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**ORIGINAL ARTICLE**

# Studies on the infection and parasitism course of sclerotia of *Sclerotinia sclerotiorum* by three different mycoparasites

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## Abstract

Mycoparasitism is an important process in microcosm of microorganisms. Understanding the mechanisms taking place may allow to effectively improved biocontrol of phytopathogens. A sequence of events during mycoparasitism process by three mycoparasites of different origin and aggressiveness was studied through an optical microscope. Additionally, both germination and entry procedure of mycoparasites on host's surface were observed through an electron scanning microscope. The development of sclerotia parasitism shows many common features in all three mycoparasites indicating very likely both common course and mechanisms. G21-3 (*Gliocladium* spp.) is the faster and more destructive mycoparasite followed by T12-9 (*Trichoderma* spp.) and FD6-15 (*Fusarium* spp.). In the present study, it was presented in a novel way both the appearance of hyphae of D6-15 isolation (*Fusarium* spp.) intra-cellularly and also the formation of chlamydospores (intra-cellularly) from G21-3 isolation (*Gliocladium* spp.). G21-3 germination on the sclerotial surface was completed within 15 h of incubation, and germ tubes will be strayed enough before entering into sclerotium. Appressoria and germ tube branches formation were not observed.

**Keywords** Mycoparasitism · Ultrastructure · *Fusarium* · *Gliocladium* · *Trichoderma*

## Introduction

*Sclerotinia sclerotiorum* (Lib.) de Bary, among other members of the *Sclerotinia* genus, has the widest host spectrum while host specification regarding it, and *S. minor* appears to be less significant. Even though they are widespread species, they are mainly found in mild climates (Pratt 1992). *Sclerotinia sclerotiorum* causes serious problems to many cultivated species of high economic interest (Bardin and Huang 2001; Hoes and Huang 1976; Huang 1980; Lee and

Wu 1986; Li et al. 1994; Tores 1990; Subbarao 1998), but, on the other hand, it is turned to be more important, especially in horticulture cultivation (Subbarao 1998; Wrather et al. 1997).

Fungus forms black irregular bodies, the sclerotia, through which it is preserved on the ground between cultivations (Adams and Ayres 1979; Coley-Smith and Cooke 1971; Merriman 1976) for many years. The primary infections are caused by the existed sclerotia in the soil. Sclerotia will be germinated either myceliogenically causing therefore infections on roots, sprouts or leaves that touch the soil or carpogenically producing apothecia and releasing ascospores.

Their well-studied structure (Willetts and Bullock 1992) justifies their ability to resist for years. Control measures like soil covering and sun heating, as well as soil rest (fallowing), which can partially control the disease, are not feasible in main cultivations. On the other hand, cost, effectiveness, environmental problems as well as health problems, due to the use of pesticides, make the development of alternative strategies of biological control important.

Quite a few mycoparasites have been evaluated and tested in both laboratory and field conditions giving very

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good results, creating therefore well-grounded hopes for biological or integrated control of the disease. Among them are *Coniothyrium minitans* (Grendene and Marciano 1999; McQuilken and Whipps 1995; Jones and Whipps 2002), *Fusarium* spp, (Rodriguez et al. 2006), *Gliocladium virens* (Budge et al. 1995; Phillips 1986a, b), *Pythium oligandrum* (Madsen and de Neergaard 1999), *Sporidesmium sclerotivorum* and *Teratosperma oligocladum* (Adams and Ayers 1983) and *Trichoderma* spp., (Aggelaki 2001; Gracia-Garza et al. 1997; Menendez and Godeas 1998; Sharma and Singh 1990; Vozenilkova et al. 1992).

Final control in both laboratory and field conditions is the result of a series of rather impressive events which starts from the germination of the parasite spore and ends at the sclerotium collapse with intense formation of mycoparasite fructifications and their journey in search of a new host. A more dramatic presentation could reveal violent pictures of invasions, pillages, collapse, chaos and absolute destruction.

Sclerotial parasitism is obviously the result of a bundle of concerted actions that remain unknown to a certain degree (Picard et al. 2000). The ability to invade the sclerotium and use the nutrients found in its environment usually relies on the possibility of the existence of enzymes and toxins, which open up the pathways and disengage nutrients. Their presence and role have been examined thoroughly (Aggelaki 1996; Dunlop et al. 1989; Correa et al. 1995; Haran et al. 1995; Lee and Wu. 1984; Madsen and de Neergaard 1999; Machida et al. 2001; McQuilken et al. 2003; Ordentlich et al. 1992; Rodriguez et al. 2006; Wolffhechel and Jensen 1992).

The ontogenesis of the mycoparasite inside the sclerotium proceeds in parallel with the cytochemical interactions at a molecular level. Hyphae intra- and inter-cellularly appeared followed by the formation of spores, cellular structure is diversified, and sclerotium starts to collapse. Anatomical changes, caused by both cytochemical interactions and mycoparasite development taking place inside a sclerotium during mycoparasitism process, have been studied for *C. minitans* (Ghaffar 1972; Huang and Kokko 1987; Tu 1984), *S. sclerotivorum* and *T. oligocladum* (Adams and Ayers 1983), *Talaromyces flavus* (McLaren et al. 1989), *T. koningii* (Aggelaki 2001), *T. virens* (Sarrocco et al. 2006), *Trichothecium roseum* (Huang and Kokko 1993), and not at full length for *G. virens* (Phillips 1986a; Tu 1980).

