BAOJ Microbiology

Tsapikounis FA, BAOJ Microbio 2015 1:1
1: 001

Research Article

An Integrated Evaluation of Mycoparasites from Organic Culture Soils as Biological Control Agents of Sclerotia of *Sclerotinia Sclerotiorum* in the Laboratory

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Abstract

Sclerotinia sclerotiorum is one of the most harmful phytopathogens in agriculture. They form sclerotia which render them resistant and persistent. Crop protection is necessary and mycoparasites are a friendly and efficient alternative to chemicals, when used as Biological Control Agents (BCAs). In this work, one hundred ninety nine candidate mycoparasites from soils of organic crops in Southwestern Greece were isolated by using the method of trapping. After preliminary evaluation the best eighteen were selected and subjected to further evaluation. The mycoparasites were applied in the form of hyphae and spore suspension and were tested in water agar, sterile and non-sterile soil. Mycoparasites produced good results to the control of sclerotia of *Sclerotinia sclerotiorum*. In particular, the isolate G21-3 (*Gliocladium* sp.) proves to be an exceptional mycoparasite and a competent antagonist as well. Mycoparasites differ in effectiveness not only among them but, moreover, each one of them demonstrates different effectiveness in different environments. Effectiveness decreases significantly from the hyphae in water agar to the impregnation by spore suspension in sterile and non-sterile soil. In non-sterile soil the effectiveness of all the mycoparasites, except for G21-3, is minimal (close to zero). The antagonism of the existing soil microflora is a highly significant factor. The ideal period to isolate aggressive mycoparasites of sclerotia of *Sclerotinia sclerotiorum* from soil paste is 15 days. Preliminary evaluation in water agar followed by a two-week period of incubation proves to be a very good instrument in the preliminary process evaluation of a large number of candidate mycoparasites. The evaluation experiments, presented in this work, constitute an integrated methodology for the evaluation of candidate mycoparasites of sclerotia of *Sclerotinia sclerotiorum* species in the laboratory. They provide an excellent, economic and fast protocol for selecting the appropriate strain as Biological Control Agent (BCA), while minimizing the work and time in the field.

Keywords: Isolation; Evaluation; Mycoparasites; Sclerotia; Biological control; *Sclerotinia*; *Fusarium*; *Gliocladium*; *Trichoderma.*

Introduction

The species *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most harmful phytopathogens of the horticulture in closed and open cultivations [1,2]. It attacks 408 plant species of 278 genera and 75 families [3]. It is considered as a "cosmopolitan" species, but it is mainly found in temperate climate territories [4]. Even though there are no official records of the damages caused by this phytopathogen in Greece, recent preliminary reports have shown that this fungus damages cultivations of green beans, tomatoes, cucumbers, lettuces in greenhouses and cabbages in the open fields with mean crop losses ranging from 5% to 30% [5]. Reports of total crop destruction have been also provided as in a case at Northern Greece (Drama, East Macedonia) where *S. sclerotiorum* completely destroyed a 0.2 hectare crop of celery [5].

The fungus forms black, irregular bodies, the sclerotia, which help its survival in the soil for many years [6]. It has been shown that approximately 90% of their life cycle is in the ground as sclerotia [6]. When climate and other environmental conditions are suitable for germination, *S. sclerotiorum* produces either apothecia or mycelium and damages the crops.

Several approaches have been employed over the years for the crop protection. Among the most popular are crop rotation system, production of resistant varieties, steam disinfestations, organic substance enrichment, plowing, subsurface irrigation, solar heating, and the use of mycoparasites as biological control agents (BCAs) [7-9]. The wide spectrum of hosts and the ability of the sclerotia to survive in the soil for a long time are the major reasons for the failure of successful plant protection based on the crop rotation system or the production of resistant varieties [4,10]. Other applied methods like the maintenance of greater distance within and between the furrows, soil coverage with black plastic in a greenhouse where the disease was widely spread, solar heating, organic substance enrichment, the use of salicylic acid, or the combination of the above mentioned methods as tested in organic greenhouse cultures at Vouprasia (Helia, Southwest Greece) presented promising and encouraging results but still failed to fully control the disease caused by this phytopathogenic fungus [5].

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Sub Date: 5 May, 2015, **Acc Date:** 31 July, 2015, **Pub Date:** 5 August, 2015

Citation: Tsapikounis FA (2015) An Integrated Evaluation of Mycoparasites from Organic Culture Soils as Biological Control Agents of Sclerotia of *Sclerotinia Sclerotiorum* in the Laboratory. BAOJ Microbio 1: 001.

Therefore, repeated sprayings with carbendazim, benomyl, iprodione, vinclozolin, dicloran, dazomet are needed in order to prevent the ascospore contamination, and disinfection with methyl bromide and steam can destroy the sclerotia, providing the solution to which the farmers are often compelled to resort [11]. However, the environmental problems caused by the chemicals used, the withdrawal of methyl bromide, the expenses and the problems concerning the application of steam, are drive forces for the development of alternative strategies of biological control [12,13].

Various mycoparasites including *Coniothyrium minitans* [14-16], *Epicoccum purpurascens* [17], *Fusarium spp., Gliocladium spp.,* [13,18], *Penicillium spp., Pythium oligandrum* [19], *Sporidesmium sclerotivorum* [20], *Teratosperma oligocladum* [21], *Trichoderma spp*., [22-24], *Trichothecium roseum* [25], as well as bacteria, actinomycetes and yeasts [9,26,27] have been tested with good results concerning the control of *S. sclerotiorum* sclerotia. The isolation and evaluation of antagonists so as to find suitable microorganisms for biological control in the field, is always a critical, time consuming and difficult procedure [28,29], which has preoccupied researchers for many decades [30]. Finding the best isolation methods and the appropriate materials could help in optimising the procedures and obtaining the most effective biological control agents [31].

Several methods have been tested and applied for the isolation of sclerotial mycoparasites, like the widely used methods of trapping in the soil in the laboratory for 30 days [18,32], in the field for 15 to 30 days [22,33], or in naturally infected sclerotia [25] and of serial dilutions [34].

