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SPORE GERMINATION AND DEVELOPMENT OF YOUNG MYCELIA IN SOME *RHIZOPOGON* SPECIES

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ABSTRACT

Spore germination in *Rhizopogon abietis*, *R. luteolus*, *R. roseolus* and *R. villosulus* was induced in the presence of *Rhodotorula glutinis* and activated charcoal, in agar medium (N6:5). In one *R. roseolus* sample, 51% of spores germinated within 35 days, allowing observation of the course of spore germination and the different developmental patterns of homokaryotic mycelia. In these plates, spores showed two times of germination. The spores that germinated early produced an apical germ tube. Later other spores germinated in proximity to young mycelium, by forming a germ vesicle. One of the hyphal growth patterns obtained (interruption-swelling-ramification) is similar to that reported for other fungi. With this technique for inducing spore germination, it is possible to obtain enough monosporic cultures to perform mating tests.

Key words: *Basidiomycotina*, Hypogeous, Monosporic Cultures.

1. Introduction

The absence of any morphological differences between some *Rhizopogon* taxa, mainly in *R. roseolus* (Corda) Th. M. Fr. complex, means that the separation into different species and varieties made by other authors is exclusively on the basis of the spore size. We thought that using the intraspecific reaction of somatic incompatibility (mating tests) may be useful in defining this species as one or more taxa. Mating tests have been used in other groups of basidiomycetes (BOIDIN, 1986; FRIES, 1983, 1985, 1987a), some of them closely related to *Rhizopogon* (FRIES, 1987b; FRIES & NEUMANN, 1990; FRIES & YU-PING, 1992).

As reported in FRIES & NEUMANN (1990), very little is known about the genetics of ectomycorrhizal basidiomycetes. The basidiospores of most of them require special environmental conditions for germination, which are still

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unknown for many species (FRIES *et al*., 1985; FRIES *et al.,* 1987). Spores only a few species have been germinated under controlled conditions, a necessary prerequisite to obtain monosporus (homokaryotic) mycelia to perfom mating tests. BULMER (1964) appears to be the first investigator to report successful germination experiments on one *Rhizopogon* sp., though he obtained a low percentage of germination (0.1%). THEODOROU & BOWEN (1987) reported higher percentages (46-69%) when roots of *Pinus pinaster* Ait. were coated with spores of *Rhizopogon luteolus* Fr. & Nordholm. Following this method, MILLER *et al.* (1993) achieved up to 11%, in the rhizosphere of *Pinus contorta* Douglas coated with *Rhizopogon rubescens* (Tul.) Tul.

Numerous ectomycorrhizal research teams are studying the effect of host-fungus specifity by inoculating roots with spore suspensions of *Rhizopogon*, which may permit them to follow the process of spore germination in the rhizosphere. We decided instead to follow Fries' technique to try to understand the spore germination process in *Rhizopogon*, as well as to obtain homokaryotic mycelia for future experiments about incompatibility patterns, that may help us to define the possible groups in *R. roseolus*, as well as in other species.

2. Material and methods

2.1 Material

Agar medium.- Germination tests were always performed in plastic petri dishes of 90 mm diameter containing 10 ml of medium. The composition of the Fries' Modified Medium 3 (N6:5) is given in Table 1 (FRIES, 1978). The trace elements ($CaCl_2.2H_2O$; $ZnSO_4.7H_2O$; $MnSO_4.4H_2O$; Fe-citrate.$3H_20$) were made up separately as a 1000 times concentrated solution of which 1 ml is added per liter of medium. The same was done with the vitamin mixture. The medium was sterilized by autoclaving at 1 Kg/cm^2 for 20 minutes.

Table 1. Composition of Fries' Modified Medium 3, diluted (and supplemented with a vitamin mixture) to serve as a medium for spore germination (N 6:5) (Fries, 1978).

Agar	15.00 g	Thiamine chlorhydrate	100.00 µg
D-Glucose	4.00 g	Pyridoxine	100.00 µg
NH_4-tartrate	1.00 g	Riboflavin	100.00 µg
KH_2PO_4	0.20 g	Nicotinic acid	100.00 µg
$MgSO_4.7H_20$	0.10 g	Calcium pantothenate	100.00 µg
NaCl	20.00 mg	p-Aminobenzoic acid	100.00 µg
$CaCl_2.2H_2O$	26.00 mg	Biotin	25.00 µg
$ZnSO_4.7H_2O$	0.89 mg	myo-Inositol	10.00 µg
$MnSO_4.4H_2O$	0.81 mg		
Fe-citrate.$3H_2O$	0.80 mg	Distilled water to	1000 ml

Spore suspensions.- Basidiospores were obtained from 41 fruitbodies of 27 collections (Table 2) belonging to six species (*R. abietis* A.H. Smith, *R. aurantiacus* A.H. Smith, *R. luteolus*, *R. roseolus*, *R. subsalmonius* A.H. Smith

Table 2. Collections used in spore germination tests, indicating the current species name, the herbarium number with subscript indicating storage conditions (A: + 26º C; B: + 4º C; C: -18º C; D: air dried material) geographic origin (*= Sweden, without *= Spain), collection date, inoculation date and germination (+: spore germination, -: no spore germination).