From the research that has been conducted until now, it was found out that there is no proper timing, and the only attempts made concern *S. sclerotivorum* and *T. oligocladum* (Adams and Ayers 1983) as well as *T. koningii* (Aggelaki 2001) and *T. virens* (Sarrocco et al. 2006). A general outlook on whether the mycoparasites follow the same route or not is also absent. It is known that the mycoparasites differ from each other in regard to their action, virulence and host specification, which presupposes and entails different cellular and molecular mechanisms. Thus, three different mycoparasites

that have been isolated using the same method (trapping method), the same host (sclerotia of *S. sclerotiorum*) and different soil samples, but belong to three different genera, were used in order to destroy the sclerotia.

## Materials and methods

### Organisms and development conditions

The mycoparasites that have been used in the present study (G21-3, T12-9 and FD6-16) were isolated from soil specimens coming from organic cultivations of south-western Greece using trap method (Tsapikounis 2015) and belong to the genera *Gliocladium, Trichoderma* and *Fusarium,* respectively (Table 1).

In detail, they were isolated among 199 candidate mycoparasites and submitted to preliminary and main evaluation in the laboratory under controlled conditions in the greenhouse (Tsapikounis 2007, 2015). *Sclerotinia sclerotiorum* originates from diseased cabbage plants in the region Brinia of the municipality of Vouprasia. All the fungi have been cultured in potato dextrose agar (PDA), while the sclerotia that have been used in the experiments originate from cultures in the same material. Both culture of mycoparasites and phytopathogenic took place in an incubatory chamber at 25 °C in the dark. The microorganisms were identified 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 systematics of the University of Athens (Department of Biology).

### Experimental conditions and inoculum preparation

Experiments were performed either in room conditions for the fixation or in an incubatory chamber for the incubation phase of sclerotia and mycoparasites, at 25 °C in the dark. Mycoparasite spores suspension was received from 10- to 15-day cultures developed in the dark at 25 °C. Inside the laminar flow and in aseptic conditions, a small quantity of deionized and sterile water (up to 20 ml) was poured into the Petri dish containing mycoparasite. By means of a small

**Table 1** Mycoparasites used in this study, name code, location and origin

| Species | Name code | Location in SW Greece | Origin |
| --- | --- | --- | --- |
| *Fusarium* sp. | FD6-15 | Kalamata, ETHIAGE | Cruciferous |
| *Gliocladium* sp. | G21-3 | Tripoli | Potato |
| *Trichoderma* sp. | T12-9 | Vouprasia Serbani | Tomato |

Mycoparasites isolated from organic cultured soils in south-west Greece by the trapping method

paintbrush, the surface was scraped gently. After that, the suspension was poured into a small beaker of 50 ml and with a haemacytometer the concentration was fixed at $10^6$ ml$^{-1}$.

## Fixative evaluation for permanent sclerotia preparations under optical microscope

The fixatives, formaldehyde (FA) and glutaraldehyde (GA), have been used in the following combinations: FA 3.7% for 12–15 h, FA 10% for 12–15 h, FA 15% for 12–15 h, GA 6.5% for 12–15 h, FA 10% + GA 2% for 12–15 h, FA 15% + GA 2% for 12–15 h, FA 4% in phosphate buffer (PB) for 12–15 h, FA 4% in PB at 4º C for 4 h, and GA 6.5% in PB at 4 °C for 4 h. Also, a preliminary experiment with double fixation (DF) was performed. During the DF, the sclerotia were put successively in GA 3% in PB for 2 h at 4 °C and afterwards in osmium acid 1% for 1.5 h at 4 °C. For every treatment, 5 sclerotia were used and produced 10 permanent preparations, which have been used in pairs in different pigmentation. For this purpose, three different pigmentations were tested: methylene blue, the combination of haematoxylin and eosin (the eosin in alcoholic or water solution) and the periodic acid–Schiff (PAS).

The sclerotia were placed in the fixative and remain at ambient temperature except the instances in which some sclerotia had to be put in the refrigerator at 4 °C. This process is followed by dehydration in water solutions of ethanol 50%, 70%, 90% and absolute ethanol, tissue clearing with ethanol/xylole (1:1) and with pure xylole, tissue infiltration with a mixture of xylole–paraffin 1:1 and then in paraffin melted at 60 °C. Sections of 3 μm depth were followed by deparaffinization and pigmentation.

## Pre-infection studies in scanning electron microscope (SEM)

The best mycoparasite (G21-3) and 25 sclerotia were used. These were immersed in spore suspension for 25 min and placed into five Petri dishes with water agar for incubation at 25 °C in the dark. Every three hours, a group of five sclerotia was put into a small glass Petri dish and this, in turn, in a glass vase containing 2–3 ml of osmium acid 1%. The vases were closed carefully with cling film and foil and left in this state for 17–24 h. After the fixation period, they were taken out of the osmium acid treatment, became metallized and finally were observed under the SEM. Observation under the SEM took place immediately.

## Mycoparasitism examination under the optical microscope

Forty sclerotia were immersed in spore suspension for 25 min and placed in Petri dishes with water agar in groups of five, for incubation. Every week, a group of five sclerotia was taken, for each mycoparasite, have been put in the fixative and remained there for 12–15 h. Glutaraldehyde 6.5% was used for the fixation process, which is the same with the one described above. The enclosure in paraffin was followed by sections (segmentation) and pigmentation made by a haematoxylin–eosin combination. Observation under the microscope took place immediately.

