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Interaction between biotrophic haustorial mycoparasite and protoplasts of host and nonhost fungi

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Abstract

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Protoplasts obtained from mucoraceous hosts *Mortierella pusilla* (susceptible), *Phascolomyces articulosus* (resistant) and nonhost, *Mortierella candelabrum* to the mycoparasite *Piptocephalis virginiana,* ranged in size from 2–8 μm. Their regeneration frequency averaged between 80–90%. Optimization of protoplast yield was achieved by using a combination of lytic enzymes Chitinase, Chitosanase and Noyozyme 234, applied to young hyphal cells. Incubation of mycoparasite spores with agarose embedded protoplasts obtained from host species exhibited higher percentage of germination than with those from the nonhost. The germ tubes of the mycoparasite attached to some protoplasts of *M. pusilla* and *P. articulosus*. Irrespective of attachment, the protoplasts became enlarged, highly vacuolated and finally collapsed in the presence of germinating mycoparasite spores. Enlargement and vacuolation of the protoplasts could not be duplicated with the culture filtrate of the mycoparasite. The protoplasts of the nonhost, *M. candelabrum,* on the other hand, did not attach and remained intact upon incubation with the germ tubes of *P. virginiana.* A change in membrane stability and permeability in host protoplasts by induced extracellular products of the mycoparasite is suggested.

Key words: Protoplasts, interaction, specificity, biotrophic, haustorial, mycoparasite.

Introduction

Interactions between the mycoparasite, *Piptocephalis virginiana,* and its susceptible host, *Mortierella pusilla,* resistant host, *Phascolomyces articulosus,* and the nonhost, *Mortierella candelabrum,* have been studied previously (Manocha 1985, Manocha et al. 1986). The mycoparasite has been shown to attach to the cell wall surface of the susceptible and resistant hosts, but not to that of the nonhost. After the initial contact and appressorium formation, the mycoparasite penetrates the hyphae of susceptible and resistant hosts, but its further growth in the latter is inhibited by the deposition of papillae and a thick sheath around the haustorium. The initial recognition and attachment of mycoparasite to the susceptible and resistant host surface seems to be mediated through carbohydrate-agglutinins (Manocha and Chen 1991).

Odermatt et al. (1988) working with plant protoplasts – *Phytopthora* system suggested that the parasite may recognize the cell wall surface of the host, but the host may only recognize the parasite when the latter reaches the host plasma membrane. Manocha et al. (1990) have recently shown that while there was no difference at the cell wall surfaces, the protoplast surfaces of the susceptible and resistant hosts, *M. pusilla* and *P. articulosus,* respectively, differed markedly in their glycosyl residues as revealed by FITC-lectin binding assay. The present study describes the interaction between the mycoparasite, *P. virginiana,* and the protoplasts of hosts (compatible and incompatible) and nonhost embedded in agarose. The purpose of this investigation was to ascertain (i) whether the loss of host cell wall results in loss of host specific reaction, (ii) whether the mycoparasite interacts with the protoplasts of nonhost, and (iii) whether the mycoparasite factors responsible for specific reaction are lost in the comparatively large diffusion space in the agarose gel.

Materials and methods

Cultures and cultural conditions: Cultures of *Mortierella pusilla* Oudeman, *Phascolomyces articulosus* Boedijn ex Benny and Benjamin, and *Mortierella candelabrum* V. Teigh and Le Monn were grown at 23 ± 1 °C on an agar medium consisting of 2% malt extract and 0.2% yeast extract. An axenic culture of spores of the biotrophic mycoparasite, *Piptocephalis virginiana* Leadbeater and Mercer, was obtained by growing the parasite on the susceptible host, *Choanephora cucurbitarum* (Berk. & Rav.) Thaxter, in complete continuous darkness which is known to inhibit sporulation of the host while the parasite sporulates normally.