Current name	Herb Nº BCC-MPM	Geographic origin	Collection date	Inoculation date	Germination
R. abietis	1557_B	Uppsala*	19.08.92	21.08.92	+
R. abietis	1872_A	Tarragona	12.10.94	15.10.94	-
R. abietis	1872_B	Tarragona	12.10.94	15.10.94	+
R. abietis	1872_C	Tarragona	12.10.94	15.10.94	+
R. aurantiacus	1560_B	Uppsala*	02.09.92	04.09.92	-
R. luteolus	1545_D	Barcelona	08.06.92	05.08.92	-
R. luteolus	1573_C	Girona	30.10.92	18.03.93	+
R. luteolus	1573_D	Girona	30.10.92	18.03.93	-
R. luteolus	1591_D	Girona	02.12.92	18.03.93	+
R. luteolus	1727_C	Girona	07.11.93	04.03.94	-
R. luteolus	1873_B	Girona	09.10.94	14.10.94	+
R. luteolus	1873_C	Girona	09.10.94	14.10.94	+
R. roseolus	1498_C	Tarragona	15.03.92	05.08.92	+
R. roseolus	1508_D	Mallorca	08.10.91	05.08.92	+
R. roseolus	1529_D	León	16.12.91	05.08.92	+
R. roseolus	1546_D	Teruel	30.05.92	05.08.92	-
R. roseolus	1570_C	Girona	30.10.92	05.05.93	+
R. roseolus	1570_D	Girona	30.10.92	18.03.93	+
R. roseolus	1572_C	Girona	30.10.92	05.05.93	-
R. roseolus	1592_D	Girona	02.12.92	18.03.93	+
R. roseolus	1625_B	Barcelona	20.03.93	26.03.93	-
R. roseolus	1656_B	Girona	26.05.93	28.05.93	-
R. roseolus	1657_B	Girona	26.05.93	28.05.93	+
R. roseolus	1797_A	Barcelona	11.03.94	17.03.94	-
R. roseolus	1797_B	Barcelona	11.03.94	17.03.94	+
R. roseolus	1797_C	Barcelona	11.03.94	17.03.94	+
R. roseolus	1861_C	Girona	25.05.94	27.05.94	-
R. roseolus	1859_C	Teruel	21.05.94	27.05.94	+
R. subsalmonius	1652_B	Girona	26.05.93	28.05.93	-
R. villosulus	1648.1_B	Barcelona	13.05.93	19.05.93	+
R. villosulus	1648.2_B	Barcelona	13.05.93	19.05.93	+
R. villosulus	1648.3_B	Barcelona	13.05.93	19.05.93	+
R. villosulus	1648.4_B	Barcelona	13.05.93	19.05.93	+
R. villosulus	1648.5_B	Barcelona	13.05.93	19.05.93	+
R. villosulus	1794_C	Barcelona	23.13.93	04.03.94	+
R. villosulus	1795_C	Barcelona	23.12.93	04.03.94	+
R. villosulus	1800_B	Barcelona	17.03.94	18.03.94	+
R. villosulus	1857_A	Barcelona	19.05.94	20.05.94	-
R. villosulus	1857_B	Barcelona	19.05.94	27.05.94	-
R. villosulus	1857_C	Barcelona	19.05.94	27.05.94	-
R. villosulus	1857_D	Barcelona	19.05.94	27.05.94	-

and *R. villosulus* Zeller). Spore suspensions were made in a laminar flow cabinet according to the method shown in Figure 1 a-e. The spore concentration was

determined, in the first experiments, using a haemocytometer; in general, 10^6-10^7 spores/ml.

2.2. Germination tests

Germination tests were carried out following the method of FRIES (1977) (Figure 1 e-h), which is based on two principles: (a) the inoculation of a germination-inducing colony of the yeast, *Rhodotorula glutinis* (Fr.) Harrison, among the spores plated on to agar medium (FRIES, 1941,1943), and (b) the addition of activated charcoal to remove inhibitory substances (FRIES, 1977). For germination tests, 0.1 ml of aqueous spore suspension was plated on to agar petri dishes and stored 24 h at room temperature (this is the time required for the excess water to evaporate). Activated charcoal was poured on one third of the medium surface and *Rhodotorula glutinis* was inoculated, at a distance of 2.5 cm from the petri dish edge, at the border between the medium and the activated charcoal overlay. Cultures were incubated at +25ºC. A minimum of 8 plates for each experiment.

