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ENDOPHYTES OF *SERAPIAS PARVIFLORA* PARL. AND *SPIRANTHES SPIRALIS* (L.) CHEVALL. (ORCHIDACEAE): DESCRIPTION OF ENDOPHYTES OF *S. PARVIFLORA*, AND IN VITRO SYMBIOSIS DEVELOPMENT IN *S. PARVIFLORA* AND *SPIRANTHES SPIRALIS*

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ABSTRACT. Endophytes were isolated from *Serapias parviflora* (Orchidaceae) roots. They are described and partially classified under microscope, after growth on PDA. Two fungi had symbiotic characters: A-Sepa-1, an ascomycete, and B-Sepa-1, a basidiomycete. At the same time, many plants of *S. parviflora* and *Spiranthes spiralis* were asymbiotically obtained by sterilisation of seeds and sowing on modified Frosch medium. After transplantation to symbiotic medium (modified Basic Oats), they were inoculated with isolated fungi. *Serapias parviflora* was used as control and *S. spiralis* was used to establish specificity between hosts and endophytes. The two fungi were able to induce symbiosis in *S. parviflora* roots *in vitro*, while only B-Sepa-1 induced symbiosis in *S. spiralis* roots *in vitro*. We conclude that specificity between these fungi and the two orchid species studied *in vitro* is different, possibly substantiating the hypothesis of a potential and ecological specificity.

RESUMEN. Hongos endófitos fueron aislados de raíces de *Serapias parviflora* (Orchidaceae). Fueron descritos y clasificados parcialmente bajo el microscopio de luz luego de ser cultivados en PDA. Dos de ellos presentaron características de simbiontes: la Cepa-A-1, un ascomicete, y la Cepa-B-1, un basidiomicete. Al mismo tiempo muchas plantas de *S. parviflora* y *Spiranthes spiralis* fueron obtenidas asimbióticamente por esterilización de semillas y cultivo en un medio Frosch modificado. Luego de transplantarlas a un medio simbiótico (Oats modificado), las plantas fueron inoculadas con los hongos aislados. *Serapias parviflora* fue utilizada como control y *S. spiralis* lo fue para establecer especificidad entre hospederos y endófitos. Los dos hongos fueron capaces de inducir simbiosis in vitro en raíces de *S. parviflora*, mientras que sólo la Cepa-B-1 indujo simbiosis *in vitro* en raíces de *S. spiralis*. Concluimos que la especificidad *in vitro* entre estos hongos y las dos especies de orquídeas estudiadas es diferente, posiblemente apoyando la hipótesis de una especificidad ecológica potencial.

KEY WORDS: Orchidaceae, *Serapias parviflora*, *Spiranthes spiralis*, symbiotic fungi, endophytes, Italy.

Introduction. Orchids need endomycorrhizae to develop: green orchids need fungi for the early developmental stages, while orchids lacking chlorophyll are always completely dependent upon fungi. Endophytes of orchids are always Mitosporic Fungi. Three classes are recognized in this group: Hyphomycetes, Coelomycetes, and Agonomycetes. To the latter class belong sterile fungi that may produce chlamydospores or sclerotia and the genus *Rhizoctonia* DC., whose members may also produce sclerotia. Most orchid endophytes are ascribed to this group. Only few traits were used to describe this genus (Curtis 1937) but unfortunately they have no taxonomic value since they do not allow discrimination between very similar *Rhizoctonia* species with very different perfect states. Rambelli (1981) suggested the presence of dolipores or clamp connections as a character to assign a fungus to the basidiomycetes when reproductive structures are absent. Moreover, Riess

and Scrugli (1987) suggested to classify orchid endophytes in four morphometrical classes: A, B, C, and D. This classification has no taxonomic value, but because fungi are studied when they are into cortical cells, it gives information on fungal ecology. Three ascomycetes strains with *Rhizoctonia*-like anamorphs were isolated from *Pterostylis* sp. but no seeds germinated when they were inoculated with these fungi (Warcup 1975). Some tropical orchids have ascomycetes as symbiont (Dreifuss & Petrini 1981 and 1984); formerly these fungi were described as *Ascorhizoctonia* Chin S. Yang & Korf, but since they are saprobe fungi and do not produce sclerotia they can not be ascribed to the genus *Rhizoctonia* DC. (Andersen 1986). Septum ultrastructure of *Leptodontidium orchidicola* Sigler & Currah (Mitosporic Fungi) isolated from tropical orchids reveals that this anamorph is related to ascomycetes in having Woronin bodies near its septum pore.

One of the first questions about the relationship between orchids and endophytes deals with the specificity. Bernard (1909) hypothesised a high level of specificity but such close relationship was soon rejected. Burgeff (1936) proposed that specificity existed between some fungi and ecological host groups, and Curtis (1937) suggested a closer relationship between fungus and habitat. Hadley and Harvais (1967) questioned Curtis' ecological specificity because not all fungi isolated from ripe plants were able to support host seed germination. Further works built up evidence in favour of the absence of specificity (Downie 1959, Hadley 1970). Riess and Scrugli (1987) observed that some orchid species (*Ophrys bombyliflora* Link. and *Ophrys tenthredinifera* Willd.) had different endophytes when collected in different sites. They also observed, in the same work, that in *Limodorum abortivum* (L.) Sw. there were simultaneously two endophytes with different morphological characters. Similar results were obtained from Curtis (1937), Downie (1943), Talbot and Warcup (1967), and Harley (1969). Masuhara and Katsuya (1989, 1994), by studying *Spiranthes sinensis* (Persoon) Ames var. *amoena* (M. Bieberstein) Hara, suggested two kinds of specificity: 1) "ecological specificity", i.e. when pelotons are into root cortical cells or into the protocorms in nature (*in situ*); and 2) "potential specificity", i.e. associations between orchids and fungi in other conditions, both *in vitro* or *ex vitro*. Masuhara and others (1993) observed ecological specificity only in some fungi with potential specificity. For example, *Microtis parviflora* R. Br. (Orchidaceae) has a narrow ecological specificity in the field, while showing a broad potential specificity in vitro. The factors that contribute to ecological specificity could be fungal growth and survival in the soil, which are influenced by environmental factors, or fungal density in the field (Masuhara and others 1995). Milligan and Williams (1988; in Masuhara & Katsuya 1995) suggested that differences between ecological and potential specificity could be due to a succession of fungi in orchid tissues, but further investigations are necessary to confirm this hypothesis.

Our study was carried out in two steps: 1) isolation and description of *Serapias parviflora* endomycorrhizal fungi; 2) description of associations, in vitro, between *S. parviflora* and fungi, and between *Spiranthes spiralis* and fungi. *S. parviflora* was used as control and *S. spiralis* was used to verify the existence of specificity between host and endophyte.

Materials and methods. *Serapias parviflora* roots samples were collected at Allerona Scalo (Umbria, Italy) on 05/16/1999. The whole plant, together with a clump of soil, was collected, in order to prevent root damage. Samples were preserved in sterilised envelopes at 5° C until fungal isolation (two days after collection). To remove fungi and bacteria from external surfaces, roots were sterilised by immersion on H_2O_2 (30%) for 4 minutes; then they were rinsed 4 times in sterile distilled water. Roots were cut, 1 cm segments were sowed on Petri dishes with PDA and streptomycin (a broad-spectrum antibiotic) and then incubated at 20° C. Fungi were observed under phase contrast microscope, scanning and transmission electron microscope and confocal laser microscope. For the latter there is no need of a particular preparation but fungi stained better when coloured with acid fuchsin. Fungi observed under TEM were

Figure 1. B-Sepa-1 monilioid cells (SEM, x 2000).

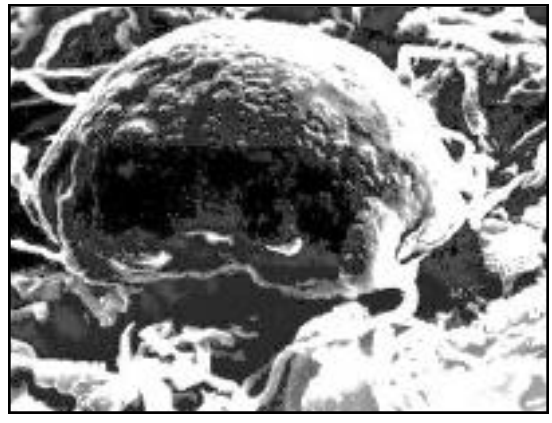

Figure 2. B-Sepa-1 sclerotium (SEM, x 1000).