# Results

## Fixatives and preparation quality

Two fixatives were used in nine combinations. The results ranged from moderate to good. The ranking (from best to worst) was as follows: GA 6.5% in PB at 4º C for 4 h, GA 6.5% for 12–15 h, FA 4% in PB at 4º C for 4 h, FA 4% in PB for 12–15 h, FA (15%) plus GA (2%) for 12–15 h, FA (10%) plus GA (2%) for 12–15 h, FA 15% for 12–15 h, FA 10% for 12–15 h and FA 3.7% for 12–15 h. At combinations in which FA was without GA and PB, breakings in the rind were observed, many vacuua in the interior and under the rind, scattered pieces, uncommon cuts and lots of folds. When 2% of GA was added, the conditions showed improved results. However, this improvement was less significant than the expected one. Addition of PB resulted in a slight improvement (at 4 °C for 4 h and not for 12–15 h) without impressive outcome. There was significant improvement only when GA (6.5%) was used as a fixative. The results were satisfactory for both treatments (remaining for 12–15 h or in PB at 4 °C for 4 h), with the second being slightly better. Preliminary tests showed that double fixation could possibly provide better outcomes (Fig. 1). As far as dyeing is concerned, the best outcomes were taken with the combination of haematoxylin–eosin. Methylene blue and PAS coloured brightly the preparations, making the observation difficult.

## Germination and penetration on the sclerotial surface

Germination and evolution study of G21-3 mycoparasite on the surface of sclerotia was made under a scanning electron microscope. The most interesting points were the time needed for the germination of mycoparasite spores on the sclerotia surface, the time needed for the penetration of the mycoparasite in the sclerotia and the formation of appressoria. Representative results are presented in Fig. 2. The germination of mycoparasite spores starts about 12 h after, generalized at 15 h later and completed 20 h later. In most cases, it was ascertained that the germ tubes would have a long time strayed before entering into sclerotium. Formation of appressoria and branches of the germ tube were not
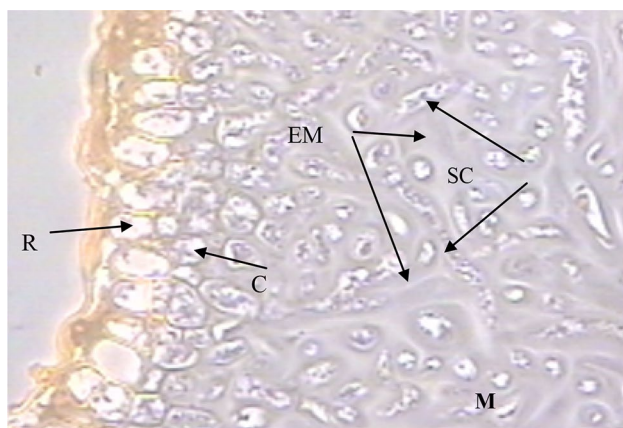
**Fig. 1** Light micrographs of *Sclerotinia sclerotiorum* sclerotia after Ga 6% for 12–15 h or double fixation. Visual parts: the rind (R), cortex (C), the medulla (M), the sclerotial cells (SC) and extracellular material (EM). Scale bar: 75 µm. (×400)

observed. Whether they are formed or not is rather difficult to be verified due to the coating of the surface and the crust created by the exudations' deflating.

## The anatomy of sclerotial parasitism

### The parasitism progression by the mycoparasite G21-3

Representative results after sclerotia infection by G21-3 mycoparasite in weekly samplings are displayed in Fig. 3. After 7 days of infection, mycoparasite hyphae were found not only in the medulla, under the rind but also inside the sclerotium cells. Furthermore, uncommon vacuua appeared in the medulla, near the rind, while a significant number of chlamydospores were formed outside the sclerotia. Fourteen days later, the presence of dense hyphae and wide vacuua was observed near the rind. As shown in Fig. 3, 21 days after the infection, some areas of the sclerotia show some changes in the cellular structure. Thirty days later, whole parts were separated, areas are evacuated, and mycoparasite hyphae are spotted in the major part of the medulla, while the presence of chlamydospores was notable under the rind. Forty-two days after the infection, the evolution was rapid and mycoparasite hyphae and chlamydospores were almost everywhere. Fifty-six days later, sclerotia reduction was important.

### Mycoparasitism progression by the mycoparasite T12-9

A similar picture was given by parasitism evaluation in sclerotia infected by T12-9 mycoparasite. The most important events are presented in Fig. 4. Seven days after the infection, areas with unusual vacuua were observed in the medulla near the rind in all sclerotia. More specifically, in some
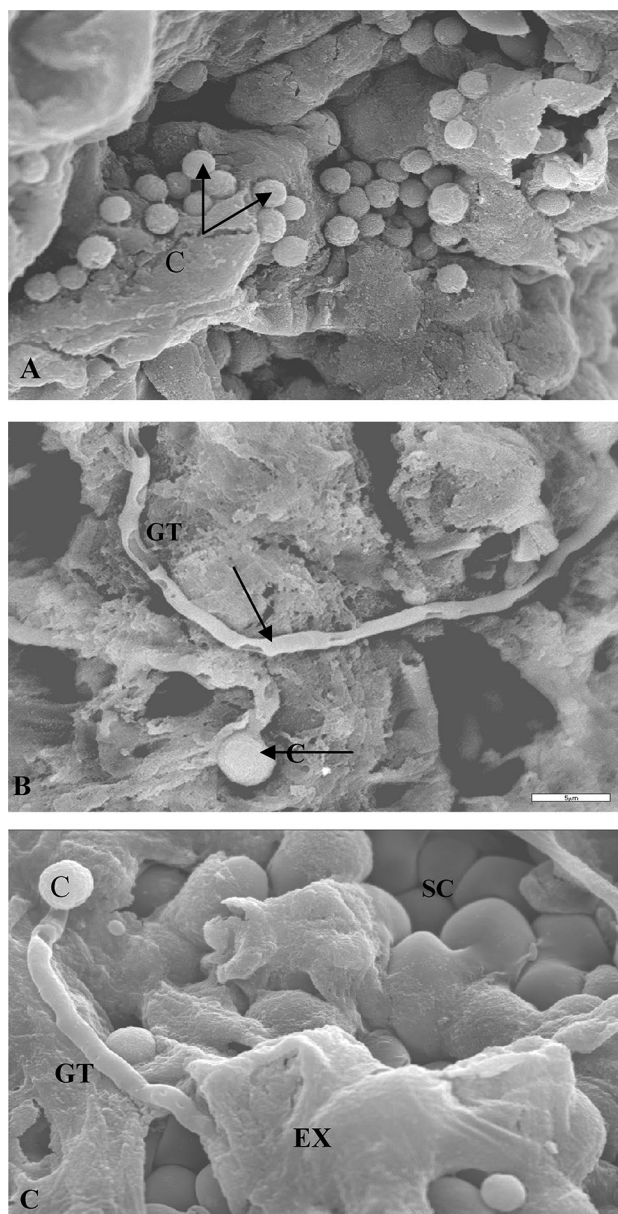


