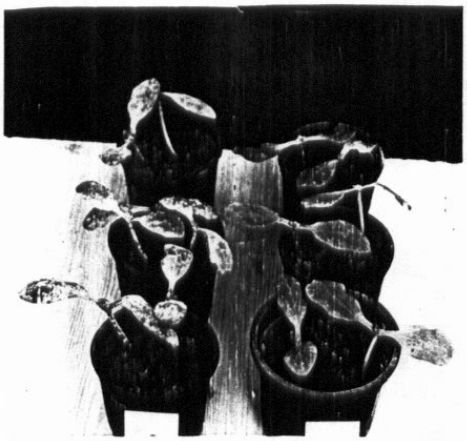


Fig. (14): Gram stained cells of the isolate No. 83 (*B. pumilus*) (X1600)



1 2

Fig. (15): Inhibition of powdery mildew disease infection on cotyledons of squash plants treated with the isolate No. 32 (*Derxia* sp.)

1. Untreated plants (control) 2. Treated plants.

Table (9): Characters of the effective antagonistic isolate No. 32 (*Derxia* sp. Jensen, Petersen, De and Bhattacharya).

The tested characters	Results
Growth on agar medium	Raised slimy massive colonies
Growth in liquid medium	Starts as a ring at the glass-liquid interface and develops into a thick surface pellicle
Aerobic growth	+
Colony color	Pale yellow at first turns to mahogany brown within ~ 10 days
Motility	+
Cell shape	Rods with rounded ends
Arrangement	Single and short chains
Size of cells	1-1.5 µm x 5-25 µm
Gram reaction	-
Presence of retractile bodies in cells	+
Capsule	Present
Catalase test	-
Indole production	-
Nitrate reduction to nitrite	-
Starch hydrolysis	- (with scant growth)
Growth on nitrogen-free glucose broth	+
Optimum temperature	25-35°C
Growth on 50°C	-
Optimum pH	7
Growth on pH 4	-

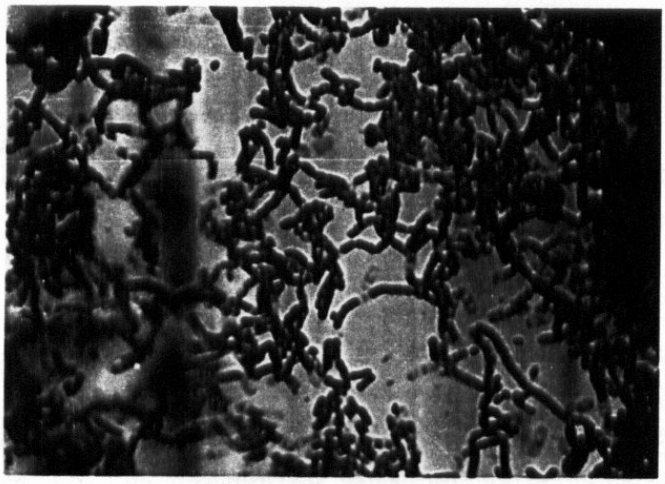


Fig. (16): Graim stained cells of the isolate No. 32 (*Derxia* sp.) (X1600)

Most of the tested microbial genus and species are known as antifungal antagonists. *Epicoccum* sp. Link has been reported as biocontrol agent against the pathogenic fungi (**Mercier & Reeleder, 1987**) and the antifungal compounds, epicorazines A and B have been extracted from its culture filtrates (**Brown *et al.*, 1987**), **Yohaleem (2000)** reported about yeast including *Rhodotorula* sp. as promising microbial tools in controlling foliar plant fungal diseases.

Derxia sp. proved its ability to control the powdery mildew disease on squash plants in both pot and field experiments (**El-Gremi, 2002**).

In addition numerous members of *Bacillus* species are known as producers of polypeptidic antibiotics (**Egorov, 1985 and Kojibski *et al.*, 1969**). Mycobacillin and Pumilin are, so far, known as antifungal antibiotics produced by *B. subtilis* and *B. pumilus*, respectively (**Majumdar & Bose, 1958 and Bhate, 1955**).

B. coagulans is used for commercial production of an enzyme for yeast cell lysis (**Parry *et al.*, 1983**).

On the other hand, in despite of the wide use of *B. thuringiensis* as bioinsecticide (**Powell, 1993**), *B. thuringiensis* in addition to *B. chitinosporus* have not been yet reported as antifungal antagonists for our up to date knowledge.

The experimental plant materials used in this study were cotyledons of squash plants as the most susceptible materials to powdery mildew infection (**McGrath & Staniszewska 1996 and Bettiol *et al.*, 1997**). In addition, prunning of plants allows cotyledons to prolong their longevity up to more than 45 days. So, experiments conducted in this manner to screen the tested control agents enable us to obtain contrast in symptoms enough to estimate the effectiveness of the tested treatments.

Since the pathogen is an important component of biological control process, **Cook and Baker (1989)** reported that the primary tests dealing with biological control of strict biotrophs (as powdery mildew pathogens) must occur on the living host. Leaf disks placed on water agar (**McGrath & Staniszewska, 1996; Hajlaoui & Belanger, 1993; Hoch & Provvidenti 1979 and Klecan *et al.*, 1990**) and detached rooted leaves (**Verhaar *et al.*, 1994, Verhaar *et al.* 1997 and Askary *et al.* 1997**) are the common techniques used in the *in vitro* experiments in biological control studies in powdery mildews on cucurbits. It is well known that leaf disks are poor representative living materials since it can not be maintained in moist petri dishes longer than 8 days (**Askary *et al.*, 1997**). In addition, the detached rooted leaves is considered as a complicated technique which necessarily needs expensive chemicals as root growth regulators (**Carver & Phillips, 1982**) which may have effects interfering with the interaction between the tested pathogen and antagonistic microorganisms.

In this study a new simple technique was used to verify the antagonistic action, of numerous isolates which were weekly applied onto destined real living plant materials durable up to at least 6 weeks old. Using this economic technique, the experimental unit needs as minor as 10 ml of antagonistic liquid culture to be applied.

4.2. The harmful actions on the pathogenic fungus:

The powdery mildew lesions treated with the tested biocontrol agents as well as those treated with the tested chemical fungicide were observed by unaided eye as fade colonies with lack appearance of sporulation. The light microscopy examination revealed significant decrease of erect conidiophores and spores per chain compared to

untreated lesions (Table 10 and Fig. 17). The scanning electron micrographs in Fig. 18 again confirmed this fact. *In situ* examination revealed bare conidiophores (Fig. 18 A) and remnants of collapsed hyphae (Fig. 18 B).

The direct inhibiting effects of either separated washed cells or their filtered metabolites were compared with the whole liquid cultures for each of the tested microbial antagonists. Data presented in Table (11) indicate that the filtered metabolites have antagonistic effects almost conincident with that of the whole liquid cultures on squash cotyledons in cases of all the tested antagonists. The disease inhibition percentages ranged from 94.46% to 100% using filtered metabolites and from 95% to 100% using whole liquid cultures.