treated as follows: growth on liquid medium, fixation for 2 hours in para-formaldehyde/glutaraldehyde (2%/2.5% v/v) in phosphate buffer 0,025 M pH 7 at 25° C, rinsed 3 times in phosphate buffer 0,025 M, postfixation in OsO4 1% in 0,05 M phosphate buffer v/v for 12 hours at 4° C, dehydration in a graded ethyl alcohol series for 15 minutes each (10%, 30%, 50%, 75%) and for 1 hour in pure ethyl alcohol, embedding on Spurr's epoxy resin and polymerisation for 8 hours at 70° C. Thin sections (70 nm), stained with uranil acetate, 10% v/v in 50% ethanol (v/v) for 7 minutes at 70° C and freshly prepared lead citrate (1% v/v on 50% ethanol v/v) for 12 minutes at 25° C were observed.

Fungi were inoculated in *S. parviflora* plants to confirm symbiosis and in *Spiranthes spiralis* plants to establish specificity. All plants were asymbiotically grown from seeds (modified Frosch medium) and transplanted on medium for symbiotic growth (modified Basic Oats medium, Riess and Pacetti, 2001) before inoculation.

Results. Many fungi were isolated from roots of *Serapias parviflora* but only two strains had characters of symbiotic fungi: mycelium with septa, monilioid cells, sclerotia, without asexual spores (grown on PDA until substrate exhaustion). Two fungi were able to form, in vitro, typical pelotons in *S. parviflora* and *Spiranthes spiralis* root cortex cells. They were called B-Sepa-1 and A-Sepa-1.

B-Sepa-1 (PDA) produces floccose to velutinous colonies, without water-soluble pigments and substrate pigmentation. Mycelium superficial and from white to light grey. On PDA, monilioid cells (fig. 1) and sclerotia (fig. 2) are differentiated. Vegetative hyphae hyaline, septated, with constriction on branched point and with smooth wall (fig. 3). Thin wall and hyaline monilioid cells, from ellipsoidal to spherical, 4,5 x 6,5 mm, organised in septated chains and branched or linear chains (fig. 4). Sclerotia torulose, 120 x 70 mm. Dolipores always present under TEM (fig. 5).

A-Sepa-1 (PDA) consisting of floccose and from light yellow to yellow colonies with white to light grey micelium on peripheral area. Uncoloured exudate, from dark grey to black water-soluble pigments and monilioid cells are produced. Vegetative hyphae hyaline, septated, with smooth walls. Hyaline monilioid cells are differentiated. Spherical to irregular, 4,5-15 x 6,5-15 mm, organised in linear and never branched chains. Thick and irregular electron dense layer around hyphae (fig. 6), Woronin bodies near septum pore (fig. 7).

Symbiosis between *S. parviflora* and B-Sepa-1 was observed under microscope. In these associations there is a massif fungal penetration of roots, preferentially through hairs (fig. 8) but in some case there is epidermal penetrations too. Subepidermal invasion is confined to the first two cortex layers. Pelotons and digested pelotons can be observed from the third to the eighth cellular layer. In some sections we can find both pelotons (fig. 9) and digested pelotons (fig. 10) in the same cell. We can also assume an infective cyclical pattern by observing mycelium intercellular connections (fig. 11).

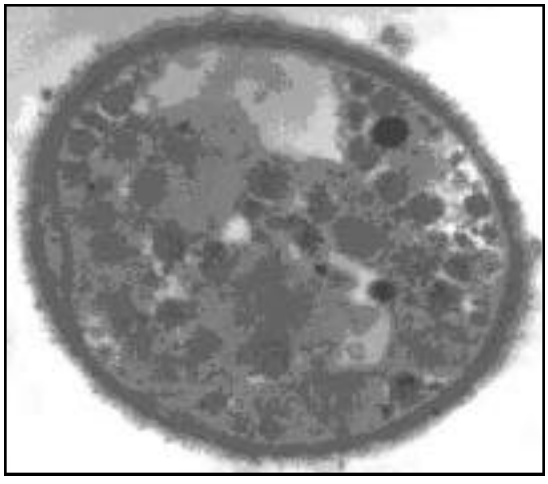

Figure 3. Trasversal section of B-Sepa-1 hypha (TEM, x 22500).

