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Specific environment of aromatic plants cultivations and native microorganisms affect the effectiveness of the mycoparasites of the genus *Trichoderma*

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Abstract
The environment has a major impact on both the physiological and the biochemical processes, even ontogenesis is a result of the interaction between the environment and the genotype. Nine mycoparasites of the sclerotia of *Sclerotinia sclerotiorum* were used in order to study the impact of the inoculum form and the environment of the experiment on their effectiveness. Organic cultivations of aromatic and medicinal plants, as well as fruiting vegetables, were used in order to exploit soil effects on mycoparasitism. The form of the inoculum (hyphae, spores-dusting, and spores-suspension) and the environment of the experiment (water agar, sterile soil, non-sterile soil) can have a decisive influence on the behaviour and mycoparasitism efficacy of the mycoparasites. As a result, there is considerable variation among the experiments ranging from 40-50% to even 100%. The antagonism in the soil, due to the microbial population, is proved a significant factor obliging the mycoparasite not only to be effective as a mycoparasite but also effective as an antagonist.

Keywords: Inoculum form, environmental, *Sclerotinia*, *Trichoderma*

1. Introduction
The soilborne diseases cause great damage to financially important cultivations all over the world. In the 1950s and 1960s, it seemed that the pesticides would solve the problem of agricultural production and this fact was faced by the scientific and industrial community with enthusiasm. However, in the next decades, it was discovered that some pesticides and the wasteful use of many others caused serious problems in the environment and the health of the citizens.

The use of resistant varieties and the rotation may provide solutions in some cases, while sun heating and soil covering can adequately control many diseases in the greenhouse. The control of the phytopathogens is often rather difficult, making it necessary to spray the plants repeatedly. But, the environmental problems, the health problems and the expenses and the problems related to the application of steam necessitate the development of alternative control strategies vitally important (Whipps and Budge, 1992; McQuilken and Whipps, 1995; Jones *et al*., 2003) [53, 30, 19]. The use of medicinal and aromatic plants alone or in combinations with tomatoes is a cultivation practice new in Greece, in order to reduce diseases and entomological attacks in cultivations. The results of such experiments will be available in a short time, but as far as they are very encouraging. The use of fungi which have mycoparasitic capacities has turned out to be an attractive and auspicious alternative for the treatment of soilborne diseases. A particularly studied group of mycoparasites is that of the genus *Trichoderma* (Tronsmo and Raa, 1977; Papavizas, 1985; Munoz *et al*., 1995; Aggelaki, 1996; Mondal *et al*., 1996; Mukherjee and Raghu, 1997; Kulling-Gradinger *et al*., 2002; Szekeres *et al*., 2004; Thorton, 2005) [46, 36, 35, 1, 33, 34, 24, 44, 45], which has been also studied extensively in order to be used as biocontrol agents against of sclerotia of *Sclerotinia sclerotiorum* (Santos and Dhingra, 1982; Zazzerini and Tosi 1985; Singh, 1991; Vozenilkova *et al*., 1991; Inbar *et al*., 1996; Gracia-Garza *et al*., 1997; Menendez and Godeas 1998; Aggelaki 2001; Escande *et al*. 2002) [40, 54, 43, 51, 22, 12, 32, 2].

The ability of a mycoparasite to control a phytopathogenic fungus will be shown through one or more experiments in the laboratory, while its effectiveness of treating a disease will be ascertained by means of many experiments in the fields. The effectiveness of an isolation is a function of its mycoparasitic and competitive efficacy depending on characteristics such as the fast germination, growth and production of enzymes e.g. chitinases, glucanases,

and proteases. However, in practice the effectiveness is affected by a number of external factors like the temperature, the humidity, the O_2, the CO_2, the presence of nutrients, the existence of organic matter and the presence of microbes that infect and parasitize the mycoparasite.

In the case of the soilborne phytopathogenic *Sclerotinia sclerotiorum*, which produces sclerotia in order to reproduce and cause the primary infections, one more series of factors affect the sclerotial parasitism either in the laboratory or the field: the origin of sclerotia (agar, sterile field carrots), (Trutmann *et al*., 1980; Turner and Tribe 1976) [47, 49], and the manner of application of mycoparasitism (as spore coating, in solid substrates and impregnation by spore suspension) (McQuilken and Whipps, 1995; McQuilken *et al*., 1997; Aggelaki, 2001) [30, 31, 2]. Furthermore, experiments conducted in the fields have indicated that both the inoculum rate and source appear to play an important role in the control of the disease (Jones and Whipps, 2002) [18].

The isolation of the mycoparasites is followed by the evaluation in the laboratory, in double cultures (Huang and Kokko, 1993; Aggelaki, 1996; Li *et al*., 2006; Rodriguez *et al*., 2006) [14, 2, 39], by immersion in spore suspension and placement in water agar, in sterile and non-sterile soil, in soaked paper filters on Petri dishes (Phillips, 1986; Whipps and Budge, 1990; Singh, 1991; Huang and Kokko, 1993; Budge *et al*., 2005; Rodriguez *et al*., 2006) [37, 52, 43, 14, 39], by placement on Petri dishes with peat or sand or soil and inoculation with spore suspension, or block or solid substrate inocula (Whipps and Budge, 1990; Aggelaki, 2001) [52, 2]. During the evaluation usually only one methodology is used while there are few data in the bibliography regarding how the inoculum type and the environment, in which the experiment is conducted, can influence the efficacy of the mycoparasite.

The first reference to the influence by the inoculum form was made by Sharma & Singh (1990) [42] and Singh (1991) [43]. At the same time, Whipps and Budge (1990) [52], have shown that the inoculum form (spore suspension and maizemeal-perlite) of *Gliocladium virens* and *Coniothyrium minitans* as well as the substrate type (sterile sand or non-sterile soil) had significant effects on the degree of sclerotial infection and viability of *Sclerotinia sclerotiorum*. A further step was made by Aggelaki (2001) [2], who placed disinfected sclerotia on a glass Petri dish with sterile peat and inoculated them with *T. koningii* developed in bran, as spore suspension (10^6 ml^{-1}) and with hyphae from a three-day culture in PDA. Finally, Tsapikounis (2007) [48], working on mycoparasites evaluation ascertained that them demonstrates different effectiveness in different environments.