For the evaluation of the candidate mycoparasites in the laboratory, a wide range of methodologies has been developed. The most significant are the evaluation at double cultivations in media like Potato Dextrose Agar (PDA), Water Agar (WA) or Malt Extract Agar (MEA) [25,35,36,37], immersion in spore suspension and placement in water agar, in sterile and non-sterile soil, on moist filter paper in Petri dishes [13,25,30,34,37], placement in Petri dishes containing peat, sand or soil, and inoculation with spore suspension or block or solid substrate inocula [30,38] in pasteurized soil in plastic pots, for 20 days on a lab bench, at 22°C, in the dark [22].

Isolation of mycoparasites must be followed by evaluation. Several different protocols have been examined and proposed the last four decades for different mycoparasites like *C. minitans*, *G. virens* and *F. oxysporum* [25,37,39,40]. Until now, these evaluations were partial, conclusive only for the particular cases studied, thus, any of these approaches is unsuitable to be proposed as a general approach. For most effective generalised results, the evaluation in the laboratory should include methodologies which will evaluate the mycoparasites under all the possible circumstances, so that the results of the mycoparasitic and the antagonistic activity of the organisms under study may safely be applied in the field for achieving the highest rates of sclerotia control in the environment. A considerable effort towards this direction was made by [30], using five combinations of inoculum form and substrate type.

This paper aims at the isolation of mycoparasites and at their assessment as far as their ability to destroy the sclerotia of *S. sclerotiorum*. Particular emphasis was given on the methods of evaluation in the laboratory so as to develop a methodology of integrated evaluation, able to present the whole "picture" of each mycoparasite and consequently limit the number of mycoparasites evaluated in the field, which is particularly time consuming and tiresome.

Methods and Materials

Fungal isolates, cultural conditions and identification

All mycoparasites used in this study were isolated by using the method of trapping from samples of soil, which came from organic cultivations in Southwest Greece (Table 1). The isolation of the pathogenic fungus *S. sclerotiorum* comes from plants of cabbage from the region of Brinia, located at the Municipality of Vouprasia in Southwestern Greece. All fungi examined were cultivated in potato dextrose agar (PDA). The identification of the microorganisms, to the level of species for the phytopathogen and to the level of genera for the mycoparasites was performed in our laboratory and confirmed in the laboratory of Mycology of the Department of Ecology and Systematics of the National and Kapodistrian University of Athens (Faculty of Biology).

Experimental conditions and inoculum preparation

All the experiments were made in Petri dishes with water agar (1%), sterile and non-sterile soil, in incubation chamber, in the dark, at 25°C. The soil came from fields of conventional cultivation of melon. It was sterilized in boiling glass of 500 or 600 ml, after the addition of 20 to 30 ml water, for two days in the row and for one hour each time. A plug of 5 mm in diameter from the apex of a developing culture was used as an inoculum for the cultivation of mycaparasites and the experiments in water agar. Spore suspensions of the mycoparasites were performed from 10 to 15 days old cultivations, developed in darkness at 25°C. 10 to 20 ml of deionized sterile water was spilled on the surface of the Petri dish and by using a small paint brush, water and spores were accumulated in a small boiling glass. With the use of a haemocytometer, the condensation was regulated at 10^6 spores per ml. For the approach of "dipping", sclerotia were sunk for 25 to 30 minutes in spore suspension, while the "impregnation" approach was achieved with the addition of 20 ml spore suspension per dish. The sclerotia used in the experiments came from cultures in PDA and they were uniform and of the same age.

Sample collection and baiting method for mycoparasites isolation

A sample was taken from two or three random spots of the cultivation, from a depth of 10-15 cm. Rocks and wood that may be on the surface were removed. Soil of 1.5 - 2 kg was selected. Until the bag was transferred to the laboratory, it stayed open for the soil to be aerated. In the laboratory, the soil was sieved (diameter of 4 mm) in order to acquire thin soil. A small, representative quantity

Table 1: Mycoparasites used in this study, Name Code, Location, Origin and Preliminary evaluation in Water Agar and Double System.

Species	Name code	Location in SW Greece	Origin	WA15[1]	DS[2]
Fusarium sp	F2-1	Tripoli, Anogia	Cruciferae	0	100
Fusarium sp	F22-2	Tripoli, Anogia	Cruciferae	16.6	100
Fusarium sp	F23-1	Tripoli, Anogia	Cruciferae	0	85
Fusarium sp	FD6-2	Kalamata	Tomato	0	100
Fusarium sp	FD6-8α	Kalamata	Bean	0	100
Fusarium sp	FD6-15	Kalamata	Cruciferae	0	100
Gliocladium sp	G20-6	Tripoli	Potato	100	100
Gliocladium sp	G20-7	Tripoli	Potato	100	100
Gliocladium sp	G21-3	Tripoli	Potato	100	100
Trichoderma sp	T3-6	Vouprasia, Brinias	Cabbage	33.2	65
Trichoderma sp	T5	Vouprasia, Almiriki	Cabbage	0	75
Trichoderma sp	T15-1	Midilogli	Cucumber	0	100
Trichoderma sp	T12-7	Vouprasia, Serbani	Tomato	0	100
Trichoderma sp	T12-8	Vouprasia Serbani	Tomato	0	90
Trichoderma sp	T12-9	Vouprasia Serbani	Tomato	100	90
Trichoderma sp	T12-10	Vouprasia Serbani	Tomato	0	80
Trichoderma sp	TD4-1	Gianitsochori	Water melon	100	100
Trichoderma sp	TD4-2	Gianitsochori	Tomato	80	100

[1]sclerotial parasitism percentage (%) after of co-incubation sclerotia and mycoparasites within water agar for 15 days (here the 18 best isolates are shown)

[2]sclerotial parasitism percentage (%) after of co-incubation sclerotia and mycoparasites in double system for one month (here the 18 best isolates are shown)

of soil (100 g) was selected in which deionized water was added to saturation point and it was placed in Petri dishes. Five sclerotia, in the shape of a cross, were planted in every dish. The sclerotia were slightly dipped so as to be covered completely and they were left to be incubated for different time periods up to 30 days. The best results were obtained after a short period of 15 days (the humidity was checked on a daily basis).