On the other hand, marked lower inhibition percentages were registered using washed cells instead of filtered metabolites in majority of the tested antagonists. These percentages descended from 100, 98, 100 and 94.88% down to 71.83, 44.62, 56.81 and 25.23% in cases of *Derxia* sp., *B. thuringiensis*, *B. coagulans* and *Rhodotorula* sp., respectively.

However, it is worthy to refer that data presented in Table (11) show that washed cell suspension of *B. chitinosporus*, applied onto the squash plants in the concentration of 10^7 cfu/ml, completely prevented the pathogen colonies to develop. This may be due to special ability to establish the leaf surfaces and/or suitability of ambient conditions (65.2 R.H and 27.3°C) for this isolate to produce its effective metabolites as enough as in laboratory culturing.

The direct effect of the tested filtered metabolites on germination of the pathogen conidial spores was insured on slides of water agar

Table (10): Effect of the antagonistic microorganisms as well as fungicide (Topas 100) on growth and sporulation of *Sphaerotheca fuliginea*

Treatments	No. of sporulating lesions/Cotyledon		Stereo light microscopy examination	
	Before treating	After treating*	No. of erect conidiophores/ lesion**	No. of new spores/ chain***
Control	17.0 b	23.00 a	54 f	5 f
B. subtilis	14.4 c	0.17 b	1 b	2 b
Derxia sp.	12.0 d	0.00 b	0 a	0 a
B. thuringiensis	19.9 a	0.20 b	1 b	2 b
B. coagulans	17.8 b	0.00 b	0 a	0 a
B. chitinosporus	19.6 a	0.00 b	0 a	0 a
Rhodotorula sp.	14.75c	0.25 b	2 c	3 c
B. pumilus	14.0 c	0.70 b	4 d	3 c
Epicoccum sp.	15.0 c	0.00 b	0 a	0 a
Fungicide (Topas 100 EC)	18.0 b	1.30 b	7 f	3 c

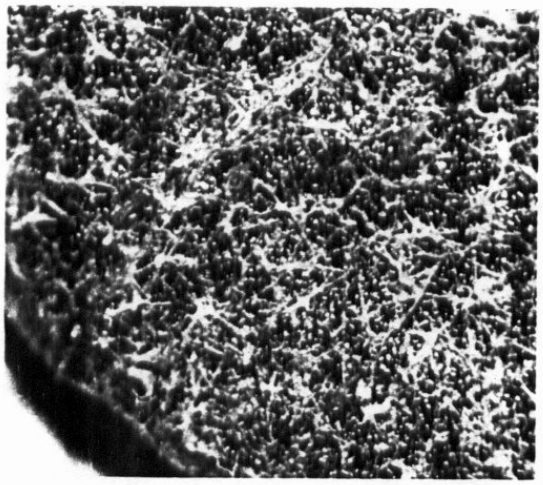
In the same column, means followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT)

* Data were taken 8 days after spraying the whole liquid culture onto mildew lesions.

** Mean of 10 lesions.

*** Mean of 10 conidiophores.

A



B

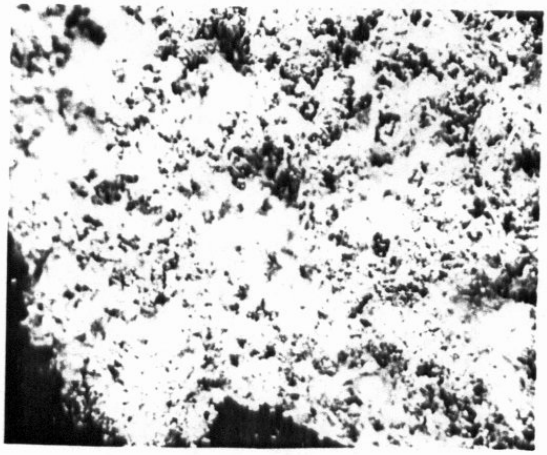


Fig. (17): The features of treated (A) and untreated (B) lesions of powdery mildew by one of the tested bioagents, observed under steriomicroscopy (50X). The difference in density of growth and sporulation is noticed.

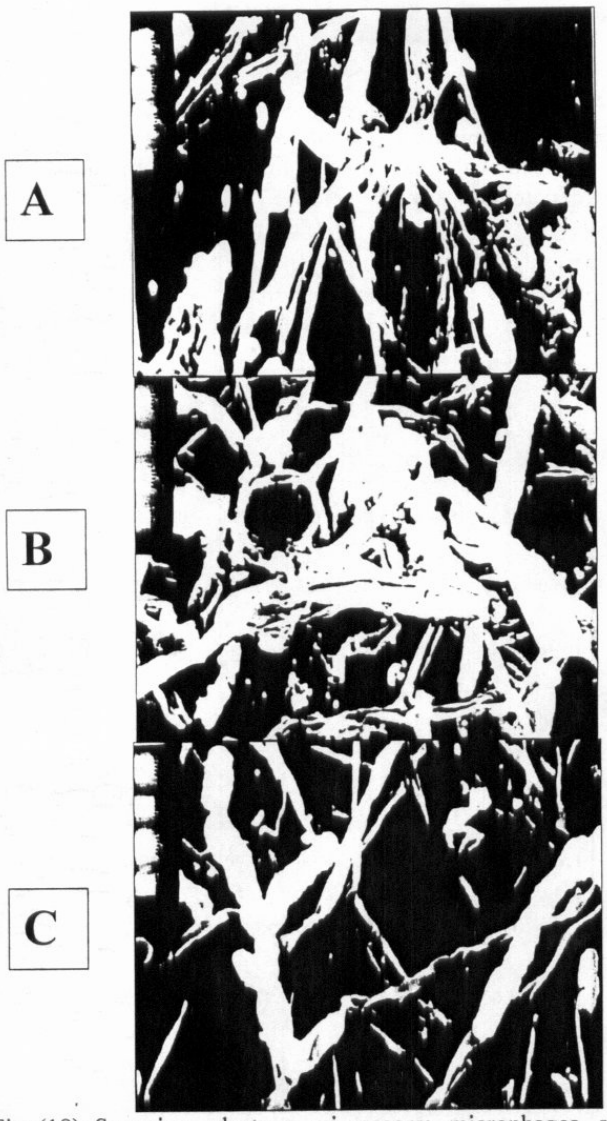


Fig. (18): Scanning electron microscopy microphages showing bare conidiophores with no spores (A) and collapsed hyphae (B) in treated powdery mildew lesions with the tested antagonists compared with normal conidiophores bearing spore-chains in untreated lesions (C). (X600)

Table (11): Effect of culture filtrates and washed cell suspensions, in comparison with the whole liquid cultures on the squash powdery mildew disease.