The existing findings suggest that the inoculum form and the environment of experiment affect the mycoparasitic ability of the mycoparasites and the parasitism development of the sclerotia of the phytopathogenic Ascomycete *Sclerotinia sclerotiorum.* Nevertheless, it has not yet been established a connection between the two previously mentioned factors and the effectiveness of the mycoparasites. The aim of this research is to study the influence of the inoculum form and the specific environment of experiment (aromatic and medicinal plants cultivations) on the mycoparasitic ability of mycoparasites of the genus *Trichoderma* and the consequent parasitism development on the sclerotia of the phytopathogenic Ascomycete *Sclerotinia sclerotiorum.*

2. Materials and Methods

2.1 Organisms, development conditions and identification

The mycoparasites were isolated from soil samples coming from organic cultures in the southwest Greece with the method of entrapping and belong to the genus *Trichoderma* (Table 1). These cultivations consisted of MAPS (medicinal and aromatic plants) such as basil (*Ocimum basilicum* L.), oregano (*Origanum vulgare hirtum* L.) and Greek mountain tea (*Sideritis scardica* L.). Briefly, the soil was sifted and a small representative quantity was selected in which de-ionized water was added to saturation point. Afterwards, this pulp was placed in Petri dishes. Five sclerotia were placed in every dish in the shape of a cross slightly dipped so as to be covered completely and they were left for incubation. After 15 days the sclerotia were removed, rinsed with tap water and disinfected in NaOCl. Then, they were placed for 24 to 48 hours 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 assessed. As long as the presence of nematodes or mites was confirmed the Petri dishes with the sclerotia are placed in the drying oven at 80º C for 4 hours. Finally, where mycelium of mycoparasite had appeared a small part of hyphae was taken into new nutrient substratum. *Sclerotinia sclerotiorum* originates from infected tomato plants in the region of Vouprasia, Greece. The identification of the microorganisms took place in our laboratory, on the level of species for the phytopathogen and on the level of genera for the mycoparasites. The identification was verified at the laboratory of systematic of the University of Athens (Department of Biology). All the fungi were cultured in (%) Bactopeptone 0.2, Yeast Extract 0.2, Maizemeal 0.25, Bran 0.25, Dextrose 1 and agar 1. The sclerotia used in the experiments originate from cultures in the same material. The cultures of the mycoparasites and the phytopathogen as well as the experiments were carried out in an incubatory chamber at 25º C in the dark.

2.2 Preparation of inoculum

All the experiments were conducted in petri dishes with water agar (WA) 1%, sterile soil or non-sterile soil. The block with the mycoparasite is taken from the edge of a developing culture. The suspension of mycoparasite spores is taken from mycoparasites which have been cultured for 10-15 days at 25º C in the dark. With the aid of a haemacytometer, the concentration regulated at 10^6 ml^{-1} spores. In the immersion, the sclerotia were placed for 25-30 minutes in spore suspension whereas during impregnation 20 ml of suspension were dispensed in each Petri dish.

2.3 Interaction of mycoparasites and sclerotia

In water agar with hyphae (I)

A block from the mycoparasite was placed in WA. Before the hyphae have covered the whole Petri dish, the sclerotia were placed peripherally on the edge of the Petri dishes, about 1-2 mm away from the hyphae and left for incubation. Five sclerotia were taken every five days which were then disinfected in NaOCl, rinsed with sterile deionized water and placed again in wa so as to evaluate their parasitism. This was followed by observation under the stereoscope at 7, 10 and 14 days later. 40 sclerotia were used and the experiment was performed three times.

In water agar with dusting (II)
The sclerotia were placed in mature cultures of mycoparasites and stirred gently for a few seconds. Afterwards, they were carried in clean Petri dishes and stirred again for a few seconds so as to remove the superfluous spores. After the dusting, the sclerotia were placed in wa and left for incubation. The rest actions were the same with those in experiment (I).

In water agar with immersion in spore suspension (III)
The sclerotia were submersed in the spore suspension of the mycoparasite for 25 minutes. Then, the attempts to drain the surplus water were followed by their placement in wa, peripherally on the edge of the Petri dishes. They were left to incubate at 25° C in the dark. The rest actions were the same with those in experiment (I).

In sterile soil and impregnation by spore suspension (IV)
The soil originated from conventional cultivations of both oregano and tomatoes and it had only traces of organic matter. It was sterilized in beakers of 500 or 600 ml, after adding a few ml of sterile deionised water, for two consecutive days (each time for one hour). Afterwards, the soil was placed on Petri dishes together with the sclerotia so that the latter are completely covered with soil. Each Petri dish was soaked with the spore suspension of the mycoparasite (10^6 ml^{-1}) and placed in order to incubate. The rest actions were the same with those in experiment (I).

In non-sterile soil and impregnation by spore suspension (V)
The process is the same with that in experiment from conventional cultivations of both oregano and tomatoes (IV). The only difference was that the soil here is non-sterile. The procedure in experiment (I) was also followed.

2.4 Parasitism Percentage in the Final Sample (PPFS)
It is the percentage of sclerotia which have been parasitised in the final sample for each mycoparasite in every experiment. This percentage, for each mycoparasite and every experiment, has been put up in table 2 in order to demonstrate the differences among the experiments.

