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Pityopus californicus: structural characteristics of seed and seedling development in a myco-heterotrophic species

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Abstract *Pityopus californicus* (Eastw.) H. F. Copel., a monotypic member of the Monotropoideae in the family Ericaceae, is a myco-heterotrophic species with distribution limited to the Pacific Northwest of the USA. Young embryos of *P. californicus* developed mycorrhizal associations in seed packets that had been buried for up to 681 days, suggesting that seeds of *P. californicus* may require the presence of a fungus to achieve germination. Samples of nongerminated seeds and early stages in embryo and root development were subsequently processed for light microscopy, histochemistry, and transmission electron microscopy (TEM). Nongerminated seeds possessed a thick testa, lacked a shoot and root meristem, and consisted of an embryo with large parenchymatous cells containing protein bodies and starch grains as storage reserves. In the earliest developmental stage (seed coat still attached), fungal hyphae were present on the testa surface and between the testa and embryo. This stage was followed by embryo elongation, the organization of a root apical meristem, and the development of a well-developed fungal mantle surrounding the elongated embryo. At least two morphotypes were identified based on structural characteristics of the mantle. One of these, with ascomycetous septa, had *Cenococcum*-like features. Late-stage embryo/early root development revealed a typical mantle and Hartig net, with fungal pegs penetrating the outer tangential walls of epidermal cells. Transfer cell-like deposits of wall material, similar to those described in *Monotropa* spp., enclosed fungal pegs. The development of a Hartig net and fungal pegs suggests that nutrient exchange interfaces are required for seedling development.

Keywords Seed reserves · Fungal colonization · Mycorrhiza · Anatomy

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Introduction

All species in the subfamily Monotropoideae (Ericaceae) are myco-heterotrophic, requiring an autotrophic host to provide photosynthates via fungal bridges (Leake 1994; Smith and Read 1997). *Pityopus californicus* (Eastw.) H. F. Copel., a monotypic member of the Monotropoideae, is found only in the Pacific Northwest of the USA. The systematics of this genus has been problematic from the earliest morphological descriptions (Copeland 1935). However, it is now clear from nuclear and plastid gene evidence that *P. californicus* is sister to *Monotropa hypopithys* (=*hypopitys*; Bidartondo and Bruns 2001).

Seeds of members of the Monotropoideae, like those of other myco-heterotrophic species such as orchids (Peterson et al. 1998), are extremely small, contain minimal storage reserves, and depend on colonization by a fungus at an early stage to germinate (Leake 1994, 2004). Germination requirements and anatomical features of initial stages of embryo development are, however, unknown for most myco-heterotrophs including *P. californicus* (Leake 1994; Bidartondo and Bruns 2005). Leake et al. (2004) did show conclusively that postgermination development of *Monotropa hypopitys* was dependent on specific fungal associates.

When seed packets were buried next to *Salix repens*, *M. hypopitys* seedlings were associated with the *Salix*-specific ectomycorrhizal fungus, *Tricholoma cingulatum*, whereas when seedlings developed in the vicinity of *Pinus sylvestris*, the fungal symbiont was *Tricholoma terreum*, a Pinaceae-specