EXAMINATION OF SPORES AND YOUNG MYCELIA OF RHIZOPOGON ROSEOLUS BY SCANNING ELECTRON MICROSCOPY

MARTA P. MARTIN & MARIA JOSE FERRAN

Rhizopogon Fr. is one of the most important genera of ectomycorrhizal fungi. Although basidiospore inocula have been used in nurseries for some years, few reports of attempts to germinate basidiospores of Rhizopogon are found in the literature. Bulmer (1964) appears to be the first investigator to report germination experiments with species of this genus.

Fries (1941) developed a method to induce spore germination in several species of gasteromycetes and ectomycorrhizal hymenomycetes. The first fruitful experiment was the result of a happy coincidence: on a malt extract agar plate with spores of Lycoperdon umbrinum Pers.: Pers. some contamination appeared, and close to one unidentified yeast colony a few germinating Lycoperdon spores were detected. This was the first step to developing a method based on the introduction of a germination-inducing colony of yeast, for instance Rhodotorula glutinis (Fr. & Harrison), among the spores plated on to agar media (Fries, 1943). The same author improved Laccaria laccata (Scop.: Fr.) Berk. germination 10 to 20% by adding activated charcoal to the agar media (Fries, 1977).

Following the methodology of Fries one of us (MPM) had the opportunity of observing spore germination and different patterns of development of young mycelia, from spore cultures of Rhizopogon roseolus (Corda) Th. M Fr., collected in Teruel, Spain (herbarium number BCC-MPM 1859).

Under the light microscope, spores of R. roseolus are cylindrico-ellipsoid to fusoid (6.5-8 x 2.5-3.5 μm), smooth and with one or two strongly light-refractive droplets. One sign of the germination process is the increment of the spore length, accompanied by the coalescence of its droplets. Twenty three days after plating, direct germination occurred by an apical germ tube, with or without development of young mycelium. After 60 days some spores produced spherical germ vesicles, without protrusion of a germ tube or development of young mycelium. One or several germ-hyphae grew out from the vesicle. In many cases, the hyphal apices ceased elongation and started swelling up forming one or more vesicles. Deacon (1988) reported that the process of interruption-swelling-ramification is frequently observed during apical growth in a great number of fungi.

To obtain more information, we studied the spore morphology under the scanning electron microscope (SEM), using both dried material and agar cultures from the same fruitbody. The principal problem, to select the best method to prepare samples for SEM, is discussed in this paper.

Methods

Dried fragments of the fruitbody used in germination experiments were prepared following three different protocols (A-C): (A) Mounted directly on standard SEM stubs using double-sided adhesive tape and coated with gold (Martin et al., 1993). (B) Fixed with 70% ethanol. Rehydrated 15 min in distilled water, 15 min in ammonia and 15 min in distilled water. Dehydrated in a graded ethanol series (50%, 60% 70%, 80%, 90%, 95%, 100%) in 15 min exchanges. Passed through four exchanges (15 min) of a graded isoamyl acetate series and dehydrated with critical point drying (CPD), using carbon dioxide as the drying fluid, mounted on standard SEM stubs using aluminium tape and coated with gold (Martin & Rocabruna, 1988). (C) Hydrated in saturated chloral hydrate for 24 h, directly fixed and dehydrated in 95% ethanol for 15 min, exchanged to 100% ethanol for 15 min. Passed through four exchanges (15 min) of a graded isoamyl acetate...

Series and dehydrated with CPD as in protocol B, mounted on standard SEM stubs using aluminium tape and coated with gold.

Samples of agar cultures were prepared by cutting 5 x 5 mm blocks of agar from areas containing germinating spores selected under the light microscope. Four different protocols were followed; one of them is the same as process B, above, and the others (D-F) are: (D) Fixed in 1.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2–7.4 for 1 h 30 min at room temperature, rinsed in four exchanges of phosphate buffer, the first very quick, and the others each 10 min postfixed in 1% osmium tetroxide in the same buffer for 1 h 30 min at 4°C, rinsed in ten 5 min exchanges of distilled water and freeze dried. Freeze-drying was performed at 10⁻² mbar for 24 h, using the Edwards Tissue-Dryer, ETD4; (E) Fixed in fumes of 1% osmium tetroxide in 0.2 M phosphate buffer and freeze dried as in protocol D; (F) Fixed and post-
fixed as described in protocol D. Samples on distilled water were dehydrated through a graded ethanol series and transferred to a graded isamyol acetate series following process in protocol B, as well as dehydrated with CPD. Samples prepared with these protocols were mounted as in protocol B.

All observations were made using a Hitachi S-2300 SEM at 15 kv. Scanning electron micrographs were recorded on Kodak 5-TM x 120.

Results and discussion
Spores of the dried fruitbody appeared very collapsed following protocol A or protocol B; however, with these methods spores of hypogeous and epigeous gasteromycetes did not appear collapsed. These samples were probably poorly hydrated when fixed.

In Rhizopogon roseolus, as in other species of this genus, fresh fruitbodies often show a high percentage of collapsed spores. We can identify these spores because they are smaller, flatter and very often hyaline. When material is dried with warm air to be conserved in the herbarium (BCC), the number of dehydrated spores increases. Thus, protocol A is not recommended for Rhizopogon species. Similar unsatisfactory results occur with protocol B.

In order to improve rehydration, we used, in protocol C, saturated chloral hydrate. According to the literature available, this product has not been employed to rehydrate samples for SEM before. It is frequently used, however, to observe the peridium and gleba of gasteromycetes, under the light microscope. Direct fixation and dehydration with 95% ethanol is recommended in Pisí & Filippini (1994). Protocol C is easier than B; moreover, this process, as is shown in Fig. 1a, seems to be optimum to rehydrate and maintain Rhizopogon spores for SEM. As Pegler & Young (1981) observed in other boletales, spores of R. roseolus present a smooth and thin perisporium; the exosporium is uniformly reticulate.

Blocks of agar prepared according to protocol B were a complete failure. Protocols D, E and F do not wrinkle the agar blocks and, moreover, it was very easy to locate spores, which were darkened by osmium tetroxide. Following protocol D, however, the agar appears torn and provides a very unstable support. Also, both ungerminated and germinated spores as well as young mycelia appear collapsed.

Things were a little different in agar blocks prepared according to protocol E (Fig. 1b–c) and F (Fig. 1d–h). The agar formed a solid support which allowed us to find the spores easily and to take many pictures. However, in protocol E, even though ungerminated spores were preserved in good condition, germinated spores appeared more or less collapsed, whether they germinated by forming an apical tube and a young mycelium (Fig. 1b) or by formation of a germination vesicle (Fig. 1c). In agar blocks prepared following protocol F, spores, vesicles and mycelia were not collapsed as shown in Fig. 1d–h. This is without doubt the best process tested to prepare solid agar cultures for SEM.

Acknowledgements
This paper is dedicated to the memory of Dr Nils Fries (Uppsala, Sweden) in appreciation of his patient guidance in initiating MPM to the mysteries of spore germination.

Many thanks are due to Dr M. Nadal and Dr A. Moret (Barcelona, Spain) for supporting laboratory facilities and to Dr M. Glenn (New Jersey, United States) for her kind English correction.

References