Isolation of protoplasts: Protoplasts from hosts and nonhost fungi were isolated as previously reported by Manocha et al. (1990) except that young germlings (24 hr) instead of 2–3 day old mycelium were used. Germlings from host and nonhost fungi obtained by incubating spores (10^6 spores/ml) in malt yeast extract medium, were filtered through Whatman No. 4 filter paper and washed twice with sterile distilled water. Samples of 0.25 g wet wt. from each fungus, pretreated in 1 ml of glycine-HCl buffer (80 mM, pH 5.5) containing 10 mM β-mercaptoethanol and 20 mM dithiothreitol for a period of 1 hr, were washed and suspended in 5 ml of lytic enzyme solution. The mycelial suspension in enzyme mixture containing 2 mg/ml chitinase (Godo Shusei Co. Ltd., Tokyo), 2 mg/ml chitosanase from *Bacillus circulans* (Lion Co., Tokyo) and 5 mg/ml Novozyme 234 (Sigma Chemical Co., St. Louis, Mo.) was incubated at 23 °C. Samples were drawn at regular intervals to check the formation and density of protoplasts. The protoplast suspension passed through 3 layers of cheesecloth and a layer of nylon mesh with 20 µm openings, was centrifuged at 1500 g for 10 min, and the pellet was suspended in glycine-HCl buffer containing 0.7 M KCl. The protoplasts were counted in a hemocytometer and their number was calculated for 50 mg mycelium.

Regeneration of protoplasts: The regenerating ability of protoplasts from the time of their isolation up to 48 h, was examined. Protoplast suspensions were transferred to 50 ml of malt yeast extract medium containing 0.7 M KCl. Germ tubes formed after incubation at 23 °C were counted in a hemocytometer. Regeneration frequency was taken as the ratio of number of germ tubes formed to the number of protoplasts added to the medium. Malt yeast extract without the osmotic stabilizer was used as the control.

Germination of the spores of the mycoparasite: Spores of the biotrophic mycoparasite, *P. virginiana,* were germinated in nutrient broth containing 1% (v/v) glycerol as described by Manocha et al. (1986). This enabled 82% germination in 24 h.

Agarose embedding of protoplasts: For agarose embedding of protoplasts and mycoparasite germ tubes, the method of Odermatt et al. (1988) was followed. A 0.1 ml suspension of germinating spores of mycoparasite (4×10^5/ml) and 0.5 ml suspension of protoplasts (2×10^5/ml) was mixed gently with 1 ml of agarose. Sterilized agarose preparation contained 75 mg of ultra low-gelling temperature agarose (Sigma Chemical Co., St. Louis, Mo.) and 1 ml of 0.7 M KCl. Four drops of

the mixture was placed on a slide and spread across the previously cleaned and acid washed slides to form a 0.5 mm thick layer. Agarose solidified in 15 min and the slides were incubated at 22 °C in Petri dish lined with moist filter paper. Controls were maintained separately on agarose for the spores of the mycoparasite and the protoplasts of hosts and nonhost species. The slides were examined after 10–15 h incubation.

Effect of culture filtrate of mycoparasite on protoplasts: The culture filtrate of mycoparasite was obtained by growing *P. virginiana* on nutrient broth as described earlier (Balasubramanian and Manocha 1986). The effect of culture filtrate on protoplasts of hosts and nonhost fungi was studied by replacing mycoparasite spore suspension in protoplast mixture with 0.1 ml of undiluted, dialysed or lyophilized culture filtrate. For lyophilization 50 ml of culture filtrate was freeze dried then dissolved in 5 ml of sterile distilled water. Protoplasts were examined for their increase in size as well as regeneration ability.

Results and discussion

Figure 1 shows the yields of protoplasts isolated from 24 h mycelium of *M. pusilla, P. articulosus* and *M. candelabrum,* susceptible, resistant and nonhost, respectively. These yields were much higher than those obtained from 2–4 days old cultures reported previously (Manocha et al. 1990). This has been the case in several fungi; e.g., *Trichoderma reesei* (Picataggio et al. 1983) and *Conidiobolus lamprauges* (Ishikawa and Oishi 1985), where maximum yield of protoplasts was obtained from 20 h old cultures. Sallen et al. (1988) obtained a higher yield of protoplasts from 24 h old cultures of *Fusarium oxysporum.* The number of protoplasts decreased with prolonged incubation in the mixture of lytic enzymes (Fig. 1). This can be explained by the fact that Novozyme contains considerable proteolytic activity which could destroy the protoplast membrane (Hamlyn et al. 1981). Similar observations were made during the isolation of protoplasts from *Microsporum gypseum* by Chadegani et al. (1989) and from *Phytophthora capsici* by Lucas et al. (1990), when Novozyme was used as one of the lytic enzymes.

