Colonization by *Psilocybe semilanceata* of roots of grassland flora

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In co-culture *in vitro* *Psilocybe semilanceata* invaded moribund cells at the periphery of the cortex of roots of the grass species *Agrostis tenuis, Poa annua* and *Lolium perenne*, and the dicotyledon *Stellaria media*. Invasion of many cortical cells of *A. tenuis, P. annua* and *S. media* was associated with the formation of papillae in cell walls adjacent to intercellular hyphae and opposite initiated penetration points. This indicated some metabolic activity in the root cells at the time of attempted penetration.

Colonization of *L. perenne* was less frequently observed than of the other three species and papilla formation was not observed. *P. semilanceata* was restricted to the outermost cortical cells of the *L. perenne* roots by a layer of cells which appeared to contain polyphenolic substances.

Key words: *Psilocybe semilanceata*, Grassland flora, Root colonization, Papillae.

*Psilocybe semilanceata* (Fr.) Kumm. is one of the few temperate species of the genus *Psilocybe*. It was first described from Europe by Fries in 1836 and subsequently in Britain by several authors, e.g. Wakefield & Dennis (1950). There are very few studies of the ecology and substrate preferences of the genus and almost none concerning *P. semilanceata*. Guzman (1983) listed the diverse substrata of 73 worldwide species of *Psilocybe* as including soil, rotten wood, herbaceous stems, humus, mosses and dung. In this list *P. semilanceata* is given as occurring in ‘clay soil’ and ‘rich soil’. *P. semilanceata* has been recorded by many authors as being common amongst meadows and well-manured pastures (Wakefield & Dennis, 1950; Pegler, 1966; Phillips, 1981). Hoiland (1978) suggested that *P. semilanceata* probably lives on decaying grass remains in the soil but gave no other ecological data. Large numbers of *P. semilanceata* fruit bodies have been reported on St Kilda, an island off the Outer Hebrides (Watling & Richardson, 1971). The species was not considered to be associated with any particular plant community but was found in nitrogenous areas where urine and water from sheep dung had been flushed downhill from sheep folds. These areas, however, also showed a correlated change in the higher plant flora (Watling & Richardson, 1971).

Guzman (1983) states that ‘all species of *Psilocybe* are saprophytic and none are (sic) known to be parasitic or symbiotic’. Seaby & McIlwaine (1982), however, reported invasion of the root tissue of grass species by *P. semilanceata* in co-culture on agar. On this observation the present investigation was made in an attempt to determine whether or not there was any relationship between *P. semilanceata* and grassland flora.

**MATERIALS AND METHODS**

**Collection of material**

Basidiocarps of *P. semilanceata* were collected in Oct. 1984 from Lady Dixon Park, Lagan Valley Park and Inner Malone Golf Course in Belfast. Identification of the fungal specimens collected was based on the descriptions of Wakefield & Dennis (1950), Phillips (1981) and Guzman (1983). The pH of soil collected from these localities was determined after preparing a suspension of soil (20 g) in distilled water (180 ml).

**Isolation and culturing of *P. semilanceata***

Isolations were made from the sporocarp tissue. The basidiocarps were cut open, small pieces of tissue were removed, placed on malt agar (2 %) and incubated at 22 °C. Cultures were maintained on either malt agar or potato dextrose agar (Oxoid). Stock cultures were maintained by placing discs cut from the leading edge of cultures in sterile distilled water in Universal Bottles. These were sub-cultured onto agar media for experimental use every 6–9 months.

**Plant material**

The grasses *Agrostis tenuis* Sibth., *Poa annua* L. and *Lolium perenne* L. and also the dicotyledon *Stellaria media* (L.) Vill. (chickweed), which was described as the dominant higher plant in areas of high density of *P. semilanceata* fruit bodies in St Kilda (Watling & Richardson, 1971), were selected for study in co-culture with *P. semilanceata*. Seeds of *A. tenuis,*
P. annua and L. perenne were obtained from B & S Weed Seed Supplies. Seeds of S. media were obtained from Agricultural Botany Research Division. These seeds had not been dressed with fungicide.