Afterwards the sclerotia were removed from the soil (the period was determined after preliminary experiments), rinsed with tap water for 3-5 min and were disinfected in NaOCl and deionized water 1:2. Tween 80 and Triton X-100 (at a concentration of 0.02% in both cases) were added, for 10 - 15 minutes. They were rinsed three times with sterile deionized water, for 5 minutes each time. Then, they were placed for 24 - 48h in Petri dishes and in 100% relative humidity, where their ability to germinate, the presence or the absence of mycoparasites along with the presence or the absence of nematodes or mites was ascertained.

As long as the presence of nematodes or mites was confirmed – by the use of a stereoscope – the Petri dishes with the sclerotia were placed in the drying oven at 80°C for 4h (a period determined after preliminary experiments). Under these circumstances, the mites and the nematodes are killed while the sclerotia and the possible mycoparasites are not affected negatively. The Petri dishes were placed again in 100 % humidity conditions for 24 - 48 h. Consequently, every sclerotium in which a mycelium (of the sclerotinia or the mycopathogen) had appeared was placed in PDA.

Preliminary Process Evaluation

Double system (DS): A sterilized slide was placed inside a Petri dish

at the bottom of which sterile filter paper is placed. A block of the candidate mycoparasite taken from the apex of a developing culture was placed in the middle of the slide. Before placing the block at its final position, it was placed 1 cm away from its final destination and it was dragged there. In this way, a thin layer of nutrient was created. This thin layer allows the hyphae to be developed and thus, reach the sclerotia. The Petri dish was left for incubation and was monitored for the development of the mycelium and the levels of moisture as well.

When the hyphae had grown on the slide up to approximately 5 mm in length, 6 sclerotia were placed at a distance of 2 - 3 mm away from the developing hyphae, in groups of three on either side. Humidity was periodically monitored. A month later the sclerotia were disinfected in NaOCl solution and rinsed with sterile deionized water for three times. Afterwards, they were placed inside water agar to be examined in the stereoscope twice, 7 and 14 days later, respectively.

A fortnightly incubation in water agar (WA15): A block of the mycoparasite was placed in water agar. Before the hyphae covered the entire Petri dish, 5 - 6 sclerotia were placed at the periphery, on the edge of the dishes, 1- 2 mm away from the hyphae. They were left there for a fortnightly incubation and then they were disinfected in NaOCl solution, rinsed with sterile deionized water for three times, and were put once again in water agar (circularly on the circumference) in order to assess their parasitism. The observation in the stereoscope for the assessment of the mycoparasitism was made 7 and 14 days later. As a control, sclerotia were placed in water agar. Three Petri dishes were used for every candidate mycoparasite.

Evaluation of the Mycoparasites in the Laboratory

In water agar along with mycelium hyphae (Evaluation experiment I): A block from the developing culture of a mycoparasite selected was placed in the center of water agar. Before the hyphae covered the entire dish, 10 sclerotia were placed peripherally, on the edge of the dishes, 1 - 2 mm away from the hyphae. They were left there for a thirty-day incubation and they were, thereafter, disinfected in NaOCl solution, rinsed with sterile – deionized water for three times and transferred once again to water agar (circularly on the circumference) in order to assess their parasitism. The observation in the stereoscope for the assessment of the mycoparasitism was made 7 and 14 days later respectively. The sclerotia at the control were placed in water agar. The experiment was repeated three times.

In sterile soil along with spore suspension (Evaluation experiment II): Sterile soil was placed in sterilized Petri dishes. The sterile soil was impregnated with the spore suspension and 10 sclerotia were planted in the soil, circularly on the circumference, 1 - 2 cm away from each other. Thirty days later the sclerotia were removed from the soil, disinfected in NaOCl solution, rinsed with sterile – deionized water and placed in water agar for observation. The sclerotia at the control were placed in sterile soil, impregnated by sterile – deionized water. The experiment was repeated three times.

In Non-Sterile Soil along with Spore Suspension (Evaluation Experiment III): The soil was placed in sterilized Petri dishes without being sterilized. 10 sclerotia were used to impregnate the non-sterile soil circularly on the circumference, 1 - 2 cm away from each other. The duration of the incubation, the reception and the treatment of the sclerotia was the same as above. The sclerotia at the control were placed in non-sterile soil and impregnation by sterile – de-ionized water followed. The experiment was repeated three times.

Evaluation in Pots

Three mycoparasites of different mycoparasitic ability –based on their evaluation so far– were chosen for further assessment in a plant experiment. These are: the best (G21-3), an intermediate (T12-9) compared to G21-3 and an inferior one (FD6-15). Two groups of plants (twenty pots per group) were created. The first group was in cultivated soil and the other in "viorgan", a composted, commercial product. In specific, "viorgan" is a composted product taken from by-products of the plant production, in the presence of earthworms. In each group four treatments were performed: the three mycoparasites and the control, with five pots for each treatment.

Every pot contained approximately 10 L of soil or viorgan. On a work bench, the content of each pot was being impregnated with 500 ml of the phytopathogenic fungus grown in a Petri dish – dissolved with a blender in water. 100 g of bran per pot was added – only in the group of the cultivated soil – watering with, approximately, one liter of water. 7 days later, the spore suspension of the antagonist was applied by watering the plants. The concentration was regulated at 10^7 spores per ml.

7 days later, one seedling of cabbage is placed inside each pot. Watering followed. In a few days a small net was placed in every pot of both groups. The nets contained 5 sclerotia, 1 to 2 cm under the surface of the soil. The phytopathogen, the cabbage plants and the net with the sclerotia were set, also, in the control. The installation of the experiment took place in October and was completed in May. The viability of the sclerotia in the net was ascertained by disinfection and placement in water agar. The experiment was performed with three replicates at the same time.