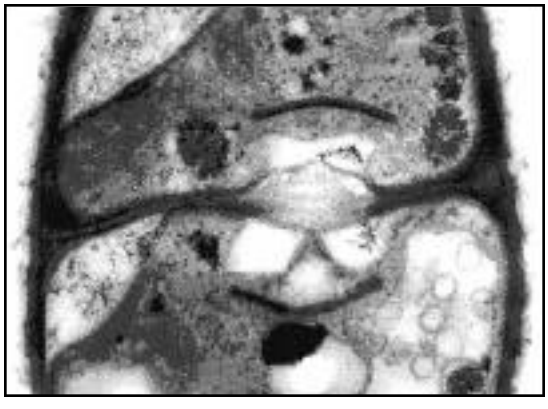

Figure 5. B-Sepa-1 septal ultrastructure with dolipore (TEM, x 45000).

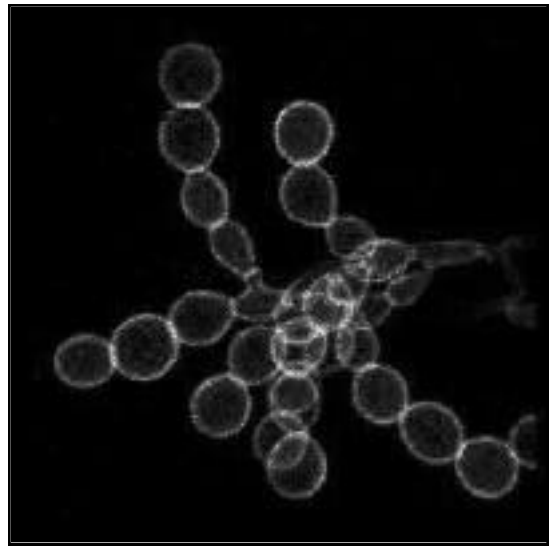

Figure 4. B-Sepa-1 monilioid cells (confocal laser microscope, x 80).

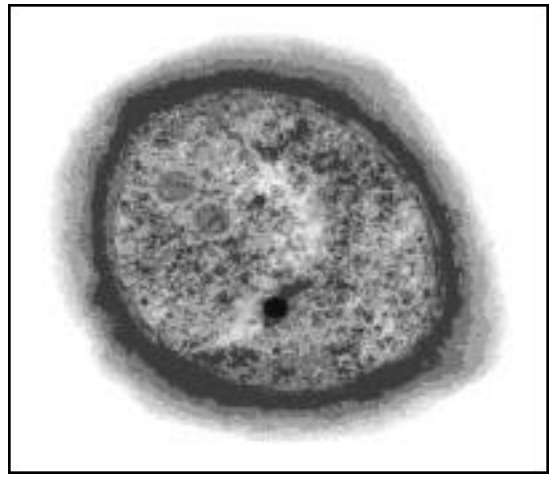

Figure 6. Trasversal section of A-Sepa-1 hypha, with a thick and irregular electron-dense layer sorrounding the cellular wall (TEM, x 30000).

In *S. parviflora* roots, inoculated with A-Sepa-1, there are few observations of epidermal penetrations (fig. 12). There is a slight subepidermal invasion and there are pelotons (fig. 13) and digested pelotons in the inner layers of the cortex (from the third to the sixth). Pelotons occupy a smaller cytoplasm portion compared to B-Sepa-1 in the same orchid.

As to *S. spiralis* inoculated with B-Sepa-1, it is possible to say that fungus preferentially penetrates through hairs (fig. 14), even if epidermal penetration is quite frequent (fig. 15). It is impossible to see subepidermal invasion because the first five layers are completely invaded by pelotons and digested pelotons (fig. 16 and 17). In this case, intercellular micelium connections are very clear. Beyond the fifth cellular layer of the cortex there is no fungus but there are many starch granules (fig. 18).

In *S. spiralis*, inoculated with A-Sepa-1, there are only very few partially or completely digested pelotons. Epidermal invasion is the only means observed for fungal penetration; hairs root invasion is never observed.