2.5 Accumulative Parasitism Percentage (APP)
It is the total percentage of sclerotia parasitised by each mycoparasite in all the samples for every experiment. Each sclerotium considered as a percentage of the total sclerotia number and in every sampling the parasitism percentage was added to this of the last sampling. Forty sclerotia were used in each experiment and the estimation of APP was made as follows. If 40 sclerotia constitute 100%, every sclerotium constitutes 2.5%. Therefore, if one sclerotium is parasitised in the first sample, the parasitism rate will be 2.5%. In the next sample, two sclerotia are parasitised and the parasitized rate is 5% but the APP is 5+2.5 = 7.5%. The APP in each sample is the result of the parasitism percentage of this sample adding the parasitism percentage of the previous sample. This accumulative parasitism percentage, for each mycoparasite and every experiment, has been put up in table 3 in order to make clearer the distinctions among the various experiments.

2.6 Statistical Analysis
Data analysis for sclerotial infection was made by SPSS 18.0 (SPSS Inc., Chicago, IL.). One-way analysis (ANOVA) was applied and treatment means were compared by Dunkan test at $P < 0.05$.

3. Results

3.1 The inoculum form, the environment and the effectiveness
The differences in efficacy among the mycoparasites, in all the experiments, are important and range from 40-50% up to even 100%. The evolution of the parasitism and the influence of the inoculum form (block-hyphae, dusting-spores, suspension-spores) and the environment (water agar, sterile soil and non-sterile soil) on this evolution are depicted in Figure 1. The inoculum form and the environment have a decisive influence on the mycoparasite efficacy. The greater efficacy is achieved using the form block-hyphae and dusting-spores in water agar (I & II) and it is decreased using spore suspension in water agar (III). In addition, the efficacy is reduced a lot more using mycoparasites with impregnation in sterile soil (IV) and is finally reduced to a minimum using them with impregnation in non-sterile soil (V). All the mycoparasites destroy the sclerotia completely over a forty-day period (for as long as the experiments last). The mycoparasites T12-9 (Figure 1e, Experiment I) and TD4-2 (Figure 1i, Experiment II) were proved to be the most efficacious, as they destroy the sclerotia within 25 days. Mycoparasites T12-10 and T15-1 destroy the sclerotia within 30 days (Figure 1f & 1g, Experiment I and II).
Appling mycoparasites with impregnation in non-sterile soil (V), there was a dramatic reduction in the efficacy. This change and its consequences can be attributed to the antagonism between the isolations and the existent microbiological flora, elevating the antagonism in the soil as a very significant factor. The efficacy was reduced to nil for six mycoparasites among them T12-9 and TD4-2 (Figure 1e & 1i). Only three mycoparasites, T12-8, TD4-1 and TD4-2 destroy 20% of the sclerotia on the 40^{th} day of the experiment. When mycoparasites applied as spore-suspension in water agar and impregnation in sterile soil, efficacy is substantially reduced in comparison to the form block-hyphae and dusting-spores in water agar and ranges at about the same level among the mycoparasites slightly higher in the first case.

3.2 Parasitism Percentage in the Final Sample (PPFS)
The percentage of sclerotial parasitism in the final sample (PPFS), for each mycoparasite in all experiments, can be seen in Table 2. The fluctuation in the mycoparasite efficacy among experiments is intense and can range up to 100%. The greatest efficacy for all mycoparasites is achieved as block-hyphae in water agar and dusting-spores in water agar as well, and the least one with impregnation in non-sterile soil. In the last case, the parasitism percentage of six mycoparasites was zero, while for all the rest mycoparasites it is very low. This is obviously due to the intense competition between the mycoparasites and the indigenous microbes. When the fungi applied as spore-suspension in water agar (III) the efficacy is lightly better than applied with impregnation in sterile soil (IV).

3.3 Accumulative Parasitism Percentage (APP)
The accumulative parasitism percentage (APP) of the sclerotia, for each mycoparasite and all the experiments, is

depicted in Table 3. In APP, we can observe large fluctuations in the mycoparasite efficacy, though smaller than those in PPFS, which reach up to 73.75%. The greatest efficacy is achieved when fungi applied as dusting-spores in water agar (II), in which six out of nine mycoparasites obtain the highest parasitism rates. When them applied as block-hyphae in water agar 3 out of nine achieve the highest parasitism rates. The lowest rates are observed when applied with impregnation in non-sterile soil in which five mycoparasites have zero parasitism rates, two mycoparasites parasitize 2.5% and a third parasitizes 7.5% of the sclerotia. This can be again ascribed to the intense antagonism between the mycoparasites and the indigenous bacteria. When mycoparasites applied as spore-suspension in water agar and with impregnation in sterile soil we see that the efficacy is higher in the first case.

3.4 The Accumulative Parasitism Percentage (APP) as graphs

The accumulative parasitism percentage (APP) of the sclerotia, for each mycoparasite and all the experiments, is presented in the form of graphs as it can be seen in Figure II. The flattening and normalization of the curves is impressive offering a simplified picture of the parasitism development in sclerotia, and by extension of the parasite behaviour. Nonetheless, the graphs in Figure 1 are more realistic. All the mycoparasites produce good results statistically significant with regard to the control. The mycoparasites T12-9 and TD4-2 were proved to be the most efficacious destroying 77.5% and 81.25% respectively of the sclerotia (Figure 2e & 2i, Experiment I & II). For the rest mycoparasites the effectiveness ranges from 38.75% to 75%.

Table 1: Mycoparasites* used in this study, Name Code, Location and Origin.

Species	Name code	Location in SW Greece	Origin
Trichoderma sp	T3-6	Vouprasia, mprinias	Oregano and tomato
Trichoderma sp	T5	Vouprasia, almiriki	Oregano
Trichoderma sp	T15-1	Midilogli	Basil and cucumber
Trichoderma sp	T12-7	Vouprasia, Serbani	Oregano
Trichoderma sp	T12-8	Vouprasia Serbani	Basil
Trichoderma sp	T12-9	Vouprasia Serbani	Basil
Trichoderma sp	T12-10	Vouprasia Serbani	Basil and tomato
Trichoderma sp	TD4-1	Gianitsixori	Mountain tea
Trichoderma sp	TD4-2	Gianitsixori	Oregano and tomato

* Mycoparasites isolated from organic culture soils in SouthWest Greece by the trapping method.