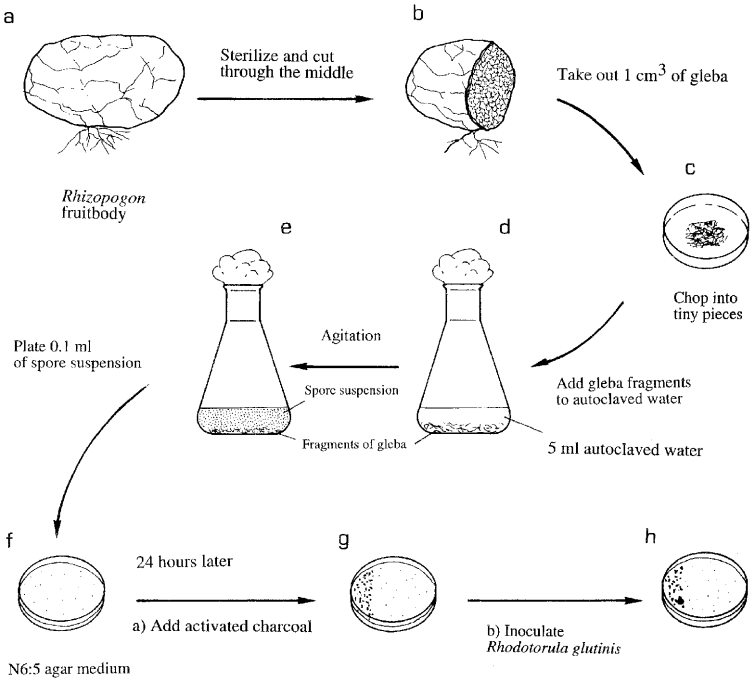

Figure 1. Schematic diagram of spore germination test protocol following FRIES (1977).

Once a week, the plates were inspected for the appearance of germinating spores or young mycelia. In some cases, microphotographs were taken directly from agar plates with a Nikon HFM unit mounted on a Nikon Labophot binocular microscope. In each plate five areas were monitored (Figure 2).

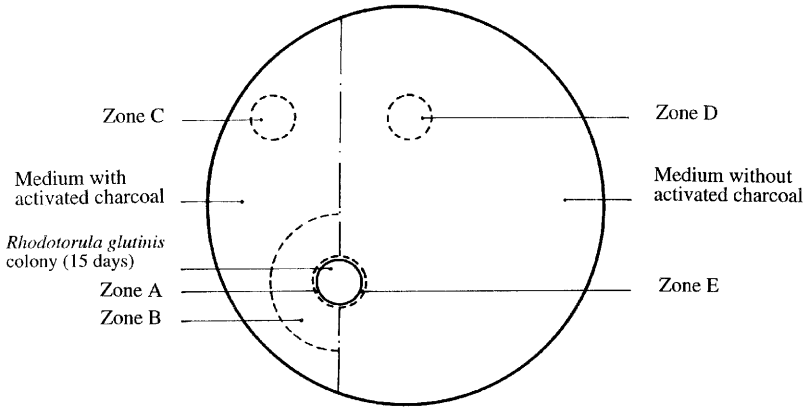

Figure 2. Schematic diagram of zones in the germination test. Zone A: Activated charcoal, within 1 mm of the edge of the *Rhodotorula glutinis* colony. Zone B: Activated charcoal, 10 mm of the edge of the *R. glutinis* colony. Zone C: Activated charcoal, 35 mm from the center of *R. glutinis* colony. Zone D: No activated charcoal, 35 mm from the center of *R. glutinis* colony. Zone E: No activated charcoal, within 1 mm of the edge of the *R. glutinis* colony.

2.3. Criteria of germination

The criteria of spore germination included not only the protrusion of the germ tube, but also some other markers indicated in SUSSMAN (1966). Morphological criteria, such as changes in the diameter, length, volume or shape of the spore were used as markers of the first irreversible stage in spore germination (FURCH, 1981). Also, according to SUSSMAN (1966) in spores that contain one or two oil droplets, such as in *Rhizopogon*, these appear to coalesce when germination occurs. When the germ tube reached a length equal or superior to the spore length it is referred to as young mycelium.

3. Results and discussion

Spore germination was observed in 61% of the experiments, which included 4 species of the six included in this study: *R. abietis*, *R. luteolus*, *R. roseolus* and *R. villosulus* (Table 2).

3.1. Storage period and temperature

The storage period was very variable (Table 2), and successful germination was detected in samples stored between 1 day to 10 months. However, the highest percentage of germination was obtained in samples stored 5 days (51%, *R. roseo-lus*, BCC-MPM 1859_C) and 3 months (25%, *R. villosulus*, BCC-MPM 1570_C).

Germination was not observed in any material stored at room temperature. In the other storage conditions, germination was observed in 68.7% of treatment B, 69% of C and 55% of D.

Though the percentage of succesful experiments in storage condition B, C and D was similar; the percentage of germinating spores varied. Table 3 shows the percentage reached in each experiment, indicating if a young mycelium developed or not. In 77.6% of experiments (100% of B and D, 33% of C), the highest percentage reached was 1.0%. In the rest of the samples (C), the percentage varied from 1.5% to 51%. Young mycelium was observed in five experiments, in four fruitbodies stored at -18ºC and one at +4ºC.