Fungal hyphae diameters are quite constant both

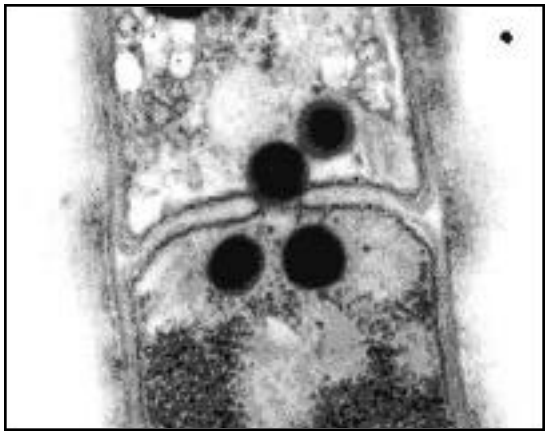

Figure 7. A-Sepa-1 septal ultrastructure, with Woronin bodies (TEM, x 45000).

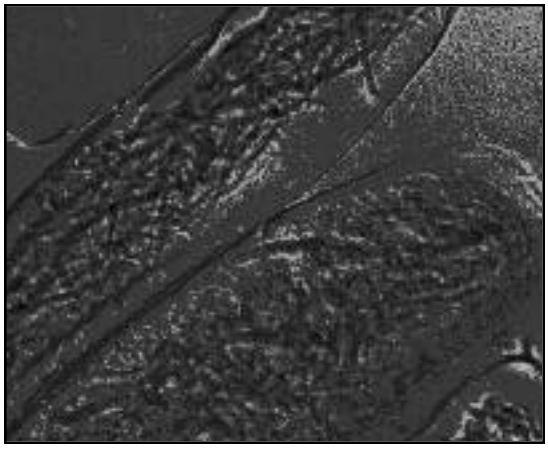

Figure 9. B-Sepa-1 pelotons in *S. parviflora* root cortex, stained by acid fuchsin (phase contrast microscope, x 40).

in pure cultures on PDA and in the hosts: in B-Sepa-1 diameters are on average 2 mm, while in A-Sepa-1 diameters are on average 3,5 mm.

Conclusion. Endophytes isolation from roots of *Serapias parviflora* reveals the presence of two fungi. Our technique allows us to determine only the division. A-Sepa-1 is an ascomycetes (presence of Woronin bodies), and B-Sepa-1 is a basidiomycetes (presence of dolipores). We can also recognise Riess and Scrugli morphometrical classes: A-Sepa-1 belongs to class B, with diameters of hyphae ranging from 2,6 to 5 mm, while B-Sepa-1 belongs to class A, having hyphae with diameters less than 2,5 mm. Molecular markers could certainly provide diagnostic tools for lower taxonomical levels and taxon fingerprints. .

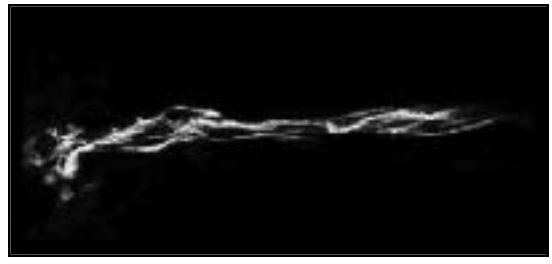

Figure 8. *S. parviflora* hair massively invaded by B-Sepa-1 (confocal laser microscope, x 40).

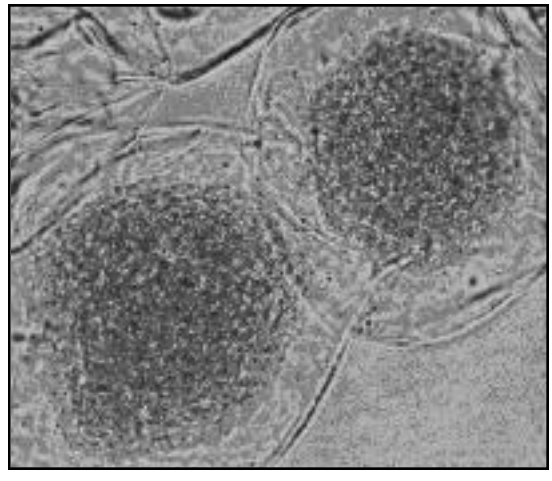

Figure 10. B-Sepa-1 partially digested pelotons in *S. parviflora* roots, stained by acid fuchsin (x 40).

We can not tell if *S. parviflor*a needs two fungi simultaneously or in succession for its development. Only observations of endophytes in samples living in the same site could maybe answer this question.