When the protoplasts isolated from *M. pusilla, P. articulosus* and *M. canbelabrum* were transferred to malt yeast extract medium containing 0.7 M KCl, germ tubes were formed within 10 h of incubation. The percentage of regeneration, however, decreased with prolonged storage of protoplasts in 0.7 M KCl at 4 °C (Fig. 2).

Figures 3 and 7 show protoplasts of *M. pusilla* and *P. articulosus* ranging in size from 3–7 μm. The mycoparasite, *P. virginiana,* spores showed an enhanced percentage of germination in the presence of the protoplasts of the susceptible host, *M. pusilla* (Table 1) than with those of the resistant host, *P. articulosus.* On the other hand, the protoplasts of *M. candelabrum* almost completely inhibited the germination of the spores of the

Table 1. Germination of the spores of the mycoparasite, *Piptocephalis virginiana,* in presence of protoplasts of hosts and nonhost fungi

Protoplast source	Mycoparasite spore germination	
	Time of germination (hrs)	Percentage of germination
Mortierella pusilla	14	96
Phascolomyces articulosus	20	72
Mortierella candelabrum	36	4

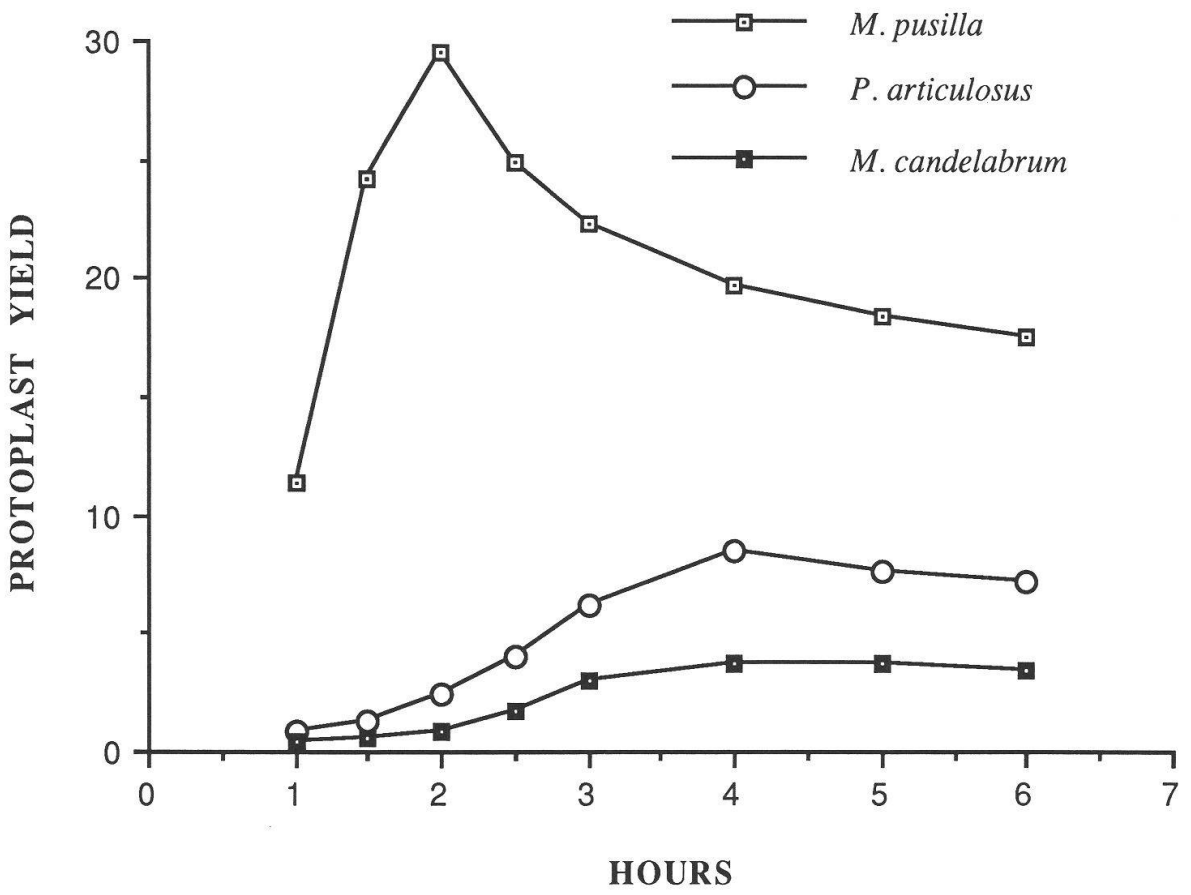


Fig. 1. Protoplast formation in 24 hr old germlings of *Mortierella pusilla, Phascolomyces articulosus* and *Mortierella candelabrum.*