Treatment	Disease severity									
	Filtrate			Cells (10^7 cfu/ml)			Liquid culture (10^7 cfu/ml)			
	At the application start	8 days after the application start	% Inhibition	At the application start	8 days after the application start	% Inhibition	At the application start	8 days after the application start	% Inhibition	Mean of inhibition
Control	17.00	23		17.00	23.00		17.00	23.00		
B. subtilis	11.60	0.25	97.84	14.80	0.83	94.39	14.40	0.17	98.80	97.1
Derxia sp.	11.25	0.00	100.0	16.25	4.66	71.83	12.00	0.00	100.00	90.61
B. thuringiensis	15.00	0.30	98.00	15.80	8.75	44.62	19.90	0.20	99.00	80.54
B. coagulans	17.90	0.00	100.00	13.50	5.83	56.81	17.80	0.00	100.00	85.60
B. chitinosporus	11.25	0.00	100.00	21.50	0.00	100.00	19.60	0.00	100.00	100.0
Rhodotorula sp.	12.90	0.66	94.88	10.70	8.00	25.23	14.75	0.25	98.30	72.8
B. pumilus	19.40	1.00	94.84	13.75	1.75	87.27	14.00	0.70	95.00	92.37
Epicoccum sp.	15.00	0.83	94.40	10.90	1.17	89.26	15.00	0.00	100.00	94.55
Mean of inhibition			97.5			71.17			98.9	

L.S.D.	5%	1%
Isolates (M)	0.81	1.073
Method of application (S)	1.316	1.752
Interaction (M*S)	2.279	3.035

Table (12): Germination of *Sphaerotheca fuliginea* conidial spores in the presence of metabolites of the selected antagonistic isolates.

Antagonists	Germination %	Inhibition of germination %
Control	67.33 f	-
B. subtilis	1.89 c	97.19
Derxia sp.	1.00 a	98.51
B. thuringiensis	1.62 bc	97.59
B. coagulans	0.62 a	99.07
B. chitinosporus	0.33 a	99.50
Rhodotorula sp.	5.51 e	91.81
B. pumilus	1.84 c	97.26
Epicoccum sp.	3.92 d	94.17

In the same column, means followed by the same letter are not significantly different according to DMRT

amended with the tested metabolites (Table 12) where the percentages of inhibition in spore germination reached 91.81-99.5% compared with the control treatment. Acting through production of metabolites with antibiotic natures is known for numerous biocontrol agents against powdery mildews. Fatty acids with antibiotic activity have been isolated from liquid cultures of *Sporothrix flocculosa* (**Benyagoub *et al.*, 1996 and Ng *et al.*, 1997**) succeeded to suppress sporulations of powdery mildew on rose using culture filtrate without spores of *Tilletiopsis pallescens*. Inhibition of *Sphaerotheca fuliginea* conidial germination have been achieved by the antifungal metabolite gramicidin S produced by *Bacillus brevis* (**Schmitt *et al.*, 1999**).

4.3. Control of the powedery mildew disease under field conditions:

Under field conditions, the selected microorganisms exhibited their efficacy to control the powdery mildew disease either on squash or on cucumber crops (Fig. 19). Data in Tables (13) and (14) indicate that treating plants three times with the tested antagonists (liquid cultures containing 10^7 cfu/ml each) completely prevented the appearance of the pathogenic lesions. It is well noticed that as the applications were repeated as the control effectiveness was greater. However, even one application of any of the tested bioagents significantly decreased the disease severity to or less than 10.0 lesions/leaf in that time in which this severity reached 72 lesions/leaf on the untreated plants. As the initial disease severity widely ranged under the open field conditions, the corrected disease inhibition percentages are suitable to realize fair comparisons. Using such equitable scale, it could be concluded that the one-application treatments were generally less effective on cucumber than on squash plants, this trend was obvious as in case of chemical as in

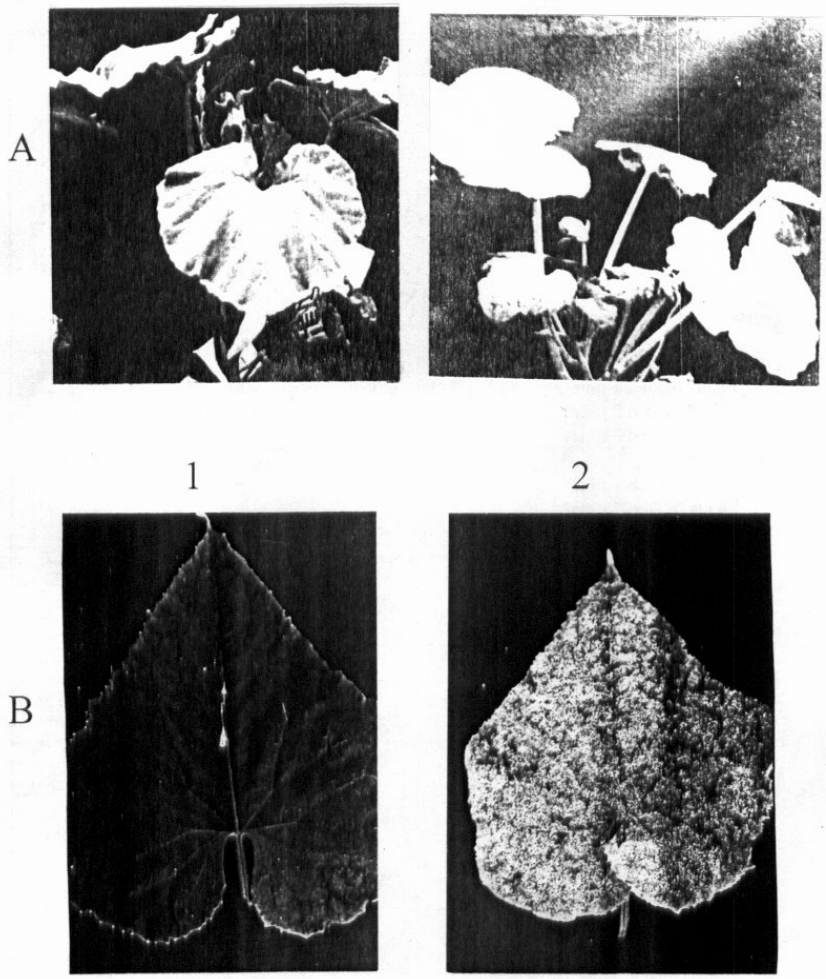


Fig. (19): Inhibition of powdery mildew disease on true leaves of squash (A) and cucumber (B) plants. (1) treated with antagonists, (2) untreated control.