Prior to the production of seedlings, seeds of A. tenuis, P. annua and S. media were surface sterilized for 10 min in 10% (v/v) sodium hypochlorite (Chloros, ICI) containing a wetting agent (Nonidet P40, BDH) and then rinsed thoroughly in sterile distilled water. Seeds of L. perenne were surface sterilized for 20 min in 20% (v/v) sodium hypochlorite and wetting agent and then rinsed in sterile distilled water. The seeds were then placed on malt agar (2% (w/v) malt, 0-5% (w/v) agar, Oxoid No. 3) to check for contamination and left in daylight in a laboratory at room temperature to germinate.

Co-culture experiments

In the first method, which was used only for A. tenuis, glass tubes (250 x 40 mm) were filled one third full with a mixture of sieved sphagnum peat (Shamrock Irish Moss Peat, Bord na Mona) and vermiculite (Dupre Vermiculite Ltd), 30 ml peat and 60 ml vermiculite per tube. A mineral solution (56 ml comprising a 0.25 strength modified Melin Norkrans solution (Marx, 1969) was added to each tube. The tubes were stoppered and autoclaved (121°, 15 min) and the pH after autoclaving was between pH 5.2 and 5.4. Tubes were inoculated with four discs (7 mm diam) cut from the leading edge of a 3-wk-old culture of P. semilanceafa on water agar. The discs were embedded in the vermiculite-peat (VP) mixture. An aseptic seedling of A. tenuis was planted in the VP substrate. Seedlings were used when 3-5 d old, by which time the radicles were 5 to 10 mm long and the coleoptile had emerged. Seedlings were planted in uninoculated VP substrate in tubes as controls. The tubes were capped with sterile beakers (100 ml) and placed in stands made from polystyrene blocks from which tube-sized wells had been cut, to shade the seedlings of S. media and P. annua and to prevent contamination. After 5 and 8 d seedlings were removed from the agar and sampled for microscopy.

Microscopy

Seedlings of A. tenuis were removed from tubes and agitated in 0.025 M potassium phosphate buffer (pH 7) to remove VP particles. The roots were dissected in the same buffer as quickly as possible to minimize damage prior to fixation. Samples were taken randomly from throughout the root system, from just behind the root tips to ca 4 cm distal to the tip. Seedlings were removed from agar plates and the roots dissected in the same manner.

Samples were fixed immediately in glutaraldehyde (4%, EM grade, Agar Aids) in 0.025 M potassium phosphate buffer (pH 7) for 2 h. Samples for transmission electron microscopy were post-fixed in osmium tetroxide in the same buffer for 2 h.

Samples for transmission electron microscopy were dehydrated in an ascending acetone series and then rotated at 2 rev. min⁻¹ for 1 h in 50:50 absolute acetone: Spurr's resin (Spurr, 1969) before transferring to pure Spurr's resin. Samples were infiltrated with resin at room temperature on a rotator (2 rev. min⁻¹) for 8 d, the resin being changed every 2 d. The resin was polymerized at 60° for 48 h. Ultra-thin sections, 70-80 nm thick, were stained sequentially in 2% (w/v) uranyl acetate and 2% (w/v) lead acetate (Reynolds, 1963) for 60 min and 10 min respectively in darkness and a carbon dioxide-free atmosphere. Sections were examined using a GEC AE1 801A transmission electron microscope.

Samples of roots of P. annua seedlings growing on water agar inoculated with P. semilanceafa were prepared for scanning electron microscopy. Root sections threaded through two fracture rivets (3 X 1 mm) were frozen rapidly in an Emscope SP2000 Sputter Cryo unit. The frozen hydrated roots were fractured and surface etched prior to surface coating with a layer of gold (15 nm). The specimens were examined in a JOEL 25 CF scanning electron microscope.

RESULTS

Location of P. semilanceafa

Basidiocarps of P. semilanceafa were found amongst the grass species A. tenuis, P. annua, L. perenne and occasionally Agrostis stolonifera L. and Holcus lanatus L. These were artificially sown grass mixtures in parkland and the weed species Ranunculus repens L., Bellis perennis L., Crepis species and mosses were also present. The stipes of fruit bodies were sometimes observed arising from dead leaf sheaths in the thatch layer. The soils in these areas were found to be in the range pH 4.4 to 5.4.

Colonization of root tissues

Agrostis tenuis. Seedlings from uninoculated tubes and tubes containing P. semilanceafa appeared similarly green and healthy. Roots were white and those from tubes containing P. semilanceafa bore no lesions or structural modifications. All tubes remained sterile; only colonies of P. semilanceafa were isolated from samples of VP transferred to malt agar.