**Fig. 2** Scanning electron micrographs of sclerotium surfaces that have been added the mycoparasite G21-3. **a** Six hours after the addition a big number of conidia (C) exist in sclerotial surface, **b** fifteen hours after the infection by the mycoparasite G21-3. The germ tube (GT) of a conidium (C) has a long time strayed before entering the interior of the sclerotium. **c** Fifteen hours after the addition of the mycoparasite G21-3. The germ tube of a spore enters the interior of the sclerotium after having strayed shortly. The crusts (EX) are a result of the exudations' deflating. Scale bar: 5 µm. (*SC* scletorial cells) (EX exudates) (**a**): SEM X1800, (**b**): SEM X2300, (**c**): SEM X2300

points it seems that mycoparasite hyphae have entered into sclerotium cells. Fourteen days later, there was an increase in the number of the areas with wide vacuua in the medulla not only near the rind but also quite far from it. Twenty-one days after the infection, the presence of mycoparasite
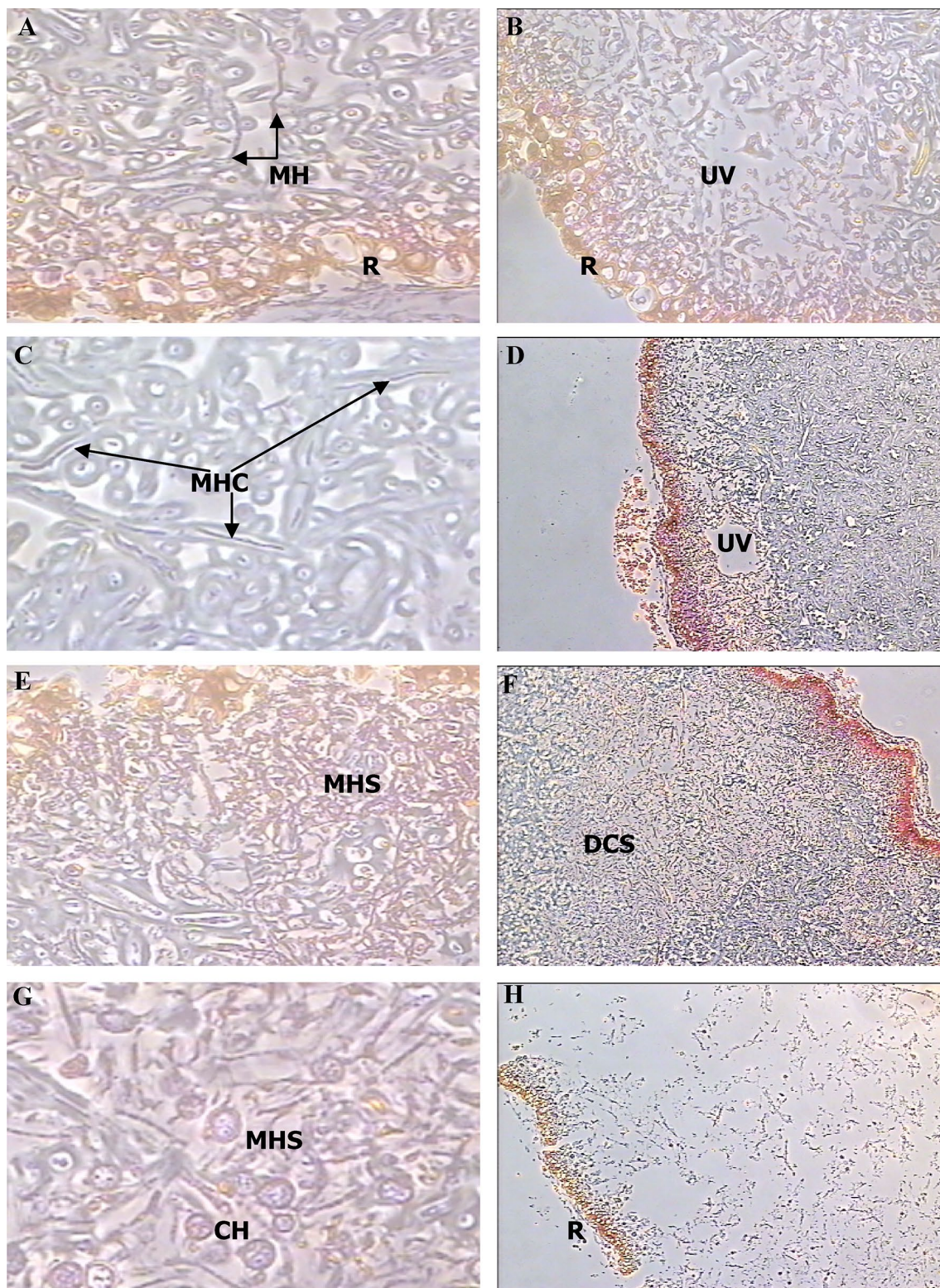
**Fig. 3** Light micrographs of sclerotial sections infected by the mycoparasite G21-3. Seven days after the infection, mycoparasite hyphae (MH) (x400) (**a**), unusual vacuua (UV) (×400) (**b**) and mycoparasite hyphae inside sclerotial cells (MHC) (×400) (c) are discerned (see arrows), scale bar: 60 μm. **d** Fourteen days later, big unusual vacuua (UV) near the rind, scale bar: 125 μm. (×200) **e** fourteen days later, the presence of mycoparasite