A-Sepa-1 and B-Sepa-1 behave differently when inoculated in plants of *S. parviflora* and *Spiranthes spiralis*: B-Sepa-1, the basidiomycetes, is a good symbiont for both orchids, i.e. it is always possible to see all typical mycorrhizal phases, their cyclical pattern and the absence of fungi in orchids root vascular tissue. A-Sepa-1, the ascomycetes, is a good symbiont only for *S. parviflora*; in this orchid we can observe fungal confinement in the first layer of root cortex, starch accumulation in the inner layers, cyclic infections and absence of fungus in vascular tissue. A-Sepa-1 is not tolerate by *S. spiralis*: there is a massif epidermal penetration but

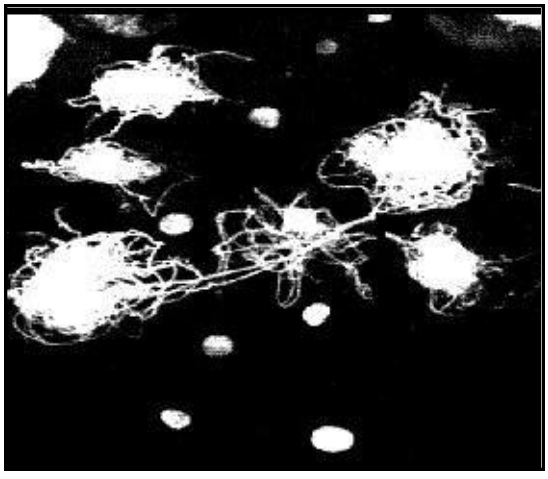

Figure 11. B-Sepa-1 pelotons with intercellular connections, stained by acid fuchsin (confocal laser microscope, x 24).

Figure 12. A-Sepa-1 epidermal penetration in *S. parviflora* (phase contrast microscope, x 40).

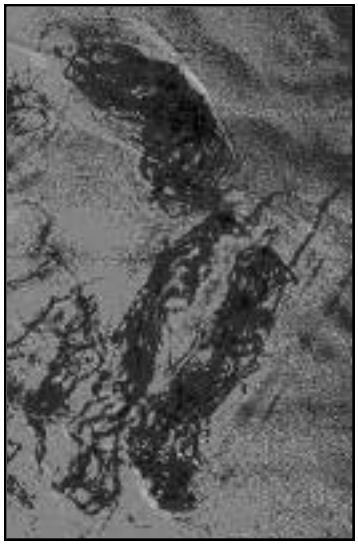

Figure 13. A-Sepa-1 pelotons in *S. parviflora*, stained by acid fuchsin (phase contrast microscope, x 20).

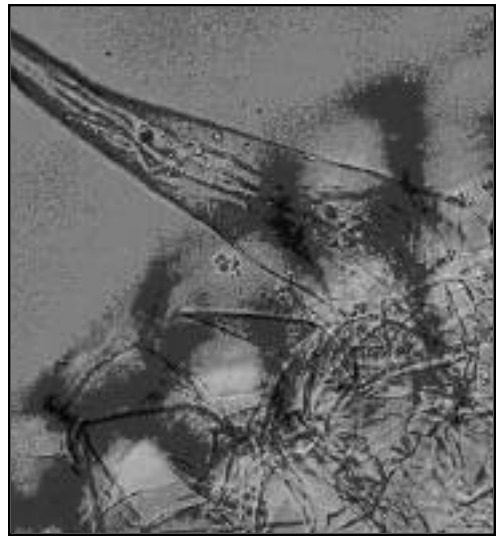

Figure 14. B-Sepa-1 penetration in *S. spiralis* root by hair (x 40).

there is no penetration through hairs. The very few pelotons are digested immediately and mycelium can not invade the other cells: typical symbiotic alternation of infection and digestion seems to be shifted toward digestion.

Symbiosis between A-Sepa-1 and *S. parviflora* and digestion of fungus in *S. spiralis* suggest a different potential specificity of the fungus for the two hosts: *S. parviflora* is micorrhizated by the two fungi and *S. spiralis* only by B-Sepa-1, *in vitro*. If it is true, inoculation of fungi in *S. parviflora* and *S. spiralis* and their infective patterns are an additional confirmation that there is no species-specificity, but potential or ecological specificity between orchids and fungi, as suggested from Masuhara and Katsuya (1989).