mycoparasite. Earlier results obtained with intact hyphae of *M. pusilla* and *M. candelabrum* showed no obvious difference in the percentage germination of mycoparasite spores in the presence of either fungus (Manocha et al. 1986). In tobacco mesophyll protoplasts, Grosset et al. (1990) have reported the release of chitinase and 1, 3 β-glucanase as a possible response to isolation procedure which is similar to wounding, thus triggering a defense reaction. There is a possibility that in the nonhost *M. candelabrum,* certain substances are induced in response to the isolation procedure which inhibit the germination of mycoparasite spores.

The germinated spores of the mycoparasite did not show any long distance attraction towards the protoplasts of either the susceptible host, *M. pusilla* or the resistant host, *P. articulosus.*

The percentage of contacts of the germinated spores of the mycoparasite with the protoplasts of *M. pusilla* (Fig. 4) and *P. articulosus* (Fig. 9) were the same. No appressoria were observed even after prolonged contact. However, with growth of the germ tubes of the mycoparasite, the protoplasts of the hosts, *M. pusilla* and *P. articulosus,* became enlarged and highly vacuolated (Figs. 5, 6 & 8). These protoplasts had an average diameter of 35–40 μm (Table 2). The decrease in the number of protoplasts during incubation with the germ tubes of the mycoparasite was probably due to the disruption of protoplasts and was not the result of fusion. The cytoplasm was seen streaming towards the periphery of the membrane and was released upon membrane rupture (Fig. 9). The