Table (13): The efficacy of all the tested microbial isolates to control the powdery mildew disease on squash plants compared with the fungicide Topas 100 under field conditions using different times of application

Treatments	% Disease severity		% disease inhibition (corrected)
	At the application start	30 days after the application start	
Control	12	72	0
B. subtilis			
Plants treated 1 time	11	7.50	88.43
Plants treated 2 times	17	3.00	97.10
Plants treated 3 times	15	0.00	100.00
Derxia sp.			
Plants treated 1 time	27	4.50	97.22
Plants treated 2 times	12	1.25	98.26
Plants treated 3 times	14	0.00	100.00
B. thuringeinsis			
Plants treated 1 time	16.00	9.25	90.12
Plants treated 2 times	19.00	4.50	96.10
Plants treated 3 times	13.00	0.00	100.00
B. coagulans			
Plants treated 1 time	11.50	7.50	89.58
Plants treated 2 times	12.70	4.25	94.63
Plants treated 3 times	7.50	0.00	100.00
B. chitinosporus			
Plants treated 1 time	19.0	7.50	93.49
Plants treated 2 times	7.00	0.00	100.00
Plants treated 3 times	9.50	0.00	100.00
Rhodotorula sp.			
Plants treated 1 time	9.00	10.00	82.64
Plants treated 2 times	12.75	4.50	94.32
Plants treated 3 times	6.00	0.00	100.00
B. pumilus			
Plants treated 1 time	15.75	4.50	95.19
Plants treated 2 times	18.00	1.75	98.38
Plants treated 3 times	11.50	0.00	100.00
Epicoccum sp.			
Plants treated 1 time	19.00	4.25	96.31
Plants treated 2 times	22.25	3.75	97.26
Plants treated 3 times	18.75	0.00	100.00
Fungicide (Topas 100)			
Plants treated 1 time	2.50	2.25	85.12
Plants treated 2 times	10.75	1.00	98.46
Plants treated 3 times	11.25	0.75	98.89

L.S.D.	5%	1%
Isolates (M)	0.356	0.488
Method of application (S)	0.356	0.497
Interaction (M*S)	1.13	1.51

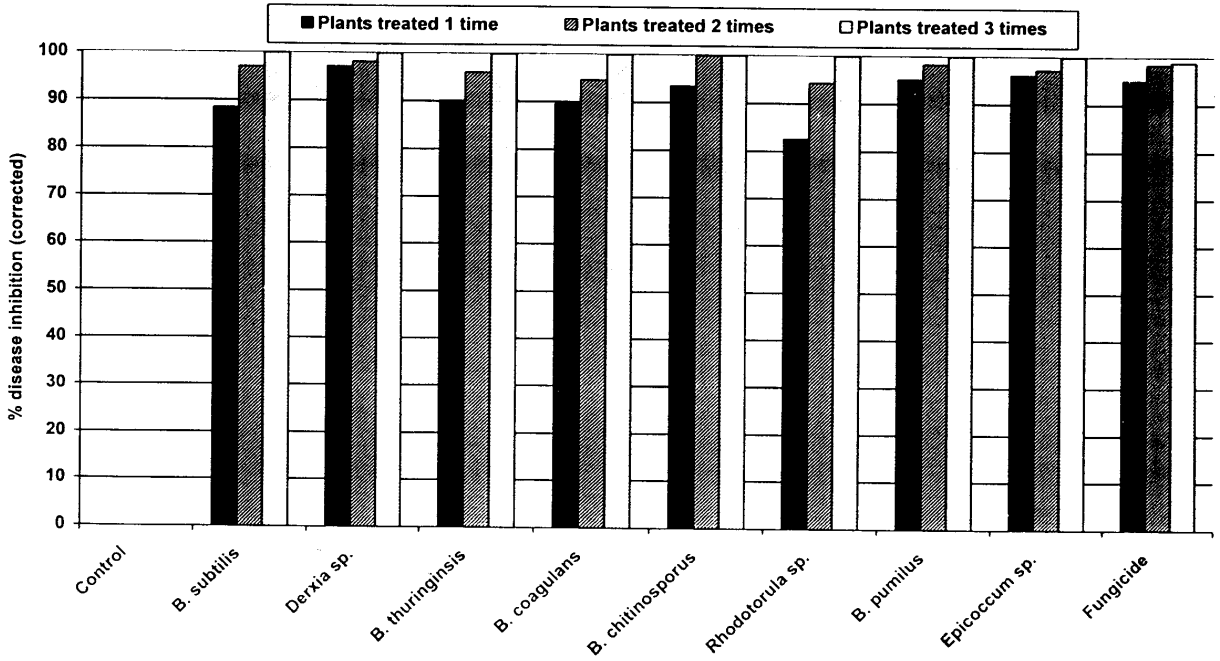


Fig. (20): The efficacy of all the tested microbial isolates to control the powdery mildew disease on squash plants compared with the fungicide Topas 100 under field conditions using different times of application.

Table (14): The efficacy of all the tested microbial isolates to control the powdery mildew disease on cucumber plants compared with the fungicide Topas 100 under field conditions using different times of application

Treatments	% Disease severity		% disease inhibition (corrected)
	At the application start	30 days after the application start	
Control	3.25	30	0
B. subtilis			
Plants treated 1 time	2.25	6.75	67.39
Plants treated 2 times	1.75	0.00	100
Plants treated 3 times	3.25	0.00	100
Derxia sp.			
Plants treated 1 time	7.00	9.00	86.4
Plants treated 2 times	3.00	1.00	96.37
Plants treated 3 times	2.00	0.00	100
B. thuringeinsis			
Plants treated 1 time	1.00	3.50	62.36
Plants treated 2 times	1.75	2.25	86.11
Plants treated 3 times	2.00	0.00	100
B. coagulans			
Plants treated 1 time	4.25	6.25	84.1
Plants treated 2 times	3.75	4.00	88.4
Plants treated 3 times	1.75	0.00	100
B. chitinosporus			
Plants treated 1 time	4.00	7.50	79.67
Plants treated 2 times	2.75	1.50	94.11
Plants treated 3 times	1.75	0.00	100
Rhodotorula sp.			
Plants treated 1 time	4.75	10.50	76.02
Plants treated 2 times	6.00	5.00	90.9
Plants treated 3 times	6.25	0.00	100
B. pumilus			
Plants treated 1 time	4.50	2.25	94.50
Plants treated 2 times	3.00	1.50	94.56
Plants treated 3 times	3.00	0.00	100
Epicoccum sp.			
Plants treated 1 time	4.50	8.75	78.86
Plants treated 2 times	5.25	2.25	95.34
Plants treated 3 times	5.00	0.00	100
Fungicide (Topas 100)			
Plants treated 1 time	1.75	5.75	63.83
Plants treated 2 times	4.75	1.50	96.57
Plants treated 3 times	5.00	0.00	100

L.S.D.	5%	1%
Isolates (M)	0.662	0.908
Method of application (S)	0.758	1.013
Interaction (M*S)	1.31	1.76