Although these results are not conclusive, storage temperature of -18ºC appeared to maintain with a greater consistency the ability of *Rhizopogon* spores to germinate and develop a young mycelium. These results agree with previous germination experiments reported in the literature. BULMER & BENEKE (1962) observed that the greatest number of germinated spores from *Calvatia gigantea* was obtained from fruitbodies stored at -18ºC. BULMER & BENEKE (1964) reported that spores of *Lycoperdon pusillum* germinated after 4 years stored at -18ºC. HESS & WEBER (1976) improved germination of *Polyporus tomentosus* basidiospores after three months storage at -18ºC, but their viability was reduced greatly after three months of storage at 0ºC. FRIES (1984) considers that *Flammula alnicola* and *F. conissans*, have their optimal germination if stored at -7ºC for 10 weeks up to one year; which fits well with the spore mission of spending a winter in the soil and attaching to a tree-root in spring or summer. SUSSMAN (1966) observed that high temperature serves as an activator in saprophytic fungi; whereas pathogens or mycorrhizal fungi respond to cold treatment.

On the other hand, storage at -18ºC seems to maintain germinability better that other storage methods mentioned in the literature. STACK *et al.* (1975) established through mycorrhizal synthesis, that spores lyophilized and stored, maintain their germinability; though attempts to germinate in three different agar media were unsuccessful.

At this moment, we do not know how the low temperatures improve spore germination; although the thermal shock may induce changes in the spore wall, modifying its permeability to substances in the medium.

3.2. Incubation time

In general, germination took place 2-4 weeks after the spore suspensions were plated (Table 3). However, spores germinated as quickly as 7 days (*R. abietis*, BCC-MPM 1557_B) and as slowly as one year *(R. villosulus*, BCC-MPM 1648.4_B). The median time of incubation reported in the literature for other fungi (using Fries's method), is very similar to that obtained here for *Rhizopogon*: (a) 2 weeks for *Amanita muscaria*, *Paxillus involutus* (FRIES, 1978) and *Cantharellus cibarius* (FRIES, 1979b) and (b) 3 or 4 weeks for *Leccinum scabrum* (FRIES, 1978). In the rhizosphere of *Pinus contorta*, MILLER *et al.* (1993) observed spore germination after 13 days in *Rhizopogon rubescens* and 15 days for *Suillus tomentosus*.

3.3. Effects of charcoal and inducer organism

In all succesful experiments, the first germinating spore was observed close to the border of *Rhodotorula glutinis* in the medium with activated charcoal (zone A). In this zone, the highest percentage of germination took place (Table 3). This

Table 3. Collections where spore germination was observed, indicating the current species name, the herbarium number with subcript indicating storage conditions (A: +26º C; B: +4º C; C: -18º C; D: air dried material), incubation time days between inoculation date and the first germination observed), percentage of spore germination (zone A) and the presence (+) or not (-) of a developed mycelium.

Current name	Herb Nº BCC-MPM	Incubation days	% spore germination	Mycelium
R. abietis	1557_B	7	1.0	-
R. abietis	1872_B	10	1.0	-
R. abietis	1872_C	10	1.0	-
R. luteolus	1573_C	10	5.0	+
R. luteolus	1591_D	25	1.0	-
R. luteolus	1873_B	15	1.0	-
R. luteolus	1873_C	15	1.0	-
R. roseolus	1498_C	20	1.0	-
R. roseolus	1508_D	20	1.0	-
R. roseolus	1529_D	20	1.0	-
R. roseolus	1570_C	23	5.0	-
R. roseolus	1570_D	41	1.0	-
R. roseolus	1592_D	60	1.0	-
R. roseolus	1657_B	25	1.0	+
R. roseolus	1797_B	92	1.0	-
R. roseolus	1797_C	11	5.0	+
R. roseolus	1859_C	21	51.0	+
R. villosulus	1648.1_B	41	1.0	-
R. villosulus	1648.2_B	30	1.0	-
R. villosulus	1648.3_B	54	1.0	-
R. villosulus	1648.4_B	365	1.0	-
R. villosulus	1648.5_B	23	1.0	-
R. villosulus	1794_C	14	1.5	-
R. villosulus	1795_C	48	25.0	+
R. villosulus	1800_B	20	1.0	-

result fits well with those reported by other authors (WILSON & BENEKE, 1966). However, in general, after two weeks it was not possible to monitor effects of charcoal and inducer organism because many spores collapsed and lysed shortly after germination, as observed by MILLER *et al.* (1993). Only in plates with spores of *R. roseolus* (BCC-MPM 1859_C), was it possible to monitor these effects 160 days. However, after 49 days of incubation, as in the other experiments, spores started to collapse making it difficult: it was not possible, in some spores, to determine whether they had germinated or not, before lysing. Because of that, to avoid misleading results, Figure 3 is based on the first 49 days.