Electron microscopy showed that in many of the roots of A. tenuis the outer cortical cells and cell layers lacked subcellular structure, were anucleate and in particular had no detectable plasma membrane around the cell walls. These cells were considered to be dead. In most roots, however, the innermost cortical cells closest to the endodermis and the stelar parenchyma cells had well-defined plasmalemma, well-ordered cytoplasm, nuclei and cell organelles, and appeared viable.
Figs 1–6. Transverse sections of *Agrostis tenuis* seedling roots grown in vermiculite–peat tubes inoculated with *Psilocybe semilanceata*. Fig. 1. Fungal hypha (h) within the intercellular space (is) and an intracellular infection of a moribund cell (c). Note absence of plasmalemma in cells and constriction of hypha as it passes through the wall (× 35 000). Fig. 2. Active fungal hyphae within root cells. The dense cytoplasm contains glycogen rosettes (g), mitochondria (m) and endoplasmic reticulum (er). (× 16 000). Fig. 3. Hypha (h) within the xylem cells of a moribund root, penetrating between cells at the region of the lateral pits (× 23 000). Fig. 4. Appositional deposits (d) along cell walls adjacent to an intercellular hypha (h) (× 23 000). Fig. 5. Hypha (h) penetrating cortical cell of the root. An electron dense deposit (d) surrounds the hyphal peg (× 16 000). Fig. 6. Hyphal peg within a cell wall surrounded by an electron-lucent zone. The cellulose microfibrils in the wall do not appear distorted (× 21 000).
In most sections cut from roots taken from tubes inoculated with *P. semilanceata*, hyphae were observed between the walls of the cortical cells in intercellular spaces but more often occurred intracellularly (Fig. 1). Hyphae at various stages of development were observed in the roots. Young sub-apical regions of hyphae were characterized by dense cytoplasm, endoplasmic reticulum, mitochondria and clusters of glycogen particles (rosettes), indicating a metabolically active condition (Fig. 2). Dolipore septa, characteristic of basidiomycete fungi, were frequently observed in the hyphae. At the time of sampling all root cells invaded by *P. semilanceata* were moribund; the viable endodermis and stele of living roots were not infected. In those few roots examined where the vascular tissue was also moribund fungal invasion had progressed within the thickened xylem cells (Fig. 3).

Although the fungus was observed only within dead or moribund cells there was evidence that at least some of these cells were alive when fungal penetration began. In many cortical cells there was a localized reaction to a hypha within a cell wall (Fig. 4) or a fungal peg penetrating the cell (Fig. 5). There was deposition of electron dense material along the cell wall, specifically opposite the fungal hypha or around the invading hyphal peg. When stained with uranyl acetate and lead citrate, these deposits were clearly distinct from the less densely stained cell walls. They were usually uneven in staining intensity, often with lighter electron opaque areas within the deposits and in some reactions the material appeared to be formed of concentric layers. When stained only with osmium tetroxide, the deposits were again more densely stained than the very osmiophobic cell walls but were not strongly osmiophilic. For these deposits to have formed in response to attempted fungal penetration, the cells must have been metabolically active; however, by the time the cells were fixed they had become moribund.

Penetration of root cells occurred both from the exterior of the root into the outermost cortical cells and by hyphae ramifying through the cortex penetrating directly from cell to cell or through intercellular spaces. The hyphae were often constricted as they passed through the cell wall and resumed normal dimensions in the cell lumen (Fig. 1). Immediately adjacent to and surrounding a penetrating hypha there was frequently an electron-lucent zone in the root cell wall. This was highly localized and there was no general distortion of the cellulose microfibrils in the wall (Fig. 6). Within the secondarily thickened cells of the stele, hyphae were observed penetrating walls at the region of the lateral pits (Fig. 3), or through the actual wall material with varying degrees of success. Hyphae which attempted to penetrate electron-dense areas, as illustrated in Fig. 5, ultimately became highly vacuolated with disorganized cytoplasm or had collapsed indicating that the hyphae had senesced during the invasion process.

**Poa annua.** Seedlings grown in co-culture with *P. semilanceata* appeared similar to those on control plates. There were no signs of lesion formation or necrosis on the infected roots and all roots remained white and apparently healthy. The shoots of all the seedlings were green and appeared healthy in both instances.