Table 2: Parasitism Percentage[1] (%) of the sclerotia in the Final Sample (PPFS), by the nine mycoparasites at five experiments.

Mycoparasite	**I** wa–hyphae	**II** wa–dusting	**III** wa-suspension	**IV** sterile soil	**V** nonsterile soil
T3-6	100	100	40_{a}	40_{a}	0_{a}
T5	100	100	40_{a}	30_{a}	0_{a}
T12-7	100	100	30_{a}	60_{bc}	0_{a}
T12-8	100	100	70_{bc}	52.5_{b}	22.5_{bc}
T12-9	100	100	77.5_{c}	67.5_{c}	0_{a}
T12-10	100	100	70_{bc}	62.5_{bc}	0_{a}
T15-1	100	100	60_{b}	40_{a}	0_{a}
TD4-1	100	100	42.5_{a}	40_{a}	20_{b}
TD4-2	100	100	60_{b}	60_{bc}	27.5_{c}

[1] As parasitism percentage defined the percentage of sclerotia gave hyphae of the mycoparasite after disinfection. The values consist the mean of three independent experiments.

$_{a, b, c, d, e, f}$: means followed by the same letter do not differ significantly at P=0.05 (Dunkan test).

Table 3: Accumulative Parasitism[1] Percentage (%) of the sclerotia (APP), by the nine mycoparasites at five experiments.

Mycoparasite	**I** wa–hyphae	**II** wa–dusting	**III** wa-suspension	**IV** sterile soil	**V** nonsterile soil
T3-6	53.75_{b}	48.75_{a}	15_{ab}	15.75_{cd}	2.5_{ab}
T5	45_{a}	48.75_{a}	15_{ab}	5_{a}	0_{a}
T12-7	38.75_{a}	61.25_{b}	8.75_{a}	20_{d}	0_{a}
T12-8	43.75_{a}	56.25_{ab}	33.75_{de}	12.5_{bc}	5_{b}
T12-9	77.5_{d}	57.5_{ab}	38.75_{e}	10_{ab}	0_{a}
T12-10	63.75_{c}	55_{ab}	38.75_{e}	17.5_{cd}	0_{a}
T15-1	65_{c}	75_{c}	18.75_{bc}	12.5_{bc}	0_{a}
TD4-1	58.75_{bc}	62.5_{b}	20_{bc}	20_{d}	2.5_{ab}
TD4-2	61.25_{c}	81.25_{c}	27.5_{cd}	18.75_{d}	7.5_{c}

[1] As parasitism percentage defined the percentage of sclerotia gave hyphae of the mycoparasite after disinfection. The values consist the mean of three independent experiments.

$_{a, b, c, d, e, f}$: means followed by the same letter do not differ significantly at P=0.05 (Dunkan test).