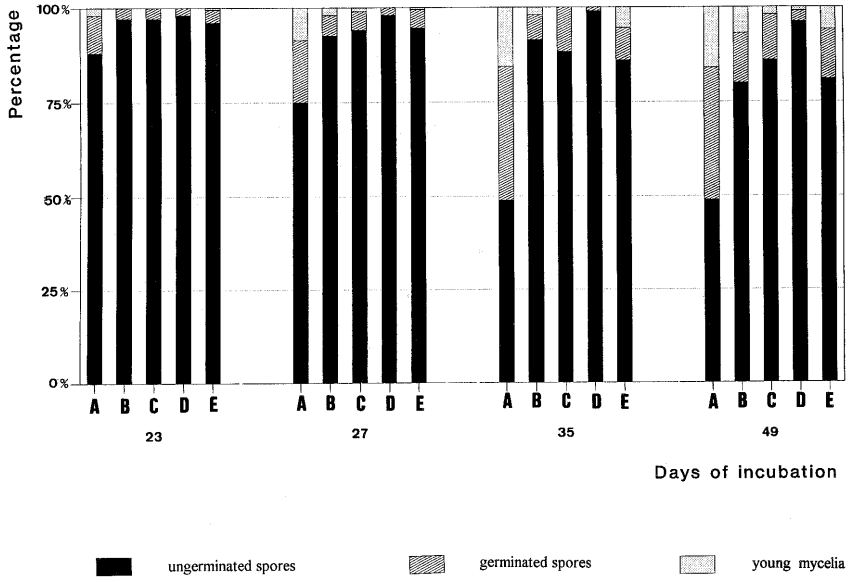

Figure 3. Bar diagram showing the percentange of ungerminated spores, germinated spores and young mycelia in the 5 zones monitored (*R. roseolus,* BCC-MPM 1859_C) .

Thus, in zone A, by 23 days, there was 10% spore germination and 2% young mycelia; whereas in the other zones, percentages were lower and similar (2-4%). With time, the percentage increased in each zone, but by different magnitudes. Thus, by 35 days, 51% germination was reported in zone A (35.4% germinating spores and 16% young mycelia); in zones B, C and E germination was 8.6%, 11.4% and 14%, respectivelly; and in zone D only 1.0 %.

After 49 days, in zone A, the percentages were similar to that at 35 days; in B, C and E zones, the percentage of germinating spores was similar (13%), but the percentage of young mycelia was triple in B and E (ca. 7%) compared to C (2%). The percentange of germination in zone D was 4% (3% germinating spores and 1% young mycelium).

FRIES (1966) suggested that *Rhodotorula* could have two different effects, not only to remove the inhibitory factor present in media containing extracts of malt, yeast or other natural products, but also to exert a stimulating influence on germination. FRIES (1976) reported that removal of ammonium from the medium and the exudation of amino acids might be the activating effect of a yeast colony.

WINKELMANN (1990) noted that germination is dependent of the presence of certain microbial siderophores (small molecular chelators, which function in transport and storage of iron), which are released and metabolized during germination of spores. The rhodotorulic acid is a typical siderophore of heterobasidiomycete yeasts (*Rhodotorula*).

Our experiments show the benefit of *Rhodotorula* not only on spore germination, but also on the development of young mycelia. Zone E (only *Rhodotorula)* has 3 times the percentage of young mycelia as zone C (only charcoal) and 6 times that zone D (without supplements). The beneficial effect of the *Rhodotorula* is higher when activated charcoal is present on the medium. Thus, in zone A, the total percentage of germination (germinating spores + young mycelia) obtained at 35 days in *R. roseolus*, is similar to that obtained in the rhizosphere of *Pinus pinaster* coated with spores of *R. luteolus* (THEODOROU & BOWEN, 1987). FRIES (1977) reported 10-20% germination in *Laccaria laccata*, but only on plate areas where *Rhodotorula glutinis* and activated charcoal were together. BIRRAUX & FRIES (1981) consider that during autoclaving, a chemically unidentified inhibitor is formed in the agar, inhibiting the spore germination in many ectomycorrhizal fungi; the presence of activated charcoal or pretreatment of the agar plate with charcoal usually counteracts the effect. However, as reported by SUSSMAN (1966) the role of some activation substances or processes must be interpreted with caution because it is not clear whether they are required as activators or for subsequent development.

3.4. Germination process and development of mycelia

Rhizopogon spores are uni or biguttulate. In many samples of *R. roseolus* and *R. villosulus* the only germination sign observed was the increment of the spore length, and the coalescence of droplets. *R. villosulus* (BCC-MPM 1795_C) had a high percentage of spores whose droplets seemed coalesced (25% in Table 3), but only 0.1% formed a germ tube.

In *R. abietis* (BCC-MPM 1557_B), some spores swelled up to form a sphere with a diameter up to three times the original, without protrusion of a germ tube. In *R. roseolus* (BCC-MPM 1797_C), this swelling was also observed in some spores, but 42 days after plating, a germ tube grew out (Figure 4a). Plates of *R. roseolus* (BCC-MPM 1797_C) show spores that undergo direct germination, generally, by an apical germ tube. This is, in general, the process observed in the rest of the successful experiments; except in *R. abietis* where it was always lateral (Figure 4b). Sometimes, germinating spores develop two or more germ

tubes (Figure 4c). As in other fungi (MACKO, 1981), the apperance of germ tubes was asynchronous (Figure 4d).