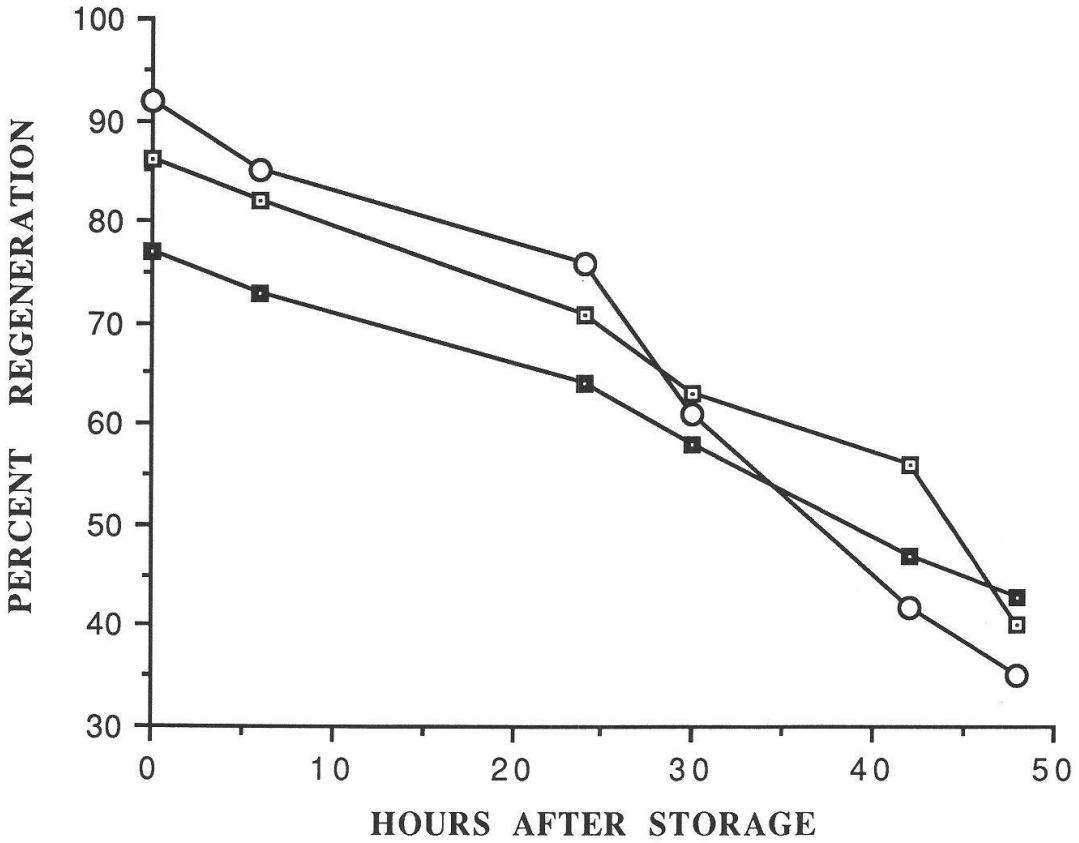


Fig. 2. Regenerating ability of protoplasts of *Mortierella pusilla, Phascolomyces articulosus* and *Mortierella candelabrum* in malt-yeast liquid medium, at different periods after isolation.

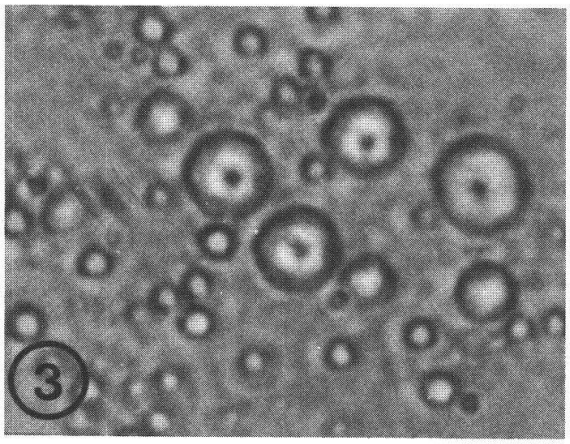
Table 2. Number and size of agarose embedded protoplasts before and after the germination of spores of *Piptocephalis virginiana*

Protoplast source	Number (size) of protoplasts/cm^2	
	before	after (4 hr)
Mortierella pusilla	22 (2–5 µm)	13 (30–55 µm)
Phascolomyces articulosus	26 (3–8 µm)	18 (25–60 µm)
Mortierella candelabrum	21 (2.5–5 µm)	17 (3– 7 µm)

Table 3. Regeneration of protplasts on agarose containing culture filtrate of *Piptocephalis virginiana*

Treatment	Regeneration frequency of protoplasts		
	M. pusilla	*P. articulosus*	*M. candelabrum*
Untreated culture filtrate	8.2 (10.3)*	6.1 (7.8)	7.3 (6.5)
Dialysed culture filtrate	2.1 (3.5)	3.3 (2.8)	2.4 (1.6)
Lyophilized culture filtrate	24.4 (27.6)	18.6 (17.3)	23.9 (29.4)