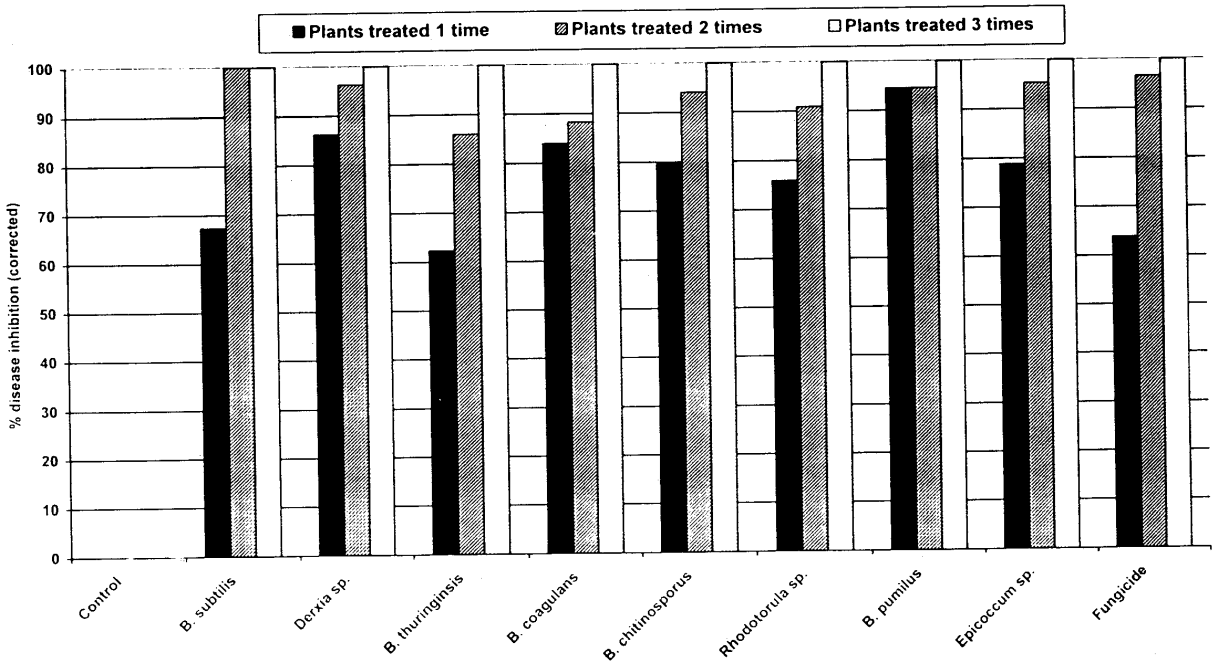


Fig. (21): The efficacy of all the tested microbial isolates to control the powdery mildew disease on cucumber plants compared with the fungicide Topas 100 under field conditions using different times of application.

biological treating (Table 13 and 14). This may be explained as a result of a specific relationship between the pathogen and the host crop plants.

Although, the used chemical fungicide (Topas, 100) and the tested biological agents are significantly comparable for controlling the powdery mildew disease on squash and cucumber, development of fungicide resistance can interfere with fungicide efficacy. **Mc-Grath and Staniszewska (1996)** reported that fungicide-treated squash plants had significantly lower powdery mildew severity than non-treated in early experiments conducted in 1991, 1992, but not in the third experiment conducted in 1993. This was most likely due to fungicide-resistance where eight resistant isolates could be collected at the end of the third experiment. The same fact have been affirmed in other previous experiments conducted by **McGrath (1991), McGrath & Ghemawat (1992) and McGrath & Staniszewska (1993)**. Furthermore, **Schroeder and Provvidenti (1969)** isolated a benomy-resistant isolate that developed profusely on fungicide-treated plants but only sparingly on untreated plants. Suggesting the possibilities that the fungus was becoming dependent upon benomyl or that benomyl was interfering with the expression of inherent tolerance within the host.

Numerous promising biological agents exhibiting more or less degrees of antagonisms towards the powdery mildew disease could be used as alternatives to chemical fungicides. Fluctuating the efficiency of the foreign "introduced" biocontrol agents creates insistent need to search for efficient local and "resident" ones. Therefore, in this work we directly began the research track from the leaf surface where the pathogen and the neighbour probable antagonists live. In addition, local antagonists isolates adapting with the ambient conditions are applicable since the major

limitation in the use of the common known antagonistic (*Ampelomyces quisqualis*, *Verticillium lecanii*, *Sporothrix* spp. or *Tilletiopsis* spp.) is the requirement of as height humidity as to free water (**Falk *et al.*, 1995b, Verhaar *et al.*, 1997 and Verhaar *et al.*, 1999**). In the present study the obtained local antagonistic isolates acts under the local ambient field conditions with relative humidity ranged from 49.5 to 83.5% and air temperature ranged from 20.3°C to 34.4°C. So, these isolates seem to be tolerant to the relatively dry conditions of Egypt.

Another limitation in the use of antagonistic microbial isolates to control plant diseases is the safety of the host plant. **Jarvis and Slingsby (1977)** achieved reduction in the cucumber powdery mildew and increment in the yield using *Ampelomyces quisqualis* , but on the other hand, they noted small, angular leaf spots and sunken lesions on fruits of the plants sprayed with the antagonist.

4.4. Influence of antagonsts on some botanical measurements:

In our study, the tested microbial antagonists were examined for any of harmful effects on the treated plants. Data in Tables 15, 16 show improvement in some detected botanical measurements. In the untreated (check) treatment, the plants were suffering from decline in chlorophyll pigments, as well as significant lower leaves, leaf area, plant height and plant weight. The powdery mildew disease causes a decline in chlorophyll contents and in the photosynthesis processes as a result of infection progresses (**Magyarosy *et al.*, 1976; Wright *et al.*, 1990 and Abo-Foul *et al.*, 1996**). In this study the improvement in plant health led to increment in the crop yield. The total yield of fruits reached 14 squash fruits/plant using *B. pumilus* and 18.3 cucumber fruits/plant using *B. thuringiensis*. These results are significantly higher than those obtained

by the tested fungicide (11.86 and 16.54 fruits/plant, respectively). The untreated plants approximately yielded 6.72 squash fruits/plant and 6.0 cucumber fruits/plant.

In this study, research for microbial antagonists against the squash powdery mildew disease is confined by local and resident antagonists. The well known traditional hyperparasites *Ampelomyces quisqualis* or *Verticillium lecanii* could not be isolated. In nature, the mycoparasite may not be observed until late in the growing season long after the pathogen of powdery mildew have formed the over wintering cleistothecia, that ensure perpetuation of the disease (**Gadory & Pearson, 1988**). In other words, hyperparasite my only help, in general, to decrease the pathogenic inoculum in nature (**Dik *et al.*, 1998**). So, hyperparasites are not the ideal means of powdery mildew diseases control in case of vegetable crops (like cucurbits), with approximate 3 months as life duration. Protective and curative treatments are needed in such case. Antagonists acting through antibiosis may achieve this purpose rather than hyperparasites.