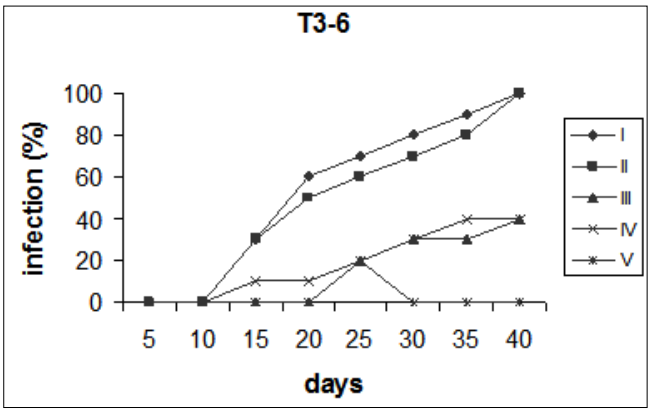


Fig 1a

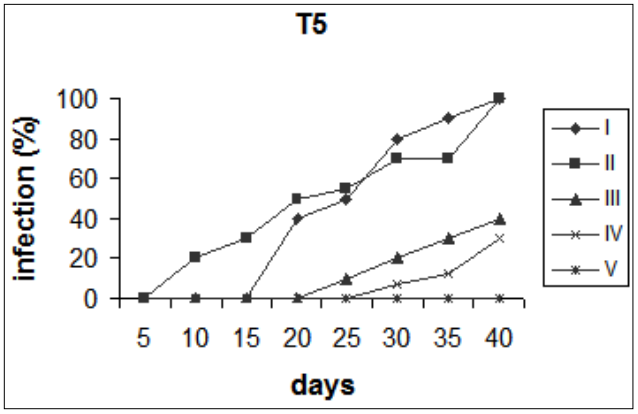


Fig 1b

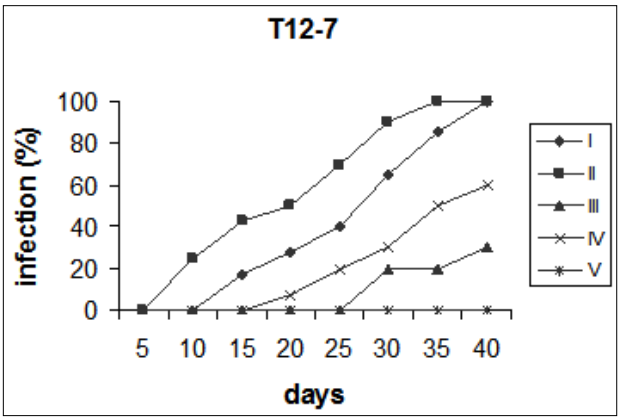


Fig 1c

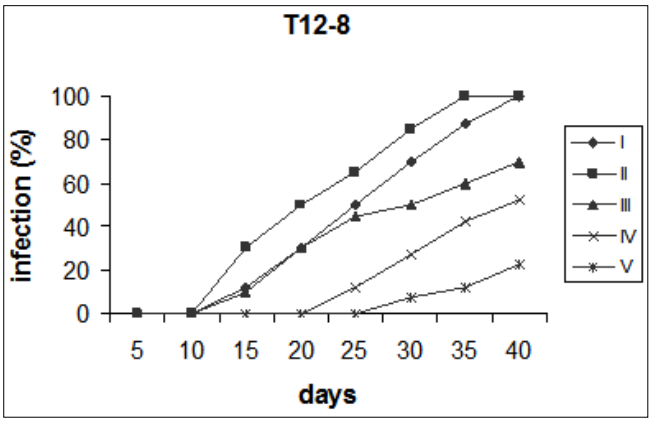


Fig 1d

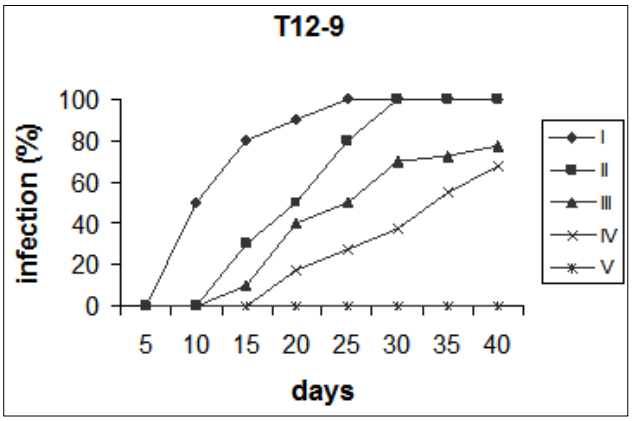


Fig 1e

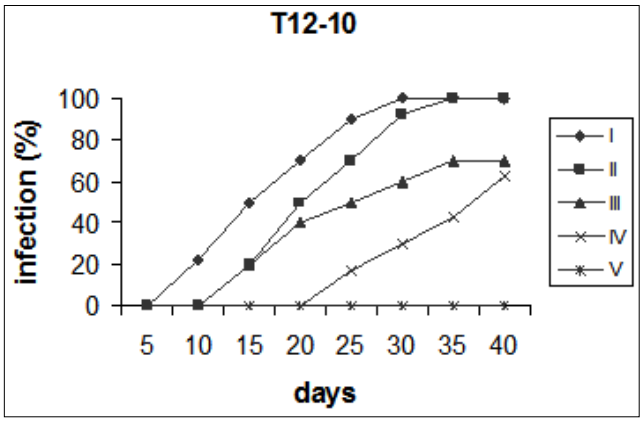


Fig 1f

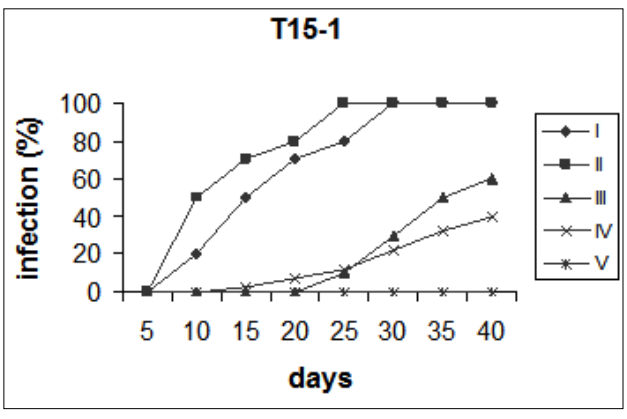


Fig 1g

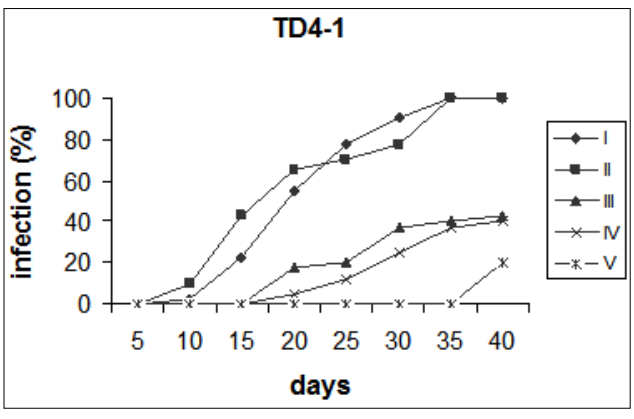


Fig 1h

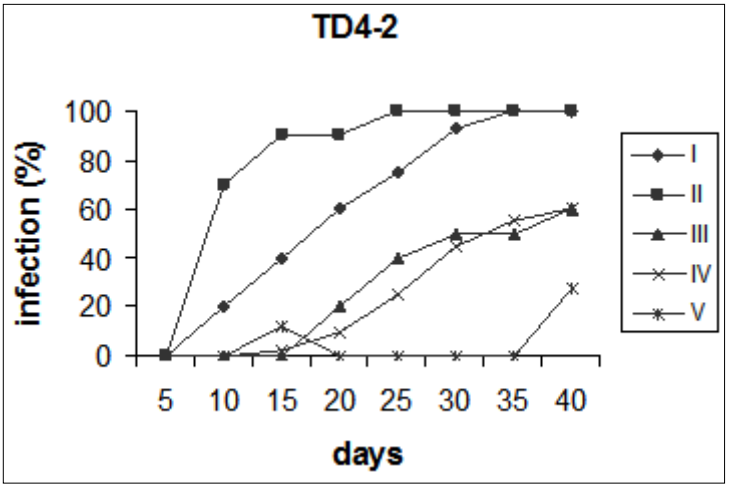


Fig 1i

Fig 1(a-i): The effectiveness of nine mycoparasites, belonging to genus *Trichoderma*, on sclerotial infection under three different inoculum source [hyphae (I), spore-dusting (II) and spore-suspension (III-IV-V)] and three different environments (water agar (I), no sterilized soil (IV) and sterilized soil (V). Each experiment repeated three times and each time forty sclerotia inoculated by a mycoparasite mycelial mat from a new growing colony (I), or sporial dusting (II), or spore suspension by immersion (III) or impregnation within sterile/nonsterile soil (IV-V). Every five days a group of five sclerotia was taken and disinfected. As parasitism percentage defined the percentage of sclerotia gave hyphae of the mycoparasite after disinfection