Microscopic examination revealed that many of the outer cortical cells were moribund, similar to those observed in *A. tenuis*. In most of the roots examined the inner cortex and stele cells were viable with normal cell contents. Only dead cells of the cortex had been colonized by the fungus (Fig. 10). Often moribund invaded cells were adjacent to uninvaded cells which were beginning to senesce.

Although the fungus was observed only in association with moribund cells, as with *A. tenuis*, there was again some evidence for fungal penetration of cells whilst they were still metabolically active. Deposits similar to those observed in *A. tenuis* (Fig. 4) were formed in root cell walls adjacent to intercellular hyphae. These were also observed in cell to cell penetration (Fig. 7). In cells where hyphal penetration was complete there frequently appeared to be a collar of electron-dense material around the hypha at the point of entry to the cell (Fig. 6). This collar stained darker than the plant cell walls to which it was closely appressed and resembled the material of similar deposits observed in *A. tenuis*, suggesting that it may represent the complete penetration of a thin deposit. This penetration may have occurred following the death of the cell or alternatively the cell may have died as a consequence of penetration. The deposits formed in *P. annua* were often smaller than those in *A. tenuis*. As in *A. tenuis* the hyphae of *P. semilanceata* within the root tissue appeared viable, and dolipore septa were seen. Electron-lucent areas were frequently observed around hyphae (Fig. 9).

**Lolium perenne.** Hyphae of *P. semilanceata* were only found in senescent or dead cells of the outer cortex of *L. perenne* roots. A layer of cells within the cortex, not infected by the fungus, was frequently observed to have uniform densely stained cytoplasm that also stained with osmium tetroxide alone. These cells were also present in uninfected roots and probably contained tannins that were precipitated during the fixation processes.

Colonization of roots of *L. perenne* was much less extensive than colonization of roots of *A. tenuis* and *P. annua*, and few instances of penetration were observed. No electron-dense deposits were observed in response to fungal invasion.

**Stellaria media.** At the time of fixation the outer cortical cells of *S. media* were beginning to senesce. Some cells were devoid of cell contents, in others the nuclei and intact plasmalemma were still visible. Some of the moribund cells had been colonized by *P. semilanceata*. Hyphae were frequently observed penetrating cell walls either from the exterior of the root or from an intercellular position (Fig. 11). A large number of these penetrations were associated with deposition of electron-dense material along the inner edge of the invaded cell wall. These deposits were very similar to those observed in *A. tenuis* cells. The deposited material was clearly visible between the host cell wall and the partly intact plasmalemma (Fig. 11) which had retracted from the wall in some cells. In some instances the hyphae were observed to have breached the wall and begun to penetrate the deposits, but in these cases the host cells and the hyphae appeared to be moribund (Fig. 12).
**Figs 7–10.** Transverse sections and scanning electron micrograph of the roots of *Poa annua* seedlings growing through a colony of *Psilocybe semilanceata* on water agar. **Fig. 7.** Hypha penetrating between cortical cells with the formation of electron-dense deposits in penetrated cells (× 25 000). **Fig. 8.** Fungal penetration of cell. At the point of entry of the cell the hypha (h) is surrounded by a small electron dense collar appressed to the host cell wall (arrowed) (× 25 000). **Fig. 9.** Small hyphal peg penetrating cell wall, surrounded by an electron-lucent zone. There appears to be no distortion of the cellulose microfibrils. The wall of the hyphal peg is indistinct (× 80 000). **Fig. 10.** Turgid root with fungal hyphae exterior to the root. Clamp connexions are present on the mycelium (large arrow). Hyphae, frozen-fractured in transverse section (smaller arrows) are visible in the outer cortical cells. Bar = 10 μm.
DISCUSSION

Psilocybe semilanceata has been shown to be capable of invading root tissue of *A. tenuis*, *P. annua*, *L. perenne* and the dicotyledon *S. media*. However, only senescing cells at the periphery of the cortex were colonized. Neither intracellular nor intercellular penetration between living cells was observed.