hyphae is strong (MHS), scale bar: 60 μm. (×400) **f** Twenty-one days later, areas, which have a distorted cellular structure (DCS), are observed, (×200) scale bar: 125 μm. **g** Forty-two days later, there is a strong presence of mycoparasite hyphae and chlamydospores (CH), (×400) scale bar: 60 μm. **h** Fifty-six days after the infection, the area under the rind is "evacuated" and its content has obviously been used as food by the mycoparasite, (×400) scale bar: 60 μm. (*R* rind)
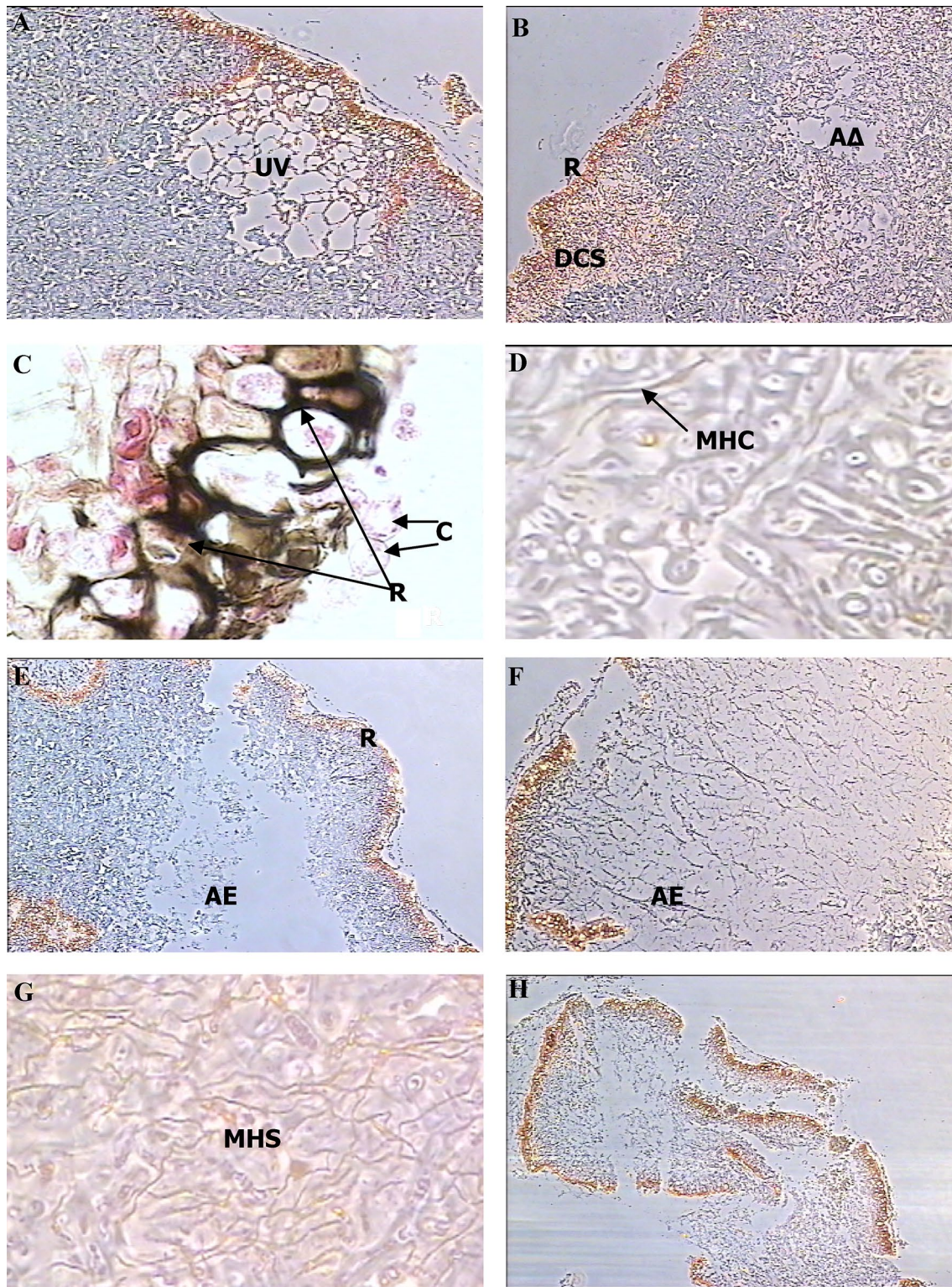
**Fig. 4** Light micrographs of sclerotial sections infected by the mycoparasite T12-9. **a** Seven days after the infection, a rather unusual vacuua (UV) can be discerned, (×200) scale bar: 125 μm. **b** Fourteen days later, unusual vacuua (×200) (UV) and distortions of the cellular structure (DCS) can be seen, scale bar: 125 μm. **c** Fourteen days later, a conidium (C) is seen to be germinated and penetrated into the sclerotium. (×1000) scale bar: 5 μm. **d** Twenty-one days later, mycoparasite hyphae are observed inside the sclerotium cells (MHC), (×400) scale bar: 60 μm. **e**, **f** Twenty-eight and forty-two days after the infection, areas being evacuated (AE) are visible, (×200) scale bar: 125 μm. **g** Forty-nine days later, a strong presence of mycoparasite hyphae can be observed (MHS), (×400) scale bar: 60 μm. **h** Fifty-six days after the infection, a part of the sclerotium is completely disrupted, scale bar: 125 μm. (×200) (*R* rind, *C* cortex)