Kinetics of the parasitism

The yield of the parasitism of the three mycoparasites FD6-15, G21-3 and T12-9 that demonstrates different parasitism effectiveness was studied by using the three evaluation tests mentioned above. Five sclerotia were submerged in spore suspension of the mycoparasites (10^6/ml) for 25 minutes and were transferred in sterile Petri dishes containing water agar (test I). In other Petri dishes containing sterile soil paste (test II) and non-sterile soil paste (test III) impregnation with suspension of the same concentration took place with the addition of five sclerotia in each Petri dish. The following incubation was scaled up in different time periods (5 - 35 days). After each incubation period, the sclerotia, were disinfected and transferred into sterile Petri dishes containing water agar. They were examined with the stereoscope for two weeks on a daily basis.

The parasitism rates, discussed in the Results section, reflect the average of three different experiments.

Statistical analysis

Data analysis for sclerotial infection was made by SPSS 9.0 (SPSS Inc., Chicago, IL.). One-way analysis of variance (ANOVA) was applied and treatment means were separated by the Dunkan multiple range test ($P < 0.05$).

Results

Isolation of mycoparasites and preparatory evaluation

Out of 23 soil samples of organic cultures, 199 mycoparasites were isolated through the baiting method. Every sample gave 8 – 9 mycoparasites in average, while none of the samples gave less than 5 mycoparasites. The most important groups of fungi within mycoparasites were *Gliocladium*, *Trichoderma*, *Fusarium* and *Phycomycetes*. The 199 mycoparasites were submitted to preliminary evaluation (Double System, Water Agar) so as the most suitable to be screened further with (*in vitro* and *in vivo*) evaluation tests (see M&M, Section 2.4). The criteria of selection were the percentage of parasitized sclerotia (i.e., >80% parasitism) and their yield after being disinfected. The ideal period to isolate aggressive mycoparasites of sclerotia of *Sclerotinia sclerotiorum* from soil paste was found to be 15 days (see M&M, section 2.3). 18 isolates gave the best results, 33 gave moderate ones and the rest gave poor results. Among the best mycoparasites, 9 fungi belonged to *Trichoderma* genus, while 6 and 3 strains were placed within the *Fusarium* and *Gliocladium* genus, respectively.

The percentage of parasitized sclerotia was increased for almost all species of mycoparasites, as incubation time increased. They ranged from 0 to 100%, and 65 to 100% for the first (i.e., WA15) and second (i.e., DS), respectively (Table 1). In other words, the mean parasitism for all 18 mycoparasites reached the values of 34.98% and 93.6% for the first and second experimental approach. For the T12-9 mycoparasite only, the percentage of parasitism decreased from one experiment to the other, from 100% to 90%. Five mycoparasites (G20-6, G20-7, G21-3, TD4-1 and T12-9) were 100% efficient even from the first experiment. More than half of mycoparasites showed no parasitism (0%) during the first experiment (WA15), while at the second approach (DS), the parasitism percentages ranged from 75 up to 100%. The remaining mycoparasites showed low percentages in the first experiment and significantly higher efficiency in the second one. These results are in accordance with those obtained from evaluation tests, in which G21-3 and T12-9 proved to be the best mycoparasites. Moreover, it is shown that for preliminary evaluation, WA15 is more sensitive and thus, ideal for identifying the most aggressive isolates in a shorter period.

Ability of mycoparasites to colonize and kill sclerotia of *S. Sclerotiorum* in water agar, sterile soil and non-sterile soil, after 30 days of co-incubation

Results obtained from 3 different evaluation tests are summarized in Table 2. The percentage of sclerotia on which hyphae of mycoparasites were developed is defined as parasitism percentage. Under the lack of antagonism all mycoparasites gave statistically significant good results in comparison to the control, but under conditions of antagonism and within the context of live soil only G21-3 provided good results, as it parasitized and killed more than 90% of the sclerotia (Table 2). Evaluation test I showed that all mycoparasites presented significantly good parasitism percentages (more than 53.3% efficiency). Data variation analysis showed that the isolate factor had a great effect ($F = 3.8$, $df = 17/36$, $P < 0.0001$). All *Gliocladium* isolates (i.e., G20-6, G21-3 and G20-7) and T12-9 from the *Trichoderma* strains presented the best possible results (100%) in colonizing and killing 100% the sclerotia. The majority of the isolates (i.e., T12-10, T3-6, T15-1, TD4-2, F22-2, FD6-2, T12-7, T12-8, F23-1 and FD6-15) from the *Trichoderma* and *Fusarium* genera provided parasitism results, which ranged from 70 to 86.6%, while isolates TD4-1, FD6-8a, T5 and F2-1 gave moderate results (parasitism percentage was 53.3 – 66.6%). All control samples showed viable sclerotia, as *S. sclerotiorum* was grown.

Evaluation test II results are in full agreement with those of evaluation test I. Even though parasitism percentages of almost all mycoparasites were lower, they were proportional to those ones from evaluation test I. Data variation analysis showed that isolation factor had a great effect ($F = 8.69$, $df = 17/36$, $P < 0.0001$). Parasitism percentage of G21-3 mycoparasite was 100% again while at the same time parasitism percentages of G20-6, G20-7 and T12-9 decreased from 100% to 93.3%, 93.3% and 73.3% respectively. The rest of the mycoparasites showed low to moderate effectiveness, giving parasitism percentages from 33.3 to 66.6%.

For evaluation test III, sclerotia infection took place on natural soil and parasitism percentage of all mycoparasites decreased drastically with the exception of G21-3, which gave high parasitism percentage (90%). This strain showed significantly higher efficiency than the respective value of the control, which was 43.3% based on parasitism by microorganisms of the soil. Five mycoparasites with representatives from the genera *Fusarium*, *Gliocladium* and *Trichoderma*, i.e., F22-2, G20-7, TD4-1, TD4-2 and T12-8 presented parasitism percentages 6.6 – 33% which are significantly lower than those of the control, while the rest of the isolates presented zero parasitism percentages. Data variation analysis showed that isolation factor had a great effect ($F = 85.3$, $df = 17/36$, $P < 0.0001$).