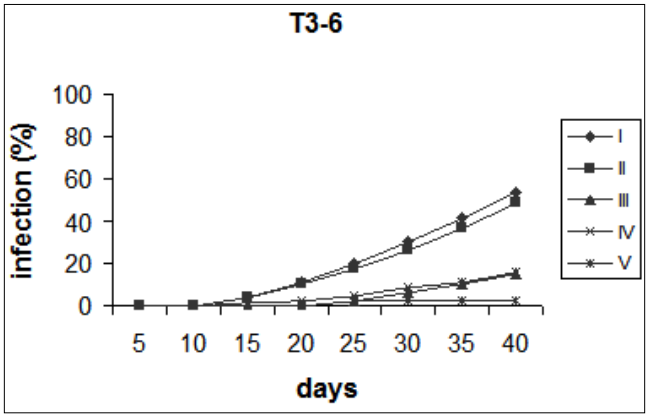


Fig 2a

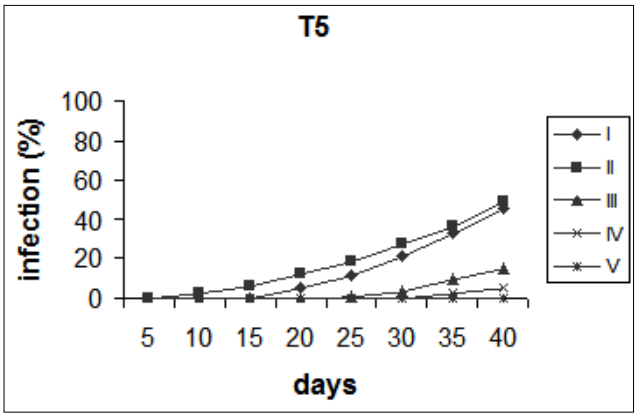
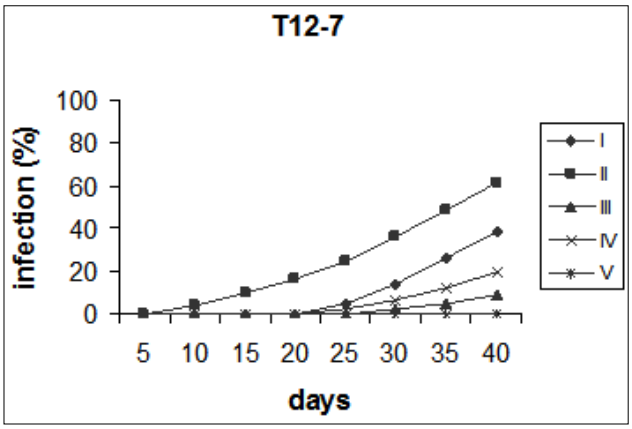


Fig 2b



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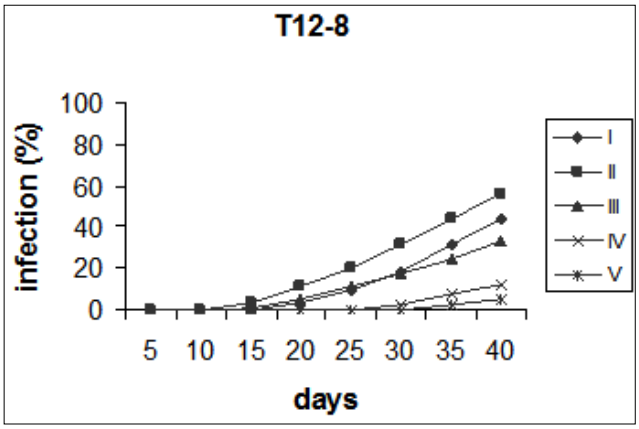
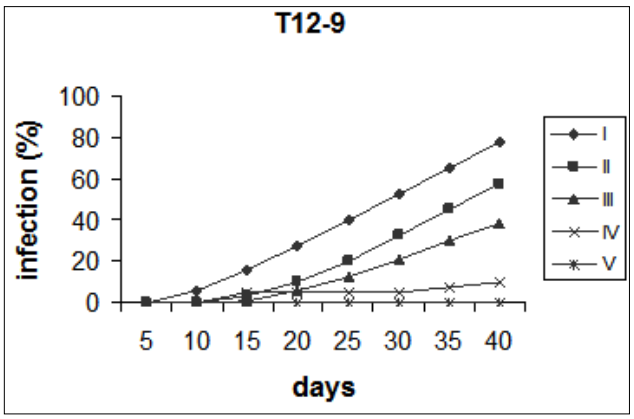


Fig 2d



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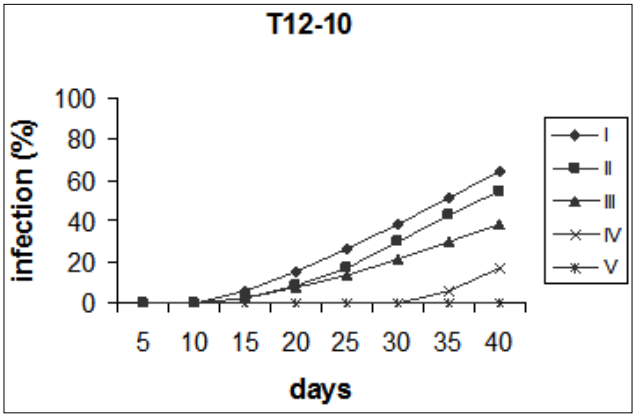