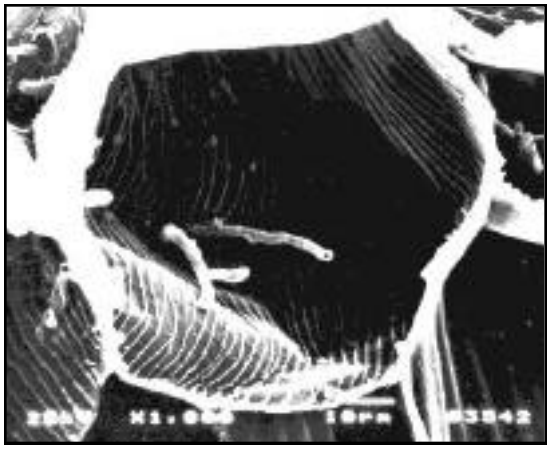

Figure 15. *S. spiralis* epidermal root cell penetrated by B-Sepa-1 (SEM, x 1,000).

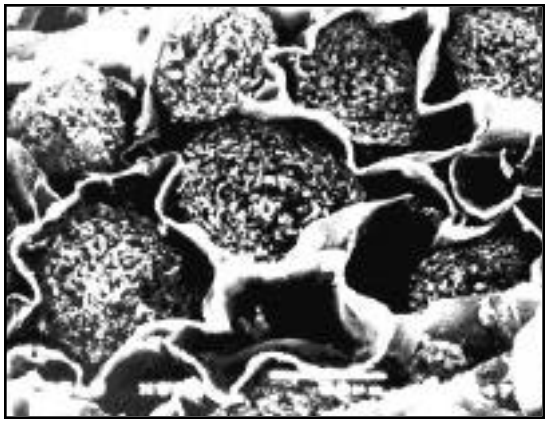

Figure 17. B-Sepa-1 pelotons in a *S. spiralis* root section (SEM, x 500).

The different pattern of infection of the two fungi in *S. spiralis* could also suggest that there is a fungal succession in this host, and that A-Sepa-1 can not induce symbiosis in *S. spiralis* in the phase of development studied.

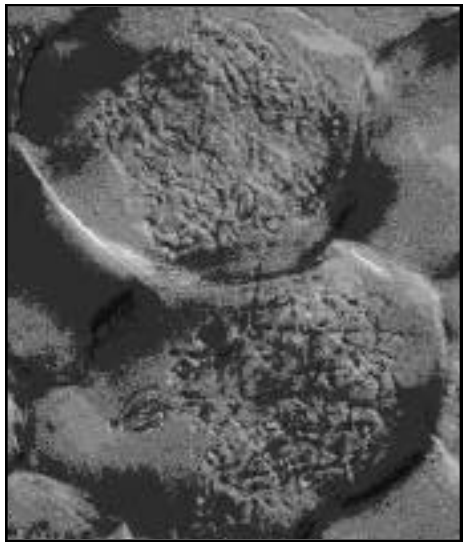

Figure 16. B-Sepa-1 pelotons in *S. spiralis* root cells (confocal laser microscope, x 40).

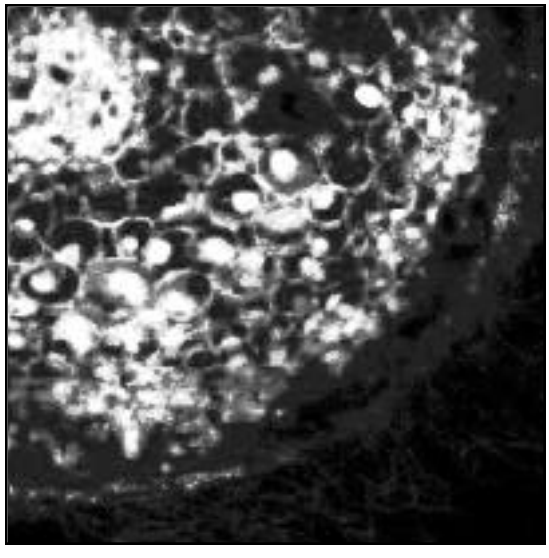

Figure 18. *S. spiralis* root section with B-Sepa-1 invasion area (confocal laser microscope).

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