From the above-mentioned results, it is obvious that mycoparasites are more effective when sclerotia infection is caused by mycoparasitic hyphae in water agar. When sclerotial infection is taking place in sterile soil by water spore suspension, parasitism percentage decreases significantly (up to 27%). For the majority of the mycoparasites, effectiveness tends to be decreased drastically or even to be eliminated when sclerotia infection is taking place in natural soil. These results display the great influence that soil's endogenous microorganisms have on mycoparasites infective ability. The average effectiveness of 18 mycoparasites, based on

Table 2: Sclerotial parasitism[1] (%), from the evaluation experiments I, II and III.

Mycoparasite[2]	Experiment I	Experiment II	Experiment III
F2-1	$53.3\pm1.45_{a}$[3]	$33.3\pm0.67_{a}$	$0.00\pm0.00_{a}$
F22-2	$73.3\pm0.67_{ab}$	$50.0\pm0.58_{abcd}$	$6.60\pm0.33_{ab}$
F23-1	$70.0\pm0.00_{ab}$	$53.3\pm0.67_{abcde}$	$0.00\pm0.00_{a}$
FD6-2	$73.3\pm0.67_{ab}$	$56.6\pm0.33_{bcde}$	$0.00\pm0.00_{a}$
FD6-8α	$66.6\pm0.88_{ab}$	$43.3\pm0.33_{abc}$	$0.00\pm0.00_{a}$
FD6-15	$80.0\pm0.58_{bc}$	$60.0\pm0.00_{bcde}$	$0.00\pm0.00_{a}$
G20-6	$100\pm0.00_{c}$	$93.3\pm0.67_{f}$	$0.00\pm0.00_{a}$
G20-7	$100\pm0.00_{c}$	$93.3\pm0.67_{f}$	$33.0\pm0.00_{d}$
G21-3	$100\pm0.00_{c}$	$100\pm0.00_{f}$	$90.0\pm0.00_{e}$
T3-6	$70.0\pm1.15_{ab}$	$46.6\pm0.33_{abcd}$	$0.00\pm0.00_{a}$
T5	$63.3\pm0.88_{ab}$	$40.0\pm0.58_{ab}$	$0.00\pm0.00_{a}$
T15-1	$80.0\pm0.00_{bc}$	$63.3\pm0.33_{cde}$	$0.00\pm0.00_{a}$
T12-7	$73.3\pm0.67_{ab}$	$60.0\pm0.58_{bcde}$	$0.00\pm0.00_{a}$
T12-8	$80.0\pm0.58_{bc}$	$66.6\pm0.67_{de}$	$1.66\pm0.67_{c}$
T12-9	$100\pm0.00_{c}$	$73.3\pm0.67_{de}$	$0.00\pm0.00_{a}$
T12-10	$86.6\pm0.88_{bc}$	$60.0\pm0.58_{bcde}$	$0.00\pm0.00_{a}$
TD4-1	$66.6\pm1.20_{ab}$	$46.6\pm1.67_{abcd}$	$10.0\pm0.58_{bc}$
TD4-2	$86.6\pm0.33_{bc}$	$60.0\pm0.00_{bcde}$	$13.3\pm0.30_{bc}$

[1]The percentage of sclerotia, which provided hyphae of the mycoparasite, after disinfection, is defined as parasitism percentage. The values are the average of three independent experiments within the same "Experiment".

[2]Mycoparasites; F: isolates classified to genus *Fusarium*, G: isolates belonging to genus *Gliocladium* and T: the isolates of the *Trichoderma* genus.

[3] $_{a,b,c,d,e,f}$:Within column (experiment) averages followed by the same letter do not differ significantly at $P<0.05$ (Dunkan test).

tests I, II and III was 78%, 60.8% and 8.57% respectively (Table 2). It is worth mentioning that G20-6 and T12-9 mycoparasites, which showed high parasitism percentages on tests I and II, showed as well, zero parasitism percentages on natural soil. On the other hand *Trichoderma* isolate TD4-1 showed low parasitism percentages on tests I and II but statistically important parasitism ability on natural soil (Table 2). Therefore, the effectiveness of a mycoparasite depends on the presence of endogenous antagonists and its efficiency is different to the same mycoparasite on a soil without endogenous microorganisms. From the 18 best isolates studied in this report, only G21-3 mycoparasite (*Gliocladium* sp.) showed high parasitism effectiveness regardless of the presence or not of soil endogenous microorganisms, a fact that makes this isolate an important candidate towards fighting the *S. sclerotiorum* on field.

Mycoparasites ability to colonize and kill sclerotia of *S. Sclerotiorum* when cabbages are planted in the pots

Tables 3 and 4 present the data of mycoparasitism of the *S. sclerotiorum* sclerotia in the pots containing cabbages in natural soil. It seems that the percentages of healthy plants are analogous with the respective ones of sclerotial parasitism. G21-3 mycoparasite, according to previous results, shows the higher sclerotial parasitism percentage and the higher plant protection at the same time. *Trichoderma* isolate T12-9 gave lower sclerotial parasitism and plant protection, while *Fusarium* sp. FD6-15 showed no difference from the control. During control experiments, endogenous mycoparasites destroyed 56% of sclerotia and protected 53% of plants. From Tables 3 and 4 it is obvious that the contribution from endogenous mycoparasites to plant protection was important at the experiments with FD6-15 and complemented the mycoparasitic action of T12-9 on the sclerotia. However, their contribution was minimal at the experiments with G21-3 mycoparasite. As for the experiments with viorgan, no plant loss occurred, while the percentage of destroyed sclerotia caused by added and endogenous mycoparasites ranged low levels (0 – 44%). These results indicate that some factors, in viorgan, prevent the development of mycoparasites as well as the growth of *S. sclerotiorum*. This result may be explained by the presence of polyphenols and tannins.