Fig 2f

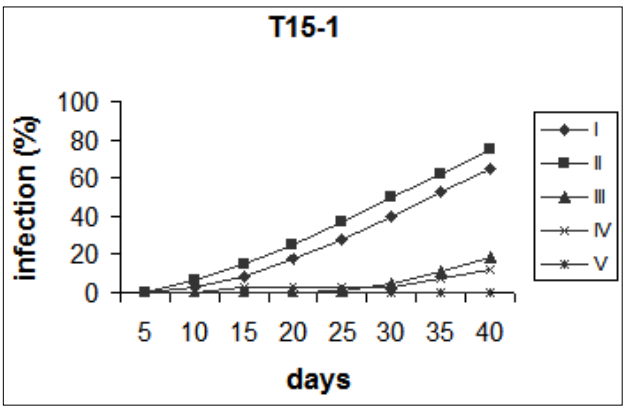

Fig 2g

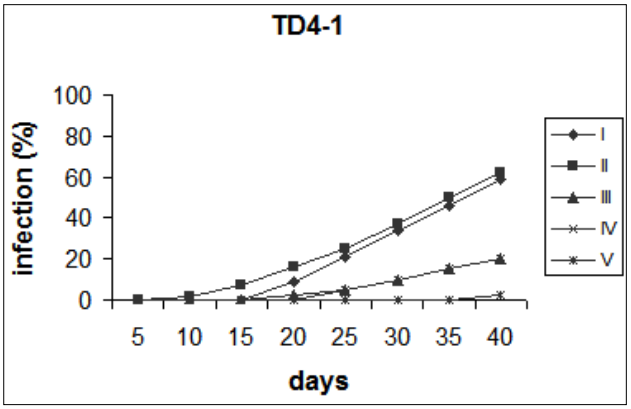

Fig 2h

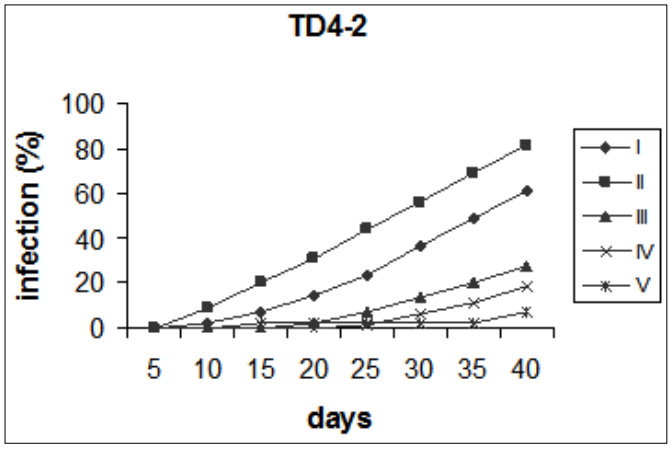

Fig 2i

Fig 2(a-i). The effectiveness of nine mycoparasites, belonging to genus *Trichoderma*, on sclerotial infection under three different inoculum source [hyphae (I), spore-dusting (II) and spore-suspension (III-IV-V)] and three different environments (water agar (I), no sterilized soil (IV) and sterilized soil (V) expressed as Accumulative Parasitism Percentage (APP). Each experiment repeated three times and each time forty sclerotia inoculated by a mycoparasite mycelial mat from a new growing colony (I), or sporial dusting (II), or spore suspension by immersion (III) or impregnation within sterile/nonsterile soil (IV-V). Every five days a group of five sclerotia was taken and disinfectived. As parasitism percentage defined the percentage of sclerotia gave hyphae of the mycoparasite after disinfection. Each sclerotium is considered as a percentage of the total sclerotia number and in every regular sampling the parasitism percentage was added to this of the last sampling

4. Discussion

The inoculum form and the environment of the experiment have a decisive influence on the efficacy of the mycoparasites. These conclusions are completely in agreement with the findings of Whipps and Budge (1990) [52]. According to them, the inoculum form of *Gliocladium virens* and *Coniothyrium minitans* and the substrate type had significant effects on the degree of sclerotial infection and viability of *Sclerotinia sclerotiorum.* In their experiments, they used two inoculum forms and two environments. They left the sclerotia to incubate for 4 weeks and disinfected them altogether. Tsapikounis (2007) [48], evaluating mycoparasites, used two inoculum forms and two environments and incubated them with sclerotia for 30 days as well. He found that, each one of them demonstrates different effectiveness in different environments. The use of

not only a different technique but also a different inoculum rate and source often leads to different results making it difficult to establish causes. The greatest efficacy is achieved by applying the mycoparasites either with dusting or as hyphae in water agar. When the experiment is conducted with submersion of the sclerotia in spore suspension and placement in water agar, the efficacy is reduced significantly. The placement of sclerotia in sterile soil and the impregnation with spore suspension entails a further decrease of the efficacy. Lastly, the placement in non-sterile soil and impregnation with spore suspension leads to the reduction of efficacy to zero. These results are completely in agreement with Tsapikounis (2007) [48]. It appears that the greatest efficiency is generally promoted by the form spore-dusting and block-hyphae, but it is eventually accepted that the best inoculum form is depends on the isolation and consequently needs investigation. Among the: hyphae/water agar (I), dusting/water agar (II) and immersion/water agar (III) approaches, the only difference probably consists in the number of hyphae approaching the sclerotium, given that the humidity, temperature and incubation time are the same. This number is likely to be smaller in system III due to the smaller number of spores adhering, as likely as not, to the sclerotia.

In the greenhouse and the field, the mycelial preparation produces better results in comparison with the spore preparation (Singh, 1991) [43], while the mycelial preparation is more efficacious than the spore one for *T. harzianum* is mentioned by Sharma and Singh (1990) [42]. In other experiments between the maizemeal-perlite and spore suspension, the best results are produced with the former indicating that the inoculum rate and source (the dominant is that of hyphae) seem to play an important role in the control of the disease (Jones and Whipps, 2002; Jones *et al*, 2003; Jones *et al*, 2004a) [18, 19, 20]. Regarding the inoculum type, more progress appears to have been made in the field experiments (McQuilken & Whipps, 1995; Jones *et al*, 2004a; Jones *et al*, 2004b; McLean *et al*, 2005) [30, 20, 21, 29].