The present study offers some local microorganisms antagonistic to *S. fuliginea* through producing metabolites which act as protective and curative agents since they inhibit spore-germination and mycelial growth and sporulation. However the characterization of these metabolites as well as the determination of the ideal conditions for these antagonists to maximize their production of the effective metabolites need further studies. Medical, veterinary and environmental permissions are also needed for their commercialization.

Table (15): Influence of the tested biological and chemical control treatments on some botanical measurements of squash plants treated with three times of 10 days interval applications.

Treatments	Total chlorophyll of leaf SPAD	No. of leaves/per plant	Leaf area cm^2	Plant height cm	Fresh weight (g) per plant	Dry weight (g) per plant	Total crop yield (fruits/plant)
Control	29.23 a	11 a	192 a	32.70 a	280.12 a	40.70 a	6.72 a
B. subtilis	36.90 c	26 e	297 e	68.90 b	425.90 h	117.25h	12.90f
Derxia sp.	38.90 c	20 d	263 c	50.50 b	377.80 c	80.15 cd	12.06 e
B. thuringiensis	37.17 c	17 bc	280 d	56.35 b	410.35 fg	82.23 de	10.92 d
B. coagulans	36.77 c	17 bc	283 d	57.25 b	415.17 gh	100.32 g	10.92 d
B. chitinosporus	39.70 c	16 bc	262 c	55.40 b	400.50 ef	80.19 cd	10.68 d
Rhodotorula sp.	35.50 bc	18 cd	280 d	60.55 b	405.14 fg	85.12 ef	8.80 b
B. pumilus	36.17 c	20 d	293 de	61.75 b	380.20 cd	87.53 f	14.00 g
Epicoccum sp.	31.80 ab	15 b	242 b	54.34 b	360.35 b	70.71 b	9.90 c
Fungicides	36.46 c	17 bc	262 c	57.93 b	390.95 de	80.20 c	11.86 e

In the same column, means followed by the same letter are not significantly different according to DMRT

Table (16): Influence of the tested biological and chemical control treatments on some botanical measurements of cucumber plants treated with three times of 10 days interval applications.

Treatments	Total chlorophyll of leaf SPAD*	No. of leaves/per plant	Leaf area cm^2	Plant height cm	Fresh weight (g) per plant	Dry weight (g) per plant	Total crop yield (fruits/plant)
Control		9.00 a	3.15 a	18.50 a	78.33 a	4.73 a	6.00 a
B. subtilis		17.00 d	67.30 cde	49.45 e	133.17 h	8.42 i	17.66 f
Derxia sp.		15.00 c	70.50 def	39.21 cd	123.18 g	8.12 g	14.40 c
B. thuringiensis		15.00 c	73.50 ef	38.55 c	103.75 bc	7.33 b	18.30 g
B. coagulans		13.00 b	63.00 bc	38.99 cd	109.52 d	7.53 d	14.50 c
B. chitinosporus		13.00 b	65.00 cd	28.45 c	107.34 cd	7.45 c	14.10 c
Rhodotorula sp.		14.00 bc	76.00 f	35.25 b	114.17 e	7.97 f	15.34 d
B. pumilus		15.00 c	57.17 b	39.15 cd	118.53 f	7.88 e	15.92 d
Epicoccum sp.		14.00 bc	63.00 bc	40.25 cd	102.42 b	7.31 b	13.40 b
Fungicides		17.00 d	73.00 ef	41.65 d	129.30 h	8.37 h	16.54 e

In the same column, means followed by the same letter are not significantly different according to DMRT

* Not calculated.

SUMMARY

This work aimed to search for effective and safe method to control an important phylloplane plant disease, the powdery mildew on cucurbits which are presented in this study by the squash powdery mildew disease caused by *Sphaerotheca fuliginea* (Schelect, ex Fr). Poll.

The most important methods, alternative to chemical ones are biological antagonists agents. So, the present study was designed to isolate microbial biocontrol agents applicable against the cucurbitacious powdery mildews. the steps of this study, and the obtained results are summarized as following:-

1. Samples of healthy and powdery mildew infected squash leaves were collected from different localities of squash plantations in Kafr El-Sheikh and El-Gharbia Governorates. the microbial survey of the squash leaf-surface resulted in distinguishing that the most frequent microbial group is bacteria (64.65%) followed by fungi (30.48%) and then by yeast and actinomycetes (2.44% each).
2. The isolated microorganisms were screened for controlling the powdery mildew disease on squash cotyledons by spraying the 8 days old liquid cultures onto cotyledons of potted plants infected with the pathogen, and the disease severity was assessed after three applications with one week intervals.

The most effective isolates were the filamentous fungal isolate No. 84. identified as *Epicoccum* sp. link , the yeast isolate No. 69

identified as *Rhodotorula* sp. Grinbergs et Yarrow, and the bacterial isolates No. 40, 58, 59, 27 and 83 identified as *Bacillus thuringiensis* Berliner, *Bacillus coagulans* Hammer, *Bacillus chitinosporus* Gordon, Haynos and Pang, *Bacillus subtilis* (Ehrenberg) Cohn, and *Bacillus pumilus* Meyer and Cottheil, respectively.

3. The selected isolates were effective and comparable to the fungicide Topas 100 in inhibition the mycelial growth and sporualation of the pathogen. The isolates of *B. subtilis*, *Derxia sp.*, *B. thuringiensis*, *B. coagulans*, *B. chitinosporus*, *Rhodotorula* Sp., *B. pumilus* and *Epicoccum* sp. inhibited the disease by 98.8, 100, 99, 100, 100, 98.3, 95 and 100 percentages, respectively, while the fungicide inhibited the disease by 92.77.

4. Treating of plants using either of whole liquid culture, filtrate or washed cells of any tested isolate did not significantly differ in disease control efficacy in cases of *B. subtilis* and *B. chitinosporus*. On the other hand, washed cells of *B. pumilus* and *Epicoccum* sp. revealed significant lower efficacy than that of the whole liquid culture or the filtrate alone.

5. The microscopic examination of powdery lesions on treated plants using light and scanning electron microscopes revealed dead hyphal remnants and bare conidiophores without spores.

6. The culture filtrates of the tested isolates significantly inhibited the spore-germination of the pathogen on water agar slides. The spore germination percentages reached 97.19%, 98.51%,

97.59%, 99.07, 99.5, 91.81, 97.26 and 94.17% using the filtrates of *B. subtilis, Derxia* sp., *B. thuringiensis, B. coagulans, B. chitinosporus, Rhodotorula* sp., *B. pumilus* and *Epicoccum* sp., respectively.

7. Under field conditions, the tested isolates proved their abilities to control the powdery mildew disease on both squash and cucumber crops. The achieved disease inhibition efficacy was as better as the application was repeated. The tested isolates sprayed three times with 10 days intervals prevented the disease lesions to appear while some lesions (3 lesions/leaf) appeared in case of the fungicide treatment on squash plants.