Parasitism kinetics

Parasitism kinetics of the examined mycoparasites are presented in figures 1, 2 and 3. Parasitism percentages represent the average values coming from 3 different experiments. These results pointed

Table 3: Plant protection ability of the mycoparasites G21-3, F12-9 and FD6-15 against *S. sclerotiorum* in natural soil.

Mycoparasites	Percentage of healthy plants (%)	Percentage of parasitism (%)*	Total percentage of parasitism (%)**
G21-3	93	84	96
T12-9	86	40	80
FD6-15	53	28	56
Control	53	56	56

*Percentage of destroyed sclerotia after the addition of the mycoparasite.

**Percentage of destroyed sclerotia after the addition of the mycoparasite and the indigenous mycoparasites.

Values presented are the average of five independent experiments.

Table 4: Plant protection ability of the mycoparasites G21-3, F12-9 and FD6-15 against *S. sclerotiorum* in 'viorgan'. Values presented are the mean average of five independent experiments.

Mycoparasites	Percentage of healthy plants (%)	Percentage of parasitism (%)*	Total percentage of parasitism (%)**
G21-3	100	0	0
T12-9	100	44	44
FD6-15	100	12	32
Control	100	36	36

*Percentage of destroyed sclerotia after the addition of the mycoparasite.

**Percentage of destroyed sclerotia after the addition of the mycoparasite and the indigenous mycoparasites.

out that *Gliocladium* isolate G21-3 colonized and destroyed sclerotia within 10 to 15 days after super-infection in water agar and sterile soil and within 25 days in non-sterile soil, with no indigenous microorganisms, at the first two tests, as well as with them at the third one. *Trichoderma* T12-9 mycoparasite presented the same speed of parasitism in water agar but lower one in sterile soil (35 days were needed for full parasitism). However, in non-sterile soil, parasitism speed/ability was eliminated. *Fusarium* FD6-15 mycoparasite presented lower enough parasitism speed in contrast to other two isolates, both in water agar as well as in sterile soil, while in non-sterile soil parasitism speed/ability was eliminated. Upon the presence of endogenous soil microorganisms both T12-9 and FD6-15 mycoparasites presented zero parasitism, a fact that indicates low antagonistic ability of those mycoparasites against endogenous microorganisms. The efficiency for every mycoparasites changed significantly when hyphae grown in water agar were soaked by spore suspension in sterile and non-sterile soils. The best results were achieved when mycoparasites were putted in contact with the host hyphae and the experiment was performed in water agar. It is also obvious that even after sterilization, the soil does not lose its controlling ability completely. Results were in complete accordance with those obtained in the evaluation experiments.

Discussion

Using baiting method, we isolated 199 mycoparasites but 18 of them presented the best mycoparasitic abilities as it is demonstrated from evaluation experiments I, II and III. Based on these results, it is also known that *Gliocladium* isolate G12-3 is an excellent mycoparasite and the second best choice is the *Trichoderma* isolate T12-9. When isolate G12-3 contacted with the host, in the form of hyphae in water agar and spore suspension in sterile soil, it destroyed sclerotia within 10 to 15 days respectively, but in antagonistic conditions, in non-sterile soil destroyed 100% of sclerotia within 25 days (Figure 1). Phillips' findings [41] are consistent with the above-mentioned duration of sclerotial degradation process, in sterile soil, caused by green fungus. According to that study, green fructifications and pale yellow droplets appeared on the surface of the sclerotia, after a 5 days inoculation of sclerotia of *S. sclerotiorum* with *G. virens* conidia. 10 days later sclerotia become soft and frequently decomposed.