The efficacy in system sterile soil/impregnation (IV) is further decreased and lacks sorely compared to hyphae/water agar (I) and dusting/water agar (II). The mycoparasites are used as spore suspension with impregnation and the environment is sterile soil. The concentration of spores is fixed and the factor that changes is the environment. Here, the variations can be attributed both to the different way of application and the origin of soil: different pH, different microbial community, metabolites produced by the microbes, fauna and allelopathy substances etc. All the above entail variations in behaviour, as a result of not only existing substances but also substances released during the sterilisation in which microbes and organisms are killed.

The results of Rodriguez *et al*. (2006) [39], verify our findings. Forty sclerotia were submerged in a suspension of *F. oxysporum* (S6) spores for 5 minutes. Half of the sclerotia (20) were planted in a Petri dish containing sterile sand and the other half in a Petri dish containing sterile soil. After 28 days, the rate of the colonised and infected sclerotia in sand was 0.00%, while in soil it was 26.67 and 6.67 respectively. Also, a possible explanation is given by Aggelaki (2001) [2]. Total loss of the sclerotia viability was observed 20 days after the application of *T. koningii* in the form of hyphae. However, for the achievement of the same results, the time was doubled when the application was made in the form of spore suspension. She ascribed it to the additional time that the spores needed in order to germinate and grow.

The environment in which mycoparasites act is not an one-dimensional system but a complex ecosystem. It is highly likely that the interaction between two or more factors takes place in this environment, affect the action of mycoparasites. Specifically, the sclerotial parasitism by hyphae is positively influenced by the presence of nutrient substrate at 10º C and not at 20º C. On the contrary, the effectiveness of the parasitism with spores is positively influenced by the absence of nutrient substrate and the increase in interaction time (Aggelaki, 2001) [2]. The sclerotia of *S. Sclerotiorum* were lysed after 20-30 days in flooded soil, but they still survive in dry soil. By contrast, they are alive in sterile flooded soil for the same period of time, with or without the addition of carbohydrates, which means that the lysis of sclerotia is probably caused by the microorganisms (Liu & Sun, 1984) [27].

The reduction, even to the zero point, of the sclerotial parasitism in system non-sterile soil/impregnation (V) for the nine mycoparasites of the genus *Trichoderma* in our experiments is also attributed to the microorganisms in the soil. Low temperatures, low water potential, heavy metals, pesticides and antagonistic bacteria are among the most stressful factors for the fungi of the genus *Trichoderma* (Kredics *et al*. 2002) [23]. The effect of the microbial community of the soil on the development and biocontrol efficacy of the antagonists is verified by Bae and Knudsen (2005) [4], who used a green fluorescent protein transformant of *T. harzianum* for this reason. Moreover, Locke *et al* (1985) [28], showed that in real conditions the conidia of an isolation of *T. viride* colonised rapidly the fumigated (82º C for 2 hours) soil mixture but did not colonise the non-fumigated.

Hubbard *et al*. (1983) [15], reported that fluorescent *Pseudomonas* species inhibited and lysed germlings of *Trichoderma spp*., under conditions of iron deprivation, resulting in failure of biological control of *Pythium* seed rots. Also, Bin *et al*. (1991) [6] observed that *Pseudomonas fluorescens* 2-79RN10 inhibited radial growth and hyphal density of *T. harzianum* on agar and in sterile bulk soil. However, in nonsterile soil, biocontrol efficacy (measured as colonization by *Trichoderma spp*. of sclerotia of *Sclerotinia sclerotiorum*) was not significantly affected by the addition of the bacterium. Hyphal growth and biocontrol efficacy of *T. harzianum* depends on its interactions with biotic components of the soil environment. Higher levels of microbial soil biomass result in increased interactions between introduced *T. harzianum* and soil microorganisms. Furthermore, that microbial competition in soil favours a shift from hyphal growth to sporulation in *T. harzianum*, potentially reducing its biocontrol efficacy (Bae and Knudsen, 2005) [4]. According to Chao *et al*. (1986) [10], the fungi of the genus *Trichoderma* do not seem to constitute effective competitors in the rhizosphere, and the successful result of a biocontrol factor depends on its efficacy in surviving, developing and multiplying in the rhizosphere. In our experiments, nine isolations which belong to the genus *Trichoderma* were not proved to be good antagonists.

The action of the mycoparasites is likely to be affected by the type of spores which are used. Larger populations of *Trichoderma* and *Gliocladium* were obtained when pellets carrying chlamydospores instead of conidia and bran instead

of kaolinite (Lewis & Papavizas, 1985) [26]. All of our experiments were conducted with conidiospores and we did not investigate the action chlamydospores. As a last remark, the nutritional environment provided by the culture medium is important, especially the carbon-nitrogen ratio (C/N) as it affects the ability of the mycoherbicidal fungus, *Colletotrichum truncatum* to infect its weed host *Sesbania exultata*, (Jackson and Bothast, 1990; Jackson and Schisler, 1992; Schisler *et al*., 1996) [16, 17, 41].

5. Conclusions

The form of the inoculum (hyphae, spores-dusting, and spores-suspension), especially the type of spores, and the environment of the experiment (water agar, sterile soil, non-sterile soil) can have a decisive influence on the specific behaviour and mycoparasitism efficacy of the mycoparasites. The nutritional environment provided by the culture medium is important, especially the carbon nitrogen ratio as it affects the ability of the mycoherbicidal fungus. As a result, there is considerable variation among the experiments ranging from 40-50% to even 100%. The antagonism in the soil, due to the microbial population, is proved a significant factor obliging the mycoparasite not only to be effective as a mycoparasite but also effective as an antagonist.

6. Abbreviations

WA: water agar, C/N: carbon-nitrogen ratio
PPFS: Parasitism Percentage in the Final Sample, APP: Accumulative Parasitism Percentage

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