8. Control of the powdery mildew diseases under field conditions led to improvement in plant health (plant weight and height, leaf area, leaf number and contents of chlorophyll) and increment in fruit yield which reached to 14 fruits/plant in squash and 18.3 fruits/plant in cucumber using *B. pumilus* and *B. thuringiensis*, respectively.

9. The above mentioned result with no noticed harmful side-effects on the tested plants candidate the tested isolates to be used in biological control of the powdery mildew disease on squash and cucumber.

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ARABIC SUMMARY

الملخص العربى

أجريت هذة الدراسة بغرض إيجاد وسيلة آمنة وفعالة كبديل للمبيدات الكيماوية المستخدمة فى مقاومة أحد الأمراض الهامة للمجموع الخضرى وهو مرض البياض الدقيقى على العائلة القرعية حيث أن من أهم الوسائل الحديثة المستخدمة فى مقاومة الآفات الزراعية هى استخدام الكائنات الدقيقة لهذا الغرض وذلك فيما يعرف بالمقاومة الحيوية. وعليه فقد تم اختيار نباتات قرع الكوسة كممثل لنباتات العائلة القرعية والتى يصيبها الفطر *Sphaerotheca fuliginea* (schlect. ex Fr.) poll مسببا لها مرض البياض الدقيقى. لإجراء هذه الدراسة.

ويمكن تلخيص خطوات الدراسة والنتائج التى تم التوصل إليها فيما يلى:

أولا: جمعت عينات من أوراق نباتات قرع الكوسة السليمة منها والمصابة من مناطق عديدة بمحافظتى كفرالشيخ والغربية بجمهورية مصر العربية ، وتم عزل عديد من الكائنات الدقيقة والتى تقطن السطح الخارجى لهذه الأوراق وتم تنقيتها وحفظها فى مزارع نقية تحمل كل منها رقما كوديا. وقد كان أكثر هذه الميكروبات سيادة فى أطباق العزل تلك التابعة لمجموعة البكتريا الحقيقية والتى وصلت نسبة تكرارها ٦٤.٦٤% من المجموع الكلى من المستعمرات المعزولة ، تليها فى ذلك الميكروبات التابعة لمجموعة الفطريات الهيفية حيث وصلت النسبة المئوية لتكرار ظهورها على أطباق العزل ٣٠,٤٨% ثم تلك الميكروبات التابعة لمجموعتى الخميره والبكتيريات الخيطية (الاكتينوميستات) حيث وصلت نسبة التكرار لكل منها ٢,٤٤%.

ثانيا: تم تنمية هذه العزلات على بيئات سائلة لمدة ٨ أيام واختبرت قدرتها على تقليل نسبة الإصابة بالبياض الدقيقى على الأوراق الفلقية لنباتات قرع الكوسة المنزرعة فى أصص والمعداة بالمسبب المرضى وذلك برش النباتات ثلاث مرات (رشة واحدة كل أسبوع). وكانت أقوى العزلات الفطرية هى العزلة التى تحمل الرقم الكودى ٨٤ وعزلة الخميرة التى تحمل الرقم الكودى ٦٩ والعزلات البكتيرية التى تحمل الأرقام الكودية ٤٠ ، ٥٨ ، ٥٩ ، ٢٧ ، ٨٣ ، ٣٢.

ثالثا: تم تعريف الثمانى عزلات السابقة على النحو التالى:

العزلة الفطرية رقم ٨٤ على أنها	*Epicoccum* sp. Link
عزلة الخميرة رقم ٦٩ على أنها	*Rhodotorulla* sp. Grinberg et Yarrow
العزلة البكتيرية رقم ٤٠ على أنها	*Bacillus thuringiensis* Berliner
العزلة البكتيرية رقم ٥٨ على أنها	*Bacillus coagulans* Hammer

العزلة البكتيرية رقم ٥٩ على أنها *Bacillus chitinosporus* Gordon, Haynes and Pang

العزلة البكتيرية رقم ٢٧ على أنها *Bacillus subtilis* (Ehrenberg) Cohn

العزلة البكتيرية رقم ٨٣ على أنها *Bacillus pumilus* Meyer and Gottheil

العزلة البكتيرية رقم ٣٢ على أنها *Derxia* sp. Jensen, Peterson, De and Bhattacharya

رابعا: تم إعادة اختبار كفاءة هذه العزلات ومقارنتها بكفاءة أحد المبيدات الكيماوية الموصى بها من وزارة الزراعة وهو المبيد "توباس ١٠٠" وذلك فى تجربة ضابطة تم إجرائها فى الصوبة الزجاجية بكلية الزراعة بكفرالشيخ. وقد أظهرت العزلات المختارة قدرتها على إعاقة نمو وتجرثم الفطر المسبب للمرض على الأوراق الفلقية لنباتات العائل وذلك بإجراء الرش بأى من المعاملات وتقدير عدد المستعمرات الفطرية على الأوراق الفلقية بعد ٨ أيام. وقد أظهرت النتائج أن العزلات *B. subtilis*، *Derxia* sp.، *B. thuringiensis*، *B. coagulans*، *B.* ، *B. chitinosporus* sp.، *Rhodotorula* ، *Bacillus pumilus* قد وصلت قدرتها فى منع ظهور المرض إلى النسب التالية: *Epicoccum* sp. ٩٨,٨١% ، ١٠٠% ، ٩٩% ، ١٠٠% ، ١٠٠% ، ٩٨,٣% ، ٩٥,٠% ، ١٠٠%. على الترتيب. فى حين كانت النسب المئوية لكفاءة المبيد فى تقليل الإصابة ٩٢,٧٧%.

خامسا: بفحص سطح الأوراق التى تم معاملتها بالعزلات المذكورة باستخدام الميكروسكوب الضوئى والميكروسكوب الالكترونى الماسح تبين تدهور النمو لهيفات الفطر الممرض وحوامله الكونيدية وما عليها من جراثيم كونيدية.

سادسا: أظهر راشح المزارع السائلة للكائنات المختبرة والتى تم خلطها كل على حده فى بيئة الأجار المائى على شرائح زجاجية قدرتها على تقليل إنبات الجراثيم الكونيدية للفطر المسبب حيث وصلت نسبة التثبيط للعزلات *B. subtilis*، *Derxia* sp.، *B. thuringiensis*، *B. coagulans*، *B. chitinosporus*، *Rhodotorula* sp.، *B. pumilus*، *Epicoccum* sp. ٩٧,١٩% ، ٩٨,٥١% ، ٩٧,٥٩% ، ٩٩,٠٧% ، ٩٩,٥٠% ، ٩١,٨١% ، ٩٧,٢٦% ، ٩٤,١٧% على الترتيب وذلك بمقارنتها بمعاملة المقارنة والتى يغيب فيها وجود الراشح الميكروبى.