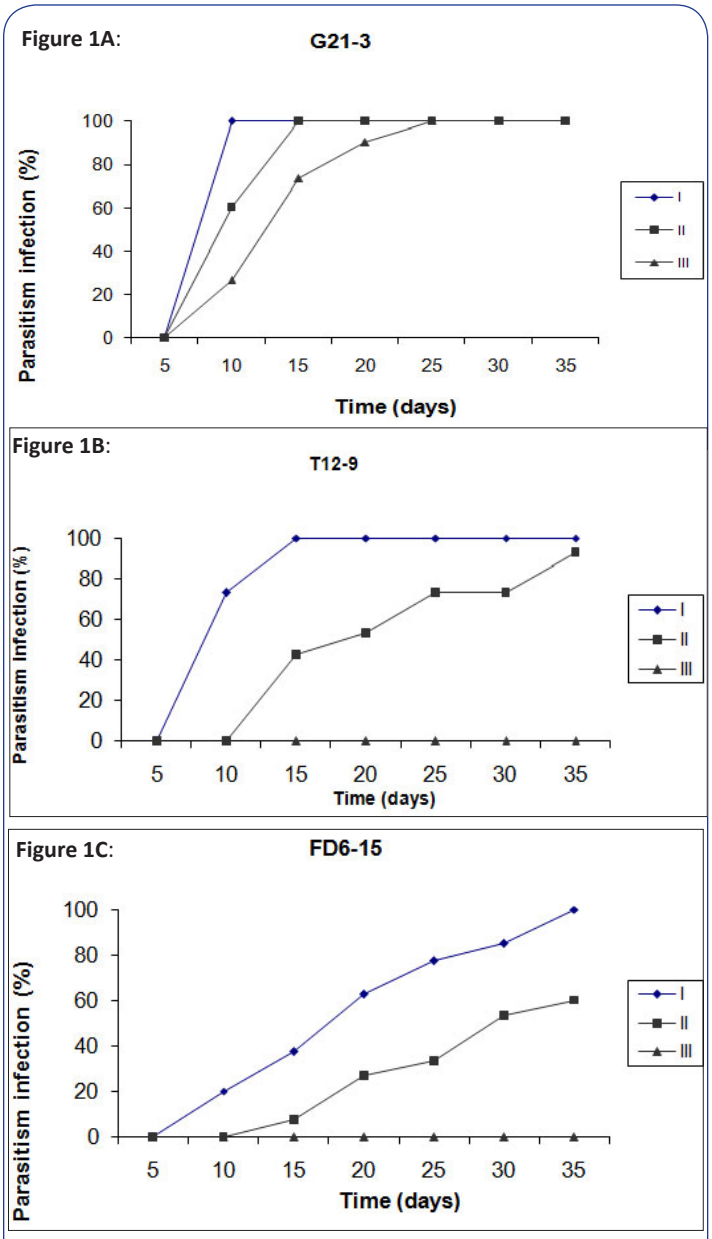

Figure 1: Kinetic of sclerotial parasitism by the mycoparasite G21-3 (A), T12-9 (B) and FD6-15 (C). Three different environments were used, water agar (I), no sterilized soil (II) and sterilized soil (III). The mycoparasite was used as hypha (I) and spore suspension (II & III). Parasitism percentage is defined as the percentage of sclerotia on which hypha of the mycoparasite was grown after disinfection.

Our results showed that the ecologically cultivated soils are rich in mycoparasitic microflora. On one hand every soil sample gave, in average, 8 – 9 mycoparasites, on the other hand none of them gave less than 5. The 18 best mycoparasites, which belong to *Gliocladium*, *Fusarium* and *Trichoderma* genera, provide, for the first time, data of the ability of *Fusarium* and *Gliocladium* species to hyperparasitize on *S. sclerotiorum* sclerotia (in Greek soil). If mycoparasites for other phytopathological fungi had been sought through the same or other methodologies, the total number of candidates from the specific soils may have increased significantly. The lack of pesticides and the use of organic matter are possibly those critical factors that favour mycoparasitic development.

Regarding mycoparasites isolation in sclerotia, several methodologies have been developed but the method of entrapment and isolation from naturally infective sclerotia is the usual approaches. In this work, the candidate mycoparasites were isolated using sclerotia, which were placed in soil paste in Petri dishes in the lab. However, this could be done in larger pots [42] or directly in the field [33] without any difference in efficacy. The only possible differences are the dispended time and the mode of action involved in each technique

This work showed that the best duration for sclerotia to remain in paste is 15 days. The same duration of incubation in paste was chosen by Garza et al. [22]. In literature [22,30,39,41], the staying duration ranged from 14 to 30 days. However, it was shown in this work that if sclerotia remain in paste beyond 15 or 20 days, then the danger of the sclerotia being colonized from non-mycoparasitic fungi increases exponentially. The lack of information in bibliography led us to develop a new protocol: by incubating sclerotia in a drying oven, at 80° C for 4 h, mites and nematodes were killed completely, but sclerotia and mycoparasites were not influenced. Sclerotia withstand higher temperatures while mycoparasites appear to be protected inside the sclerotia cells.

Preliminary evaluation was based on both, double system (DS) and incubation for 15 days in water agar (WA15). The results demonstrate that the WA15 evaluation is a good approach for this preliminary evaluation of many candidate mycoparasites of *S. sclerotiorum* sclerotia. From these results it was observed that the mycoparasites with the best results, during evaluation tests, were those which achieved 100% parasitism of sclerotia at WA15. The first step in an evaluation of candidate mycoparasites must be a quick sorting out of real and non-real mycoparasites. Further analysis need to be performed in order to evaluate in-depth their hyperparasitic capability and to find the best of them.

Mycoparasites evaluation methodology until now (in lab) consists of many alternative ways. The most common approaches are double cultures [43], laying of antagonist as a block at the center of Petri dish and of sclerotia roundly at the edge [33], immersion of sclerotia in spore suspension and laying in water agar [44], peat, soil [34] and sand, in Petri dishes, or laying of sclerotia followed by their saturation with suspension. Moreover, mycoparasites can be developed inside solid materials and then be mixed with soil [13,19], or peat [35,38] before being placed inside the sclerotia. A 25 μl spore suspension of a mycoparasite on top of each sclerotium may be an alternative [40]. Even if evaluation methodology is enriched, rarely more than one method is applied during mycoparasitic evaluation of sclerotia in lab. This leads to less information concerning the mycoparasites' behaviour. Furthermore, it is known that spores react differently from hyphae in experimental conditions [35,38],

while at the same time environment influences as well the growth state influence the results.

Additionally, when the time of evaluation comes, a number of different factors must be addressed. For instance, the way of application of the mycoparasite (through blocks or soak), the incubation time of both sclerotia and mycoparasites, or the experimental environment that influences their effectiveness have to be included in the interpretation of the data. Whipps and Budge (1990) [30] provided some data towards this goal. They found that inoculum form and substrate type had significant effects on the degree of sclerotial infection and viability. In order to provide more answers, in this work two inocula form of mycoparasites and three different environments were used. Mycoparasites under the form of hyphae and spore were evaluated in water agar, sterile, and non-sterile soil.

The results provided a variety in efficiency between 18 mycoparasites on three evaluation tests. The effectiveness declined significantly from hyphae in water agar, in spore suspension, in sterile soil, and finally in spore suspension in non-sterile soil. Our results are in accordance with the respective data of Whipps and Budge (1990) [30]. However, the following questions still remain: are hyphae more effective than spores or is the environment that defines the behaviour, given that incubation time is stable? Why sterile soil diminishes effectiveness, as long as we have greater nutrients' solubility? For the first question, data from this work indicate that hyphae are more effective, while for the second query, it is likely that the presence of metabolites, released by the dead microorganisms, may explain the diminished efficiency. However, more experiments with more combinations are necessary in order fully address these questions.

In water agar and in sterile soil, *Gliocladium* isolate G21-3 parasitised the 100% of sclerotia, indicating thus, that a good mycoparasite must function equally effectively. In a non-sterile soil it is well documented that microorganisms' addition will face competition from indigenous microorganisms, showing therefore an expected decrease of mycoparasitic effectiveness. More than half of the examined mycoparasites (in our experiments) have zero effectiveness, and they are, therefore, inappropriate for field usage as biological control agents. A biological control agent for sclerotia must be a good mycoparasite and a capable antagonist. In order to provide this data, a potential mycoparasite should be evaluated in different environments by applying different types. From our data, the *Gliocladium* isolate G21-3 seems to be consistent to those requirements.