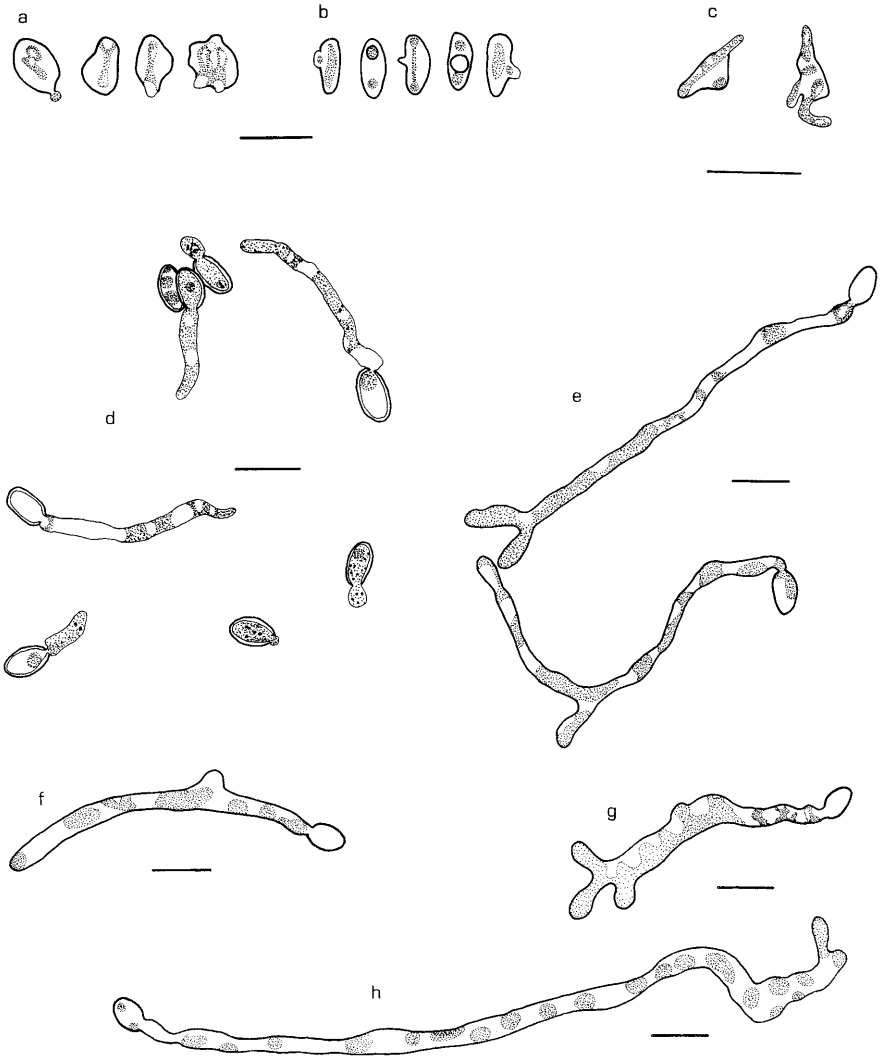

Figure 4. Spore germination and development of young mycelium in some *Rhizopogon* species. **a**: *R. roseolus* BCC-MPM 1797_C, Zone A, 42 day culture, spores with apical germ tubes. **b**: *R. abietis* BCC-MPM 1557_B, Zone A, 7 day culture, spores with lateral germ tube. **c**: *R. roseolus* BCC-MPM 1797_C, Zone A, 34 day culture, spores with more that one germ tube. **d**: *R. roseolus* BCC-MPM 1859_C, Zone A, 23 day culture, ungerminated spores close to those with germ tube or even with short young mycelia. **e-h**: *R. roseolus*, BCC-MPM 1859_C, Zone A, 35 day culture, some aspects of the mycelium development. (bar= 10 µm).

The information obtained about the effect of activated charcoal and *Rhodotorula* in the germination process and the development of homokaryotic mycelia, is very scant, due to the lysis of spores after germination. Spores of *R. luteolus* (BCC-MPM 1573_C), *R. roseolus* (BCC-MPM 1657_B, BCC-MPM 1797_C) and *R. villosulus* (BCC-MPM 1795_C) developed young mycelia in zone A, but they died 14 days after plating. In all cases, young mycelia did not develop a thick wall, it was continuous with the inner spore wall. Only in plates with *R. roseolus* (BCC-MPM 1859_C), have we observed the process of germination and the subsequent hyphal development in the different control zones up to 160 days after plating:

Zone A.- Twenty three days after plating, germination occurred by apical germ tube, with or without development of young mycelium (Figure 4d). By 35 days young mycelia started to branch dichotomously (Figure 4e), laterally (Figure 4f) or, as ROBERTSON (1965) reported, as "a two-dimensional Christmas tree" (orthotropically), developing an acropetal series of branches (Figure 4g). In general, mycelia look very vacuolized (Figure 4h). Forty-nine days after plating, the still ungerminated spores started producing spherical germ vesicles, that appear strongly light-refractive in contrast to the spores (Figure 5a). In the light microscope, these vesicles seem thick walled; however, as reported in MARTÍN & FERRÁN (1995), the vesicle wall easily breaks when observed under SEM. Once this vesicle is formed it seems that different models of behaviour occur: (a) the vesicle increased in size without developing hyphae (Figures 5b and 7a); (b) one or several germ-hyphae grew out from the vesicle and finally died; (c) a vigorous mycelium developed from the vesicle (Figure 5c). In some cases, the mycelium ceased elongation and started swelling up forming one or more vesicles (Figures 5d and 7b); 93 days after plating, these hyphae branched from the vesicle (Figure 5e). In some hyphae, the vesicles grew up to 20 mm diameter and produced subapical branches (Figure 5f).