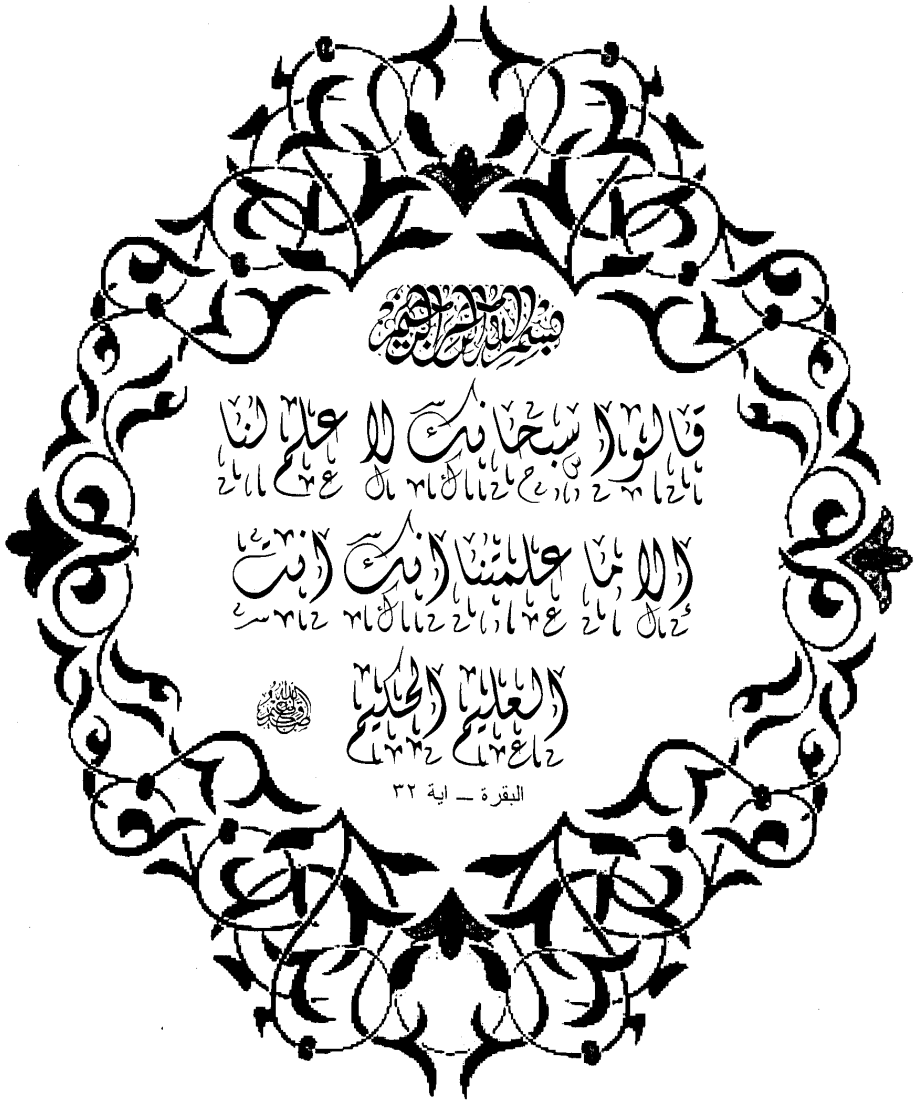
سابعا: أوضحت التجارب أن معاملة النباتات بأى من المزرعة السائلة ككل أو الراشح والخلايا بكل منهما على حده أدى إلى نتائج متشابهة معنويا من حيث الكفاءة فى

مكافحة البياض الدقيقى على نباتات قرع الكوسة وذلك فى حالة العزلة ٢٧ *B. subtilis* ، العزلة ٥٩ *B. chitinosporus* أما فى حالة العزلات ٤٠ *B. thuringiensis* ، والعزلة ٥٨ *B. coagulans* والعزلة ٦٩ *Rhodotorula* sp. ، العزلة ٨٣ *B. pumilus* ، العزلة ٨٤ *Epicoccum* sp. فقد أظهرت المعاملة بالمزرعة السائلة ككل وراشح المزرعة المفصول عنه الخلايا كفاءة أعلى معنويا فى مكافحة المرض عنها فى حالة استخدام الخلايا المفصولة من الراشح.

ثامنا: فى تجارب الحقل المفتوح أظهرت العزلات السابقة كفاءتها فى مكافحة مرض البياض الدقيقى على كلا من محصولى قرع الكوسة والخيار وإزدادت هذه الكفاءة بزيادة عدد مرات معاملة النباتات بهذه العزلات فقد أدى رش النباتات فى الحقل ثلاث مرات بين كل رشة والأخرى ١٠ أيام لأى من العزلات المستخدمة إلى منع ظهور أعراض المرض تماما طوال فترة المعاملة فى نفس الوقت الذى ظهرت فيه بعض مظاهر الإصابة ــ ولو أنها قليلة ــ فى حالة استخدام المبيد الفطرى توباس ١٠٠ (٣ مستعمرات/ورقة من أوراق قرع الكوسة).

تاسعا: أدت مكافحة مرض البياض الدقيقى باستخدام العزلات تحت الدراسة إلى زيادة إنتاجية محصولى قرع الكوسة والخيار تحت الظروف الحقلية حيث وصل المجموع الكلى لعدد الثمار لكل نبات إلى ١٤ ثمرة لكل نبات قرع الكوسة ، ١٨,٣ ثمرة لكل نبات خيار باستخدام العزلة ٨٣ *B. pumilus* والعزلة ٤٠ *B. thuringiensis* على الترتيب بزيادة قدرها ١٠٨% ، ٢٠٥% على التوالى وذلك بالمقارنة بالنباتات الغير معاملة.

عاشرا: ولقد صاحب مكافحة مرض البياض الدقيقى باستخدام العزلات السابقة إلى تحسن معنوى فى الصفات النباتية للنباتات المعاملة مثل الوزن الجاف والرطب وطول النبات ومساحة الورقة وعدد الأوراق للنبات الواحد والمحتوى الكلى للورقة من الصبغات مما يقوى ترشيح استخدام هذه العزلات الواعدة فى مكافحة مرض البياض الدقيقى فى المستقبل وذلك بعد إجراء البحوث التى تسمح بإنتاجها على النطاق التجارى.


بسم الله الرحمن الرحيم
قالوا سبحانك لا علم لنا
إلا ما علمتنا إنك أنت
العليم الحكيم
صدق الله العظيم
البقرة – اية ٣٢

التأثيرات التضادية لبعض ميكروبات المجموع الخضري لنباتات قرع الكوسة تجاه الفطر المعرض

رسالة مقدمة من

سعيد محمد حسن كامل

بكالوريوس العلوم الزراعية والتعاونية ١٩٩٥

دراسات تكميلية بكلية الزراعة بكفرالشيخ ١٩٩٨

للحصول على درجة الماجستير

فى

العلوم الزراعية

الميكروبيولوجياالزراعية

قسم النبات الزراعى

كلية الزراعة بكفرالشيخ

جامعة طنطا

٢٠٠٣