hyphae near the rind was more intense, while a high number of mycoparasite hyphae had entered into sclerotium cells. On the 28th day, whole parts of sclerotium surface were visible to be separated and areas evacuated. Thirty days later, the presence of mycoparasite hyphae inside the sclerotium was even stronger. As it can be seen in Fig. 4, on the 42nd day large areas of the sclerotium were reduced in terms of content and the presence of mycoparasite hyphae is discovered not only all over the section surface, but also inside the sclerotium hyphae. The same picture was also observed in

the next sampling 49 days later. In the last sampling, 56 days later, chlamydospores were discovered near the rind and the sclerotia depletion has been extensively completed.

## Mycoparasitism progression by the mycoparasite FD6-15

Representative findings after the infection of sclerotia by FD6-15 mycoparasite (in different time) are presented in Fig. 5. In 7 days, mycoparasite hyphae were observed inside the sclerotium near the rind, as well as uncommon
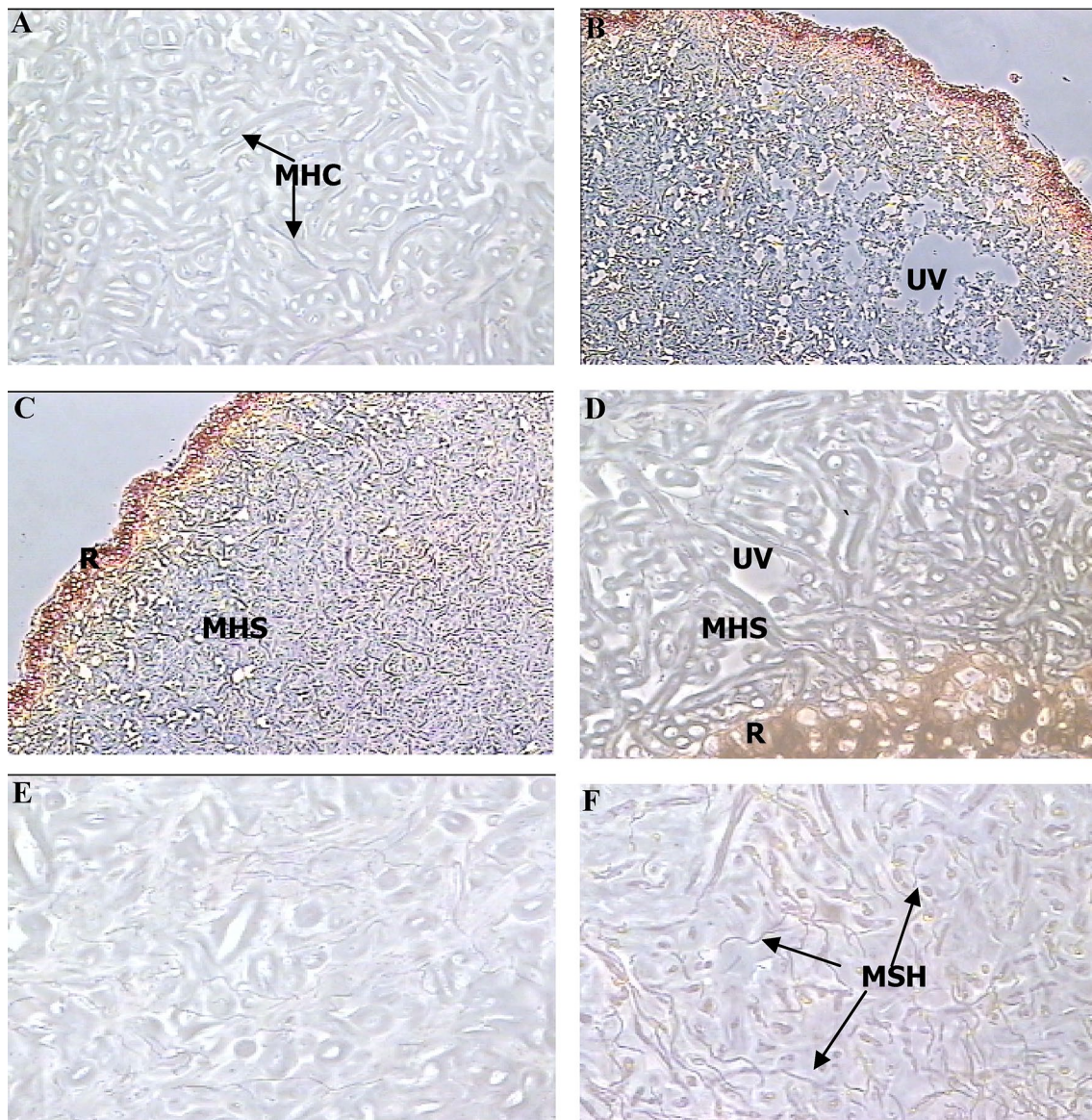