* Figures in parentheses show control in nutrient broth treated in same fashion as culture filtrate. For concentrations of culture filtrate, see 'Materials and methods'.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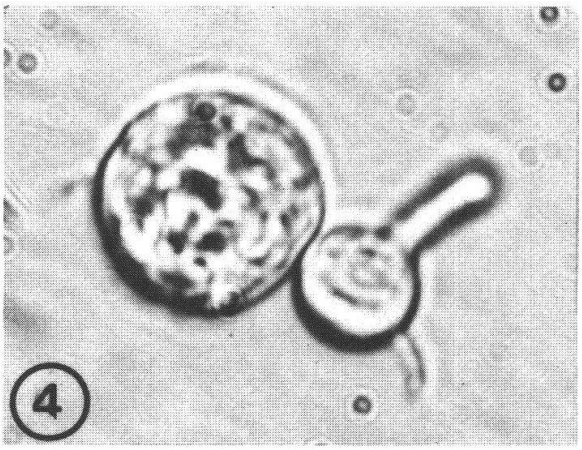
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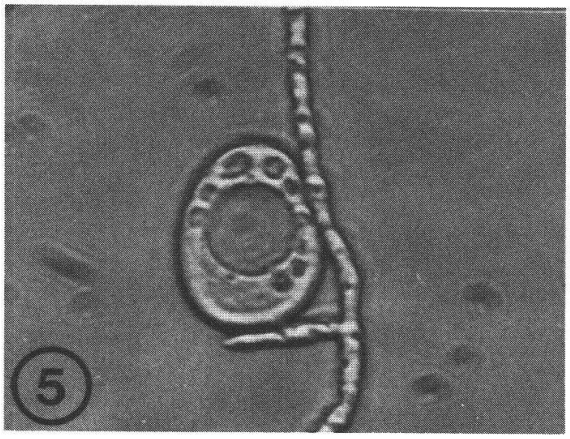
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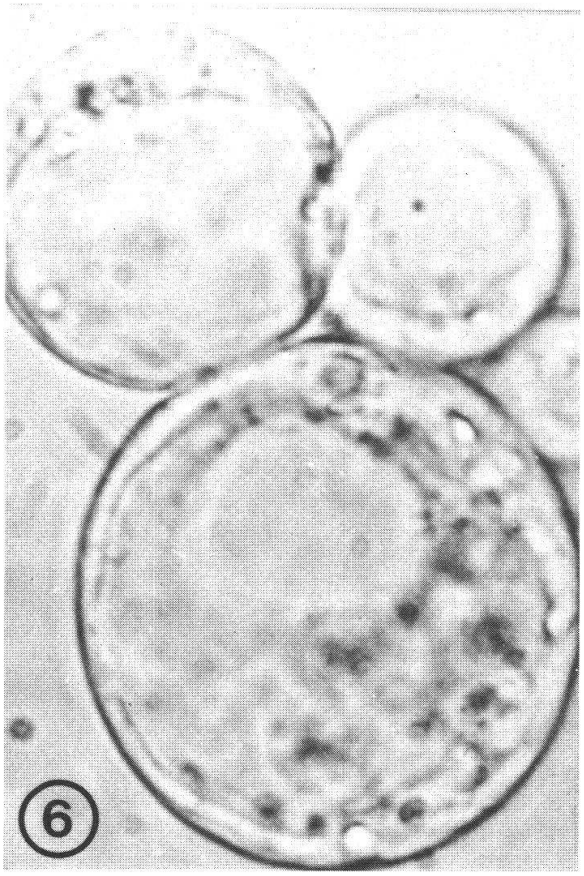
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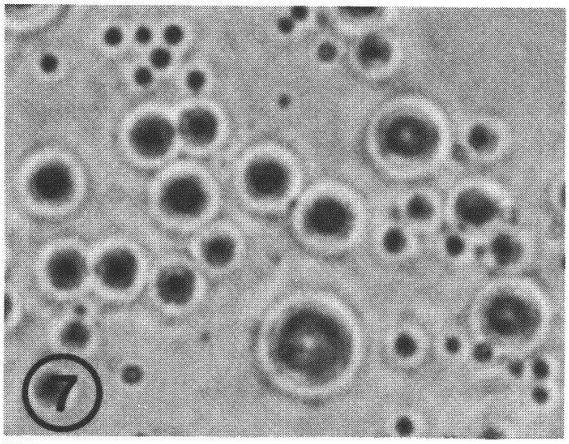
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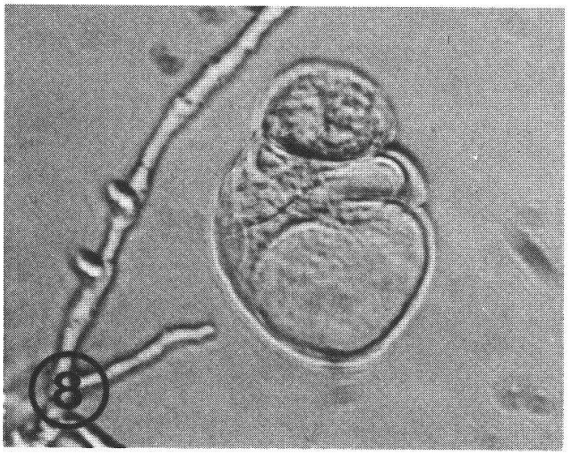
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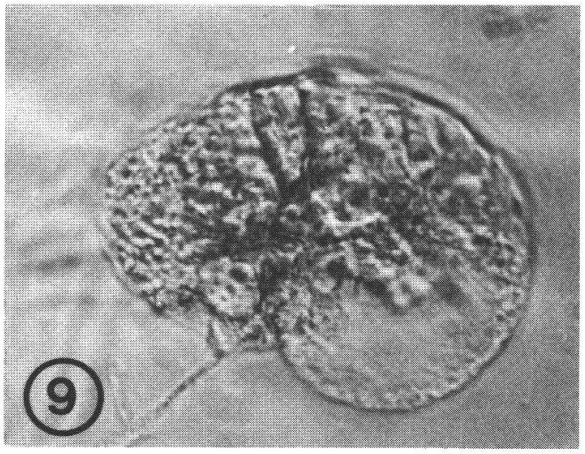
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protoplasts of the nonhost, *M. candelabrum,* remained intact. The enlargement of the protoplasts in *M. pusilla* and *P. articulosus* occurred irrespective of their contact with the mycoparasite (Figs. 6 & 8), thus suggesting the involvement of some extracellular product released upon mycoparasite spore germination. The control slides for protoplasts without the spores of the mycoparasite did not show these changes.