Zone B.- Germination first occurred by apical germ tube. Forty nine days after plating, as in zone A, the still ungerminated spores formed a vesicle, branched (Figure 6a) or not. Young mycelia were observed, some of them branched monopodially and orthotropically, reaching up to 350 mm length (Figure 6b).

Zone C.- Up to 60 days after plating, germination always occurred by apical germ tube, without vesicle formation (Figure 6c); but after this time, hyphae swelled forming vesicles (Figure 6d) and branches. 144 days after plating the still ungerminated spores produced spherical germ vesicles (Figure 6e), as observed in zone A after 68 days.

Zone D.- In general, spores germinated by apical germ tube, but 60 days after plating some spores that had germinated by forming a vesicle, the vesicle appeared branched (Figure 6f).

Zone E.- The germination patterns and posterior development of young mycelia were similar and occurred at the same time as that in zone B.

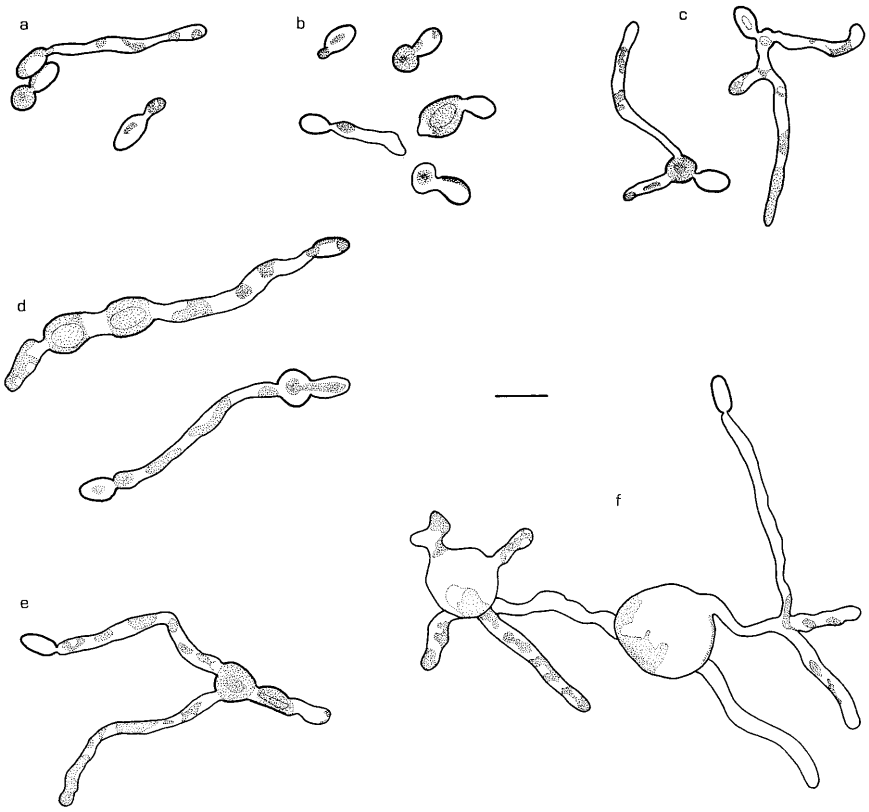

Figure 5. Spore germination and development of young mycelium in *R. roseolus* BCC-MPM 1859_C, Zone A. **a-b**: Spores showing spherical germ vesicles (→), 60 day culture. **c**: Hyphae growing from the germ vesicles, 93 day culture. **d**: Vesicles formed once the mycelium ceased elongation, 60 day culture. **e**: Hyphae branched from the vesicle, 93 day culture. **f**: Vesicles that have grown up to 20 µm and have developed many hyphae, 93 day culture. (bar= 10 µm).

As reported by FRIES (1979 a) in *Leccinum scabrum*, in all zones of plates BCC-MPM 1859_C, germination first occurred by apical germ tube. Later, a second wave of germination was observed in the vecinity of young mycelium (after 49 days: A, B and E; after 60 days: C,D), that was related to the higher percentage of young mycelia obtained. FRIES (1981) called this effect, the inductor reaction; spores respond by forming a germ vesicle. He reported that