It is well-documented that the root cortices of grass and cereal plants become moribund and eventually slough off (Troughton, 1957; Russell, 1977). In natural conditions this is accompanied by the growth of fungi and bacteria in the root tissues (Marchant, 1970; Old & Nicolson, 1975). Despite this the cortex generally remains intact, attached to the roots and the roots appear white and healthy (Holden, 1975). Henry & Deacon (1981) suggested that root cortical death is endogenously controlled and found no evidence that the process was enhanced by either soil micro-organisms or necrotrophic parasites. It has, however, also been suggested that when weak parasites or saprophytes invade naturally senescing root cortices rotting of the whole root is hastened (Salt, 1979). These minor pathogens colonize only the cortical cells, not penetrating the vascular stele; some will also survive in the soil as saprophytes (Sale, 1979). Results presented here suggest that in *in vitro* infection, *P. semilanceata* occupies a similar position within senescent root tissues to that of these saprophytes or weak pathogens.

Although *A. tenuis*, *P. annua* and *S. media* belong to very different families, similar reactions in their roots to the penetrating hyphae of *P. semilanceata* were observed. Fungal invasion of many of the cortical cells of all three plant species was associated with a localized deposition of electron-dense material along the host wall. The material appeared to be paramural, deposited between the cell wall and the plasma membrane, and in *S. media* the electron-dense material was clearly observed to be between the plant cell wall and the separated but intact plasmalemma of the cell. These reactions were observed in cell walls adjacent to intercellular hyphae and opposite initiated penetration points. Where the fungus had breached either the cell wall or both the wall and the reaction material, the reaction material had the appearance of a sheath or collar around the invading hypha. This may indicate growth of the hypha through a previously intact deposit or possibly a later deposition of material around the hypha after invasion, although the latter would necessitate a living plant protoplast.

To have formed the reaction material, considered to be papillae (Aist, 1976; Bushnell, 1972), to the fungus the root cells must have been capable of metabolic processes. Virtually all cells in which this response to infection was observed had no cellular contents and were presumed to be dead. The role of fungal invasion in the death of these cells is, however, debatable. At the time the root material was fixed, progressive senescence of the cortical tissues was also occurring in uninfected roots (data not presented). Thus the majority of intracellular penetrations observed were probably in cells already moribund or too senescent to react to fungal invasion. Those cells in which papillae formed may have been infected when already senescing but were still sufficiently active to respond to invasion. It is likely that cell death would have proceeded whether or not the cell had been invaded.

Colonization of *L. perenne* was much less frequent than colonization of the three plant species already discussed and papilla formation was not observed. *P. semilanceata* colonized only the outermost moribund cells of the cortex. The hyphae...
may have been restricted in colonizing the roots by a layer of cells that appeared from their staining properties to contain polyphenolic material and were present in both infected and uninfected roots.

Penetration of root cell walls by *P. semilanceata* caused very little distortion of cellulose microfibrils, suggesting that enzymes were involved rather than mechanical force. However, hyphae were frequently constricted as they passed through the wall and this has been advanced as evidence for mechanical penetration (Cooper, 1983). It is also possible that such constriction was the consequence of very localized enzymic degradation of the wall. Within the walls hyphae of *P. semilanceata* were observed associated with localized electron-lucent zones which are characteristic of penetration of host walls by some fungal pathogens, e.g. *Gaumannomyces graminis* (Sacc.) v. Arx & Olivier (Faull & Campbell, 1979). The papillae in cell walls adjacent to points of attempted penetration by *P. semilanceata* also resembled reaction materials (lignitubers) which form in roots of wheat in response to penetration by *G. graminis*.

The electron-lucent areas in cell walls around the penetration pegs of *P. semilanceata* may have represented areas of degraded pectin (Maeda, in Bracker 1978). The presence of such light areas surrounding *P. semilanceata* hyphae within the pectin-rich middle lamella suggests that pectic enzymes were produced by the fungus. In the intact cell walls cellulose fibrils were clearly visible, but they were not discernible within the electron-lucent areas surrounding the hyphae, suggesting cellulase activity.

It appears from the observations described that the association between *P. semilanceata* and some of the flora of its habitat resembles that of a weak pathogen colonizing almost senescent root tissues and sometimes evoking a resistance reaction from cells that are still sufficiently functional. Saprophytic growth was also observed in dead cells as the hyphae ramified through the tissues penetrating cell walls. It is possible that *in vivo* the fungus may occupy a similar niche on the dead and dying grass tissues with which the fruit bodies are often associated.

The authors wish to thank Mrs N. Shields for her assistance in the preparations of the plates presented here. We also thank Dr T. W. Fraser and Professor J. P. Blakeman for discussions of results. S. M. K. wishes to thank the Natural Environment Research Council for providing support for this work.

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(Received for publication 9 January 1989)

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