Incubation of protoplasts of hosts and nonhost fungi with the culture filtrate of the mycoparasite showed no enlargement or vacuolation of protoplasts. These protoplasts regenerated after 4 days of incubation. Their regeneration frequency did not differ from that on nutrient broth which served as control (Table 3). Dialysis of culture filtrate or nutrient broth markedly decreased the regenerating ability of the protoplasts. Maximum regeneration of protoplasts in all three fungi occurred in malt yeast extract medium within 24 h of incubation (Fig. 2).

These results show that the culture filtrate of the mycoparasite does not induce enlargement, vacuolation or bursting of protoplasts as observed in the presence of germinating spores of the mycoparasite. Wakabayashi et al. (1985) observed the formation of giant protoplasts of *Pleurotus cornucopiae* in the presence of cell wall lytic enzyme cellulase "ONOZUKA" RS. These protoplasts increased in size not from the fusion of protoplasts but from physiological growth of the protoplast which is inhibited from regenerating into normal hyphae by consistent degradation of cell wall component by the lytic enzyme. It is possible that some similar product of the mycoparasite induced upon incubation with host (susceptible and resistant) protoplasts may be responsible for enlargement of the latter. The germinating spores of the mycoparasite, *P. virginiana,* are known to produce higher levels of proteinase, chitinase and chitosanase activities in the cell extract than in the culture filtrate (Balasubramanian and Manocha 1986). This may explain the absence of enlargement in culture filtrate due to very low concentration of these enzymes.

Interaction of mycoparasite with the protoplasts of the susceptible and resistant hosts, *M. pusilla* and *P. articulosus,* respectively, did not exhibit the specificity observed

Fig. 3. Protoplasts of *Mortierella pusilla* × 400.

Fig. 4. Attachment of germinating spore of the mycoparasite *Piptocephalis virginiana* to *Mortierella pusilla* protoplast on agarose × 1000.

Fig. 5. Vacuolation of agarose embedded *Mortierella pusilla* protoplast upon prolonged contact with the mycoparasite × 400.

Fig. 6. Swelling and vacuolation of agarose embedded *Mortierella pusilla* protoplasts incubated with the germinating spores of the mycoparasite. Increase in protoplast size is observed irrespective of contact with the mycoparasite × 1000.

Fig. 7. Protoplasts of *Phascolomyces articulosus* × 400.

Fig. 8. Dumbbell shaped highly vacuolated protoplasts of *Phascolomyces articulosus* on agarose after apparent fusion. Increase in protoplast size seen after incubation with germ tubes of the mycoparasite × 400.

Fig. 9. Bursting of agarose embedded *Phascolomyces articulosus* protoplast after prolonged contact with the mycoparasite. The cytoplasm is seen streaming out of ruptured protoplast × 1000.

with the intact hyphal cells of these fungi (Manocha and McCullough 1985). The mycoparasite did not interact with the protoplasts of the nonhost, *M. candelabrum*. Similar results were obtained for the intact cells as well. Whatever the mycoparasite released in the vicinity of protoplasts embedded in agarose was not lost, instead it induced enlargement and vacuolation of protoplasts of host fungi.

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