**Fig. 5** Light micrographs of sclerotial sections infected by the mycoparasite FD6-15. Mycoparasite hyphae are very small and difficult to be discerned. Seven days after the infection, mycoparasite hyphae can be discerned inside the sclerotial hyphae (MHC) ($\times 400$) (**a**) (scale bar: 60 μm) and unusual vacuua (UV) (**b**) can be discerned, ($\times 200$) scale bar: 125 μm. **c** Twenty-one days later the presence of the mycoparasite hyphae is strong (see arrows), ($\times 200$) scale bar: 125 μm. **d** After forty-two days, mycoparasite hyphae (see arrows) and unusual vacuua (UV) can be discerned, ($\times 400$) scale bar: 60 μm. As can be seen, the mycoparasite hyphae are throughout the medulla ($\times 400$) (**e**), scale bar: 60 μm. **f** Fifty-six days later, no significant change can be observed, scale bar: 60 μm. ($\times 400$) (*R* rind)

vacuua near the rind. In 14 days, the mycoparasitism has evolved a little bit more. On the 21st day, the presence of mycoparasite hyphae is quite strong especially under the rind. In the next two samplings, the parasitism evolution was relatively slow and without significant new events. In 42 days, the presence of mycoparasite hyphae was as perceptible near the rind as it was in the interior. Lastly, in the eighth sampling (56 days), no significant change was ascertained.

### Possibly a common route and cytochemical changes

The comparison of the aforementioned findings showed clearly that the fastest and most destructive mycoparasite was G21-3 followed by T12-9, while FD6-15 parasitizes and depletes the sclerotium at a slower rate. The presence of hyphae in the medulla, the presence of chlamydospores and the changes in the cellular structure of sclerotia took place faster as regards infections by G21-3 and more slowly with T12-9 and FD6-15. In 56 days, the sclerotia infected by G21-3 were completely destroyed; the ones infected by T12-9 were semi-destroyed, whereas those ones infected by FD6-15 were in a rather good condition. The evolution of sclerotia parasitism showed many common features for all three mycoparasites. Initially, mycoparasite hyphae were observed under the rind and later in the medulla. The first chlamydospores are formed near the rind in parallel with the appearance of hyphae in the medulla, while a few vacuua appear in the medulla of some sclerotia. In the end, the mycoparasite hyphae were settled in the whole sclerotium resulting in disorganization, with obvious changes in the cellular structure of sclerotia. Finally, the sclerotia collapsed and great areas disappeared. Total progress of parasitism is presented in Fig. 6.
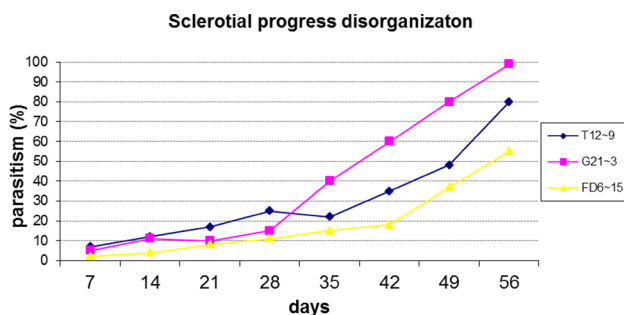


**Fig. 6** Progress of *S. sclerotiorum* sclerotia degradation by three mycoparasites of different mycoparasitic capacity

## Discussion

In the present study, three different mycoparasites were recognized (*Fusarium* spp., *Gliocladium* spp., and *Trichoderma* spp.) of different aggressiveness followed the same route in order to destroy the sclerotia. Also, glutaraldehyde 6.5% (for 12–15 h at room temperature) was found to be an excellent fixative for the creation of fixed sclerotia sections, used for the observation of mycoparasitism under an optical microscope. A preliminary experiment showed that double fixation might produce better results. The advantage of double fixation was due to the fact that osmium acid "locks" fatty acids while aldehydes "lock" only proteins. In double fixation, a greater number of molecules contribute to the cell fixation if compared with conventional fixation. Moreover, the combination of haematoxylin–eosin was proved to be the best pigmentation. Methylene blue and PAS colour brightly the preparations which cause difficulties during observation. Observation of sclerotia mycoparasitism can be conducted under an optical microscope with permanent sections using formaldehyde for the fixation (Adams and Ayers 1983; Phillips 1986a) or directly with freeze substitution using lactophenol blue (Aggelaki 2001). Also, under the transmission electronic microscope (TEM) with double fixation (Huang and Kokko 1993) and under SEM with double fixation (Tu 1984), the final results were considered only satisfactory. Glutaraldehyde (6.5%) was used by Bullock et al. (1980), to study the structure and histochemistry of sclerotia of *Sclerotinia minor,* by Backhouse and Willetts (1984), to study the anatomy and histochemistry of sclerotia of *Sclerotium cepivorum,* and by Backhouse and Willetts (1984) (3%), to study the histochemistry of sclerotia of *Botrytis cinerea* and *B. fabae*. With the use of conventional fixation, problems arise only in histochemical studies (Young et al. 1993); in this case, it is advisable to use freeze substitution.