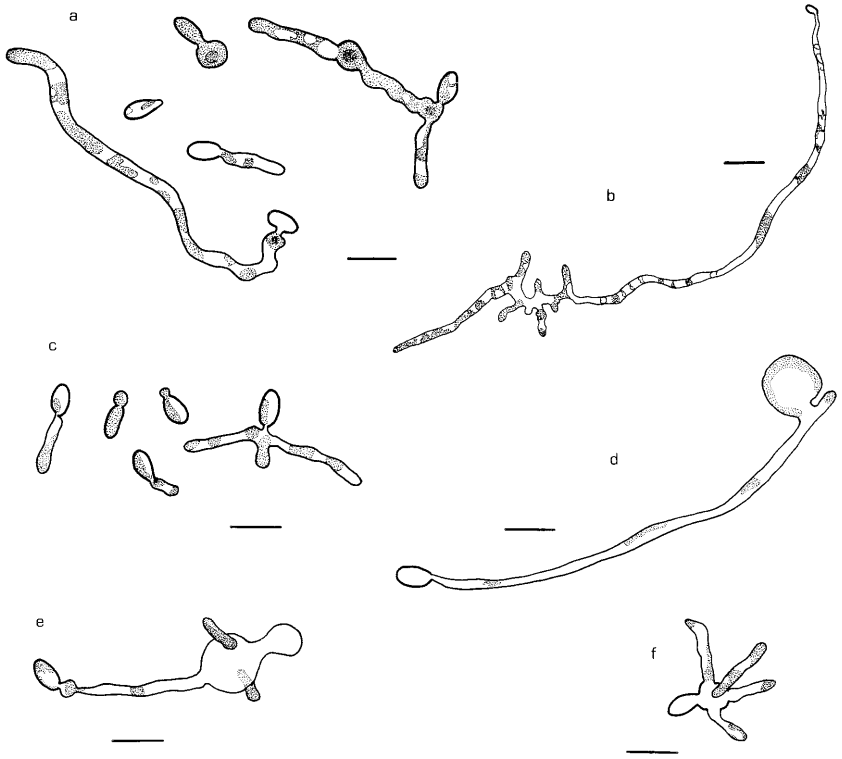

Figure 6. Spore germination and development of young mycelium in *R. roseolus* BCC-MPM 1859_B, Zone B. **a**: Germ vesicle (→), 49 day culture. **b**: Young mycelium branched orthotropically, 93 day culture. **c**: Spore germination by apical germ tube, 49 day culture. **d**: Hypha swelled showing a vesicle, 93 day culture. **e**: Hypha developed from a spherical germ vesicle, 144 day culture. **f**: four hyphae developed from a spherical germ vesicle, 93 day culture. (bar= 10 µm).

there is a high degree of specifity between the inductor mycelia and the basidiospores. This inductor reaction could be used to check whether the mycelia and the spores plated on one petri dish belong to the same species or not. However, more studies are required before using the inductor reaction as a test in *Rhizopogon*.

The hyphal growth patterns observed in *R. roseolus* are similar to those reported by other authors. ROBERTSON (1965) considers that the hyphal patterns are modified by the nutritional state of agar on which the colony is growing. According to DEACON (1988), the process of interruption-swelling-ramification is a behaviour very frequent in the apical growth in a great number of fungi. HICKMAN (1969) reported that in some parts of old (dikaryotic) hyphae of *Phytophthora cinnamomi* the protoplasm acumulates food reserves and secretes a

thick wall cutting off the hyphae on each side, and forming a resistant vegetative cell or chlamydospore. However, the swelling observed in Figures 5d and 7b, belongs to a young homokariotic mycelium. LACEY *et al.* (1991) reported that the swelling observed in mycelia of *Aspergillus flavus* is caused by *Bacillus amyloliquefaciens;* these hyphae often appeared granulated or vacuolated with malformations. In our experiments swelling was detected not only in zone A, B and E, close to the *Rhodotorula* colony; but also in zone C and D, where the presence of *Rhodotorula* on the plate did not affect too much the percentage of germination.

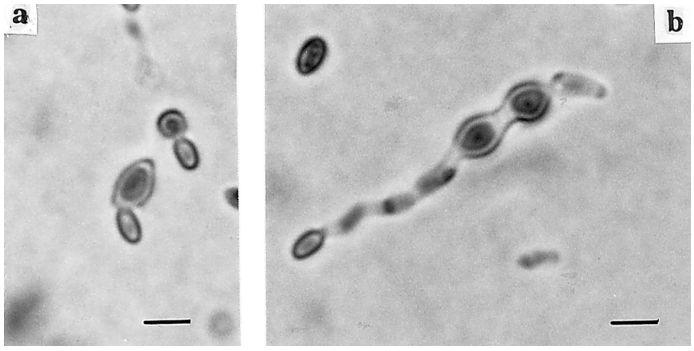

Figure 7. Spore germination and development of young mycelium in *R. roseolus* BCC-MPM 1859_C, Zone A, 60 day culture. **a**: Spores with a well developed germination vesicle. **b**: Young mycelium showing two vesicles. (bar= 10 µm).

4. Conclusions

Basidiomes of *Rhizopogon* stored at -18ºC germinated and developed young mycelium with the greatest consistency.

Germination and the development of young mycelia was enhanced in media inoculated with *Rhodotorula glutinis,* and further enhanced when activated charcoal is also present on the medium.

With the simple technique for inducing spore germination in *Rhizopogon* described here, a sufficient number of monosporic cultures can be obtained for use in mating tests and other biological investigations, to contribute to the solution of certain problems regarding the taxonomic status of some complex groups.

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