Whipps and Budge (1990) [30] performed their experiments by using spore suspension and maizemeal perlite (1%) into sterile sand and non-sterile soil. In this study, hyphae and spore suspension in water agar, sterile and non-sterile soil. Hyphae were applied as plugs in water agar but not on nutritious substrate as maizemeal. The reason for that approach is that lab experiments must be consistent to the mode of application of mycoparasites in the field. Mycoparasitic application in greenhouses or fields for experimental reasons would require preparation of about ten kg and some hundred kg or more, for commercial purposes. This would be difficult in practice; therefore, methodology must be quick and easy. Packages of one or two Kg, which soil surface or potting mixture are sprayed, are recommended. Under that perspective, similar experimental methods should be developed at a lab scale.

The protective abilities of mycoparasites FD6-15 (*Fusarium spp*), G21-3 (*Gliocladium spp*) and T12-9 (*Trichoderma spp*) were examined on pots with cabbage under field conditions. Our results showed that G21-3 mycoparasite provided high protection (93%) to plants. Significant plant protection (86%) was noticed in T12-9 while at the same time FD6-15 showed zero plant protection. Plant protection percentages were consistent to those of sclerotia parasitism that have been placed in pots. These results are in accordance with those described above indicating that G21-3 might be used as an effective biocontrol agent of *S. sclerotiorum* in field.

Experiments with composted substrate viorgan, did not provide any useful evaluation of plant protection with mycoparasites as controls (in absence of mycoparasites) presented complete plant protection (100%) from phytopathogenic *S. sclerotiorum*. A possible explanation is that 'viorgan' contained antagonists of *S. sclerotiorum* able to protect plants. However, sclerotium culture, placed in pots, showed absence of those antagonists in most cases. Therefore, compost appears to contain some factors (polyphenols and tannins, possibly), which suspended the development of phytopathogenic fungi.

In both groups of plants the presence of new mycoparasites was observed. An impressive element is that, in natural soil, 41 of 100 sclerotia provided new mycoparasites, the majority (13 out of 41) belonging to the *Coniothyrium* genus. The used soil originated from conventional cultivation of melon, where pesticide application was particularly frequent for controlling diseases caused by *S. sclerotiorum* and *Alternaria alternata*. These results are in contrast to the data provided by Tondje et al [45]. According to that work, *Trichoderma* strains, quite ubiquitous in most agricultural soils, were not easily isolated or were below detection levels, in the cacao-agroforestry system. The use of broad-spectrum fungicides, such as Ridomil, suppresses saprophyte's populations of this soil but more detailed studies are needed to confirm the above suggestions.

The application time of antagonist in relation to application time of phytopathogenic agent seems to play some role, but findings are controversial and are likely to be related directly with the microorganism species as well as the conditions [46,47]. In pots, coexistence time of both phytopathogen and mycoparasite (*T. koningii*) before seeding, seems to play no positive role. Mycoparasite's prior establishment shows no positive influence to its effectiveness, while its increased quantity in brans influences rather negatively mycoparasite's effectiveness [38]. Under simultaneous incorporation of those two, mycoparasite's quantity increase and co-existence time with phytopathogen, appears to favour positively

mycoparasites effectiveness, while, when establishing mycoparasite a week earlier, its increase of quantity and co-existence time with phytopathogenic seems to influence positively mycoparasite effectiveness. However, between prior and simultaneous incorporation, there is no statistically significant variation [38]. Moreover, addition of nutritious substrate in experiments with mycoparasites is considered to be important by many researchers although further investigations are needed, namely on application timings and quantities. The influence of nutritious substratum appears to be negative, when it is added in large quantities [35, 38]. A positive influence was caused by the addition of brans in the case of *T. harzianum* [34,48], various isolates of *Trichoderma* and *Gliocladium* [46] and chosen materials concerning the activity of *T. koningii* and *T. harzianum* against *Pythium ultimum* [49]. Except brans, corn flour [13,15] and oats have been used as well [50], with similar results.

In this study, a phytopathogen was established firstly, seven days later a mycoparasite, and after seven days the plants were added. In the group with natural soil 100 g brans were added. Phytopathogen early establishment was made aiming to its successful establishment. The results from the group of cultivated melon soil are in complete accordance to those ones from evaluation experiments, indicating that brans as well as the time of addition of phytopathogen and mycoparasites showed no negative or positive influences on mycoparasites effectiveness. It is well established that for each mycoparasite there are specific organic compounds and in specific quantities which favor its activity [34,35,38,46,48,49]. Therefore, for each new mycoparasite, all requirements should be addressed before using it as biopesticide, in the field.

This work shows that mycoparasitic effectiveness for hyphae in water-agar is different from that of spore suspension impregnation in sterile and non-sterile soils. Hence, mycoparasites that overcome preliminary evaluation must be evaluated in different environments so as their real possibilities can be revealed. Based on these results, G21-3, appears to be an excellent mycoparasite and a capable antagonist as well. In evaluation III the whole idea was "to bring the field into lab", so scaling up to an intermediate phase allowing to select the mycoparasite(s) worth to proceed to field experiments. So, all three experiments (I, II, III) recommend a proposal for a complete evaluation of mycoparasites in lab, as they combine different types of mycoparasite inocula and different experimental environments.

Acknowledgements

The author is greatly thankful to Assist. Prof. Vassili Kouveli (Dept. of Genetics and Biotechnology, National and Kapodistrian University of Athens), Dr. Anastasios Mintzas (University of Patras), Dr. Nikolaos Papadopoulos (University of Thessaly) for their assistance with the manuscript and statistical analysis, Dr. Evangelia Kapsanaki-Gotsi and Dr. Ioanna Pyrri (Dept. of Ecology and Systematics, National and Kapodistrian University of Athens) for their assistance with the identification of fungi.

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