Parasitism progress showed many common characteristics, revealing therefore a common route and possibly common mechanisms. Adams and Ayers (1983) observed that *S. sclerotivorum* and *T. oligocladum* presented the same behaviour during the parasitism of sclerotia. According to our findings, mycoparasite hyphae were firstly observed under the rind and later in the medulla. At the same time, the first chlamydospores were formed near the cortex and vacuua appeared. Finally, mycoparasite hyphae were settled in the whole sclerotium resulting in disorganization, with obvious changes in the cellular structure in certain points. The sclerotium collapses and large areas disappear, which evidently supplied mycoparasites with food. The presence of hyphae in the medulla, the existence of chlamydospores as well as the changes in the cellular

structure took place in a more rapid way in the case of G21-3 (*Gliocladium* spp.*)* and more slowly as far as T12-9 (*Trichoderma* spp.) and FD6-15 *(Fusarium* spp.) were concerned. Chronological correspondence of the events is presented in detail in the results. Several researchers have attempted to discover the sequence of events that take place during the sclerotia parasitism of *S. sclerotiorum*. In experiments with spores and hyphae of *T. koningii* in sclerotia of *S. sclerotiorum*, it was discovered that *T. koningii*'s hyphae develop between and into the cells of the rind the first 20 days. (The chlamydospores are formed simultaneously, inside and between the cells.) Forty days later, the hyphae were traced inside the medulla cells as well. (Yet neither conidia nor chlamydospores were observed.) Finally, the sclerotia became soft, lost their tenacity and were disrupted (Aggelaki 2001). Sarrocco et al. (2006), working with fluorescent microscopy, found out that 9 and 14 days after inoculation of *S. sclerotiorum* sclerotia by a GFP-transformed *Trichoderma virens*, antagonist's mycelium being in the medulla and 20 days later the medulla was completely colonized. In our experiments, hyphae of all three mycoparasites (FD6-15, G21-3 and T12-9) used were verified within the medulla during the first days.

Macroconidia of *S. sclerotivorum* and *T. oligocladum* usually need 3–5 days in order to germinate and infect the sclerotia. Hyphae often branch on the surface of the sclerotium leading to multiple penetrations. The germ tubes penetrate the rind and multiply under the surface of the sclerotium (5th–10th day). Under the cortex, they can be detected on the 10th day and in the medulla on the 21st day. However, widespread development is observed only on the 43rd day. Mycoparasite does not enter into cells, but into intercellular spaces taking their form. After extensive propagation in the medulla, mycoparasite continues to develop on the surface of the sclerotium, where it bears plenty of fructifications (Adams and Ayers 1983).

*Trichothecium roseum* enters either with direct penetration or from the contact points of the rind cells. When penetration is used, lysis of the cell walls takes place, followed by hyphae branching and development inside the sclerotium, cortex and medulla. Mycoparasite hyphae branch throughout the sclerotium resulting in its destruction (Huang and Kokko 1993).

As parasitism by *C. minitans* progressed, the mycoparasite increased the colonization of both surface and interior of the sclerotia. In the last stages of parasitism, the hyphae of *C. minitans* multiplied inside the sclerotium and formed pycnidia near the surface while the sclerotia were softened and shattered. Moreover, our study under the scanning electronic microscope revealed that the mycoparasite hyphae develop both inter- and intra-cellularly as it was referred previously (Tu 1984). It has been observed that pycnidia were formed not only externally (on the surface of infected

sclerotia) but also interiorly (Ghaffar 1972). Finally, Phillips (1986a) provided a short description of *G. virens* on sclerotia. Some sclerotia were softened after 10 days of incubation. The rind was sloughed easily off the infected sclerotia due to the decay of the outer layers of the medulla. When the infected sclerotia were cut in half, the medullary tissues appeared water-soaked and discoloured.

The presence of hyphae was verified for all kinds of isolations (G21-3, T12-9 and FD6-15) inside the sclerotia cells. Mycoparasite hyphae inside the sclerotial cells were observed by Phillips (1986a), regarding *G. virens*, by Tu (1980), regarding *C. minitans*, by Huang and Kokko (1993), regarding *T. roseum,* and by Aggelaki (2001), regarding *T koningii*. In the present study, the presence of hyphae of the isolated FD6-15 (*Fusarium* spp.) was verified inside the sclerotia cells and this is probably mentioned in bibliography for the first time. Sarrocco et al. (2006) ascertained that *T. virens* grew intra-cellularly and inter-cellularly in *Sclerotium rolfsii* sclerotia but only inter-cellularly in *S. sclerotiorum* sclerotia.

It is not known what exactly happens when mycoparasite hyphae penetrate in the sclerotial hyphae. The events that take place might be the same with those ones described by Huang and Hoes (1976), Huang and Kokko (1988) and Tu (1980) during the penetration of *C. minitans* hyphae inside the *S. sclerotiorum* hyphae in double cultures. Mycoparasite hyphae infected the *S. sclerotiorum* ones after about 5 days of incubation. The infection peg tips penetrated the cell walls of the host, the cytoplasm was granulated or shattered, and the cell walls collapsed when the mycoparasite hyphae branch turned from outwards to inwards (external to internal penetration).

Many studies refer to the creation of chlamydospores inside the sclerotium by genus *Trichoderma* (Aggelaki 1996, 2001; Lee and Wu 1984) but not by *Gliocladium*. In our experiments, G21-3 (*Gliocladium* spp.) was verified to create chlamydospores in the sclerotia on the 14th day. This is probably mentioned in bibliography for the first time. T12-9 (*Trichoderma* spp.) also creates chlamydospores on the 21st day. On the contrary, FD6-15 (*Fusarium* spp.) does not create chlamydospores. For as long as the experiment lasted (56 days), there was not any presence of conidia or macroconidia in the sclerotium as well. *T. koningii* and *T. virens* form chlamydospores within the sclerotium on the 20th day (Aggelaki 2001; Sarrocco et al. 2006).

Mycoparasites G21-3 and T12-9, but not FD6-15, caused changes in the cell structure, which were obviously the result of either enzymes or toxins and even both of them. *T. roseum* caused granulation and evacuation of the host hyphae without being at immediate contact with the mycoparasite hyphae indicating the presence of toxic metabolites (Huang and Kokko 1993). The sclerotial disruption has started since the 20th day, without being at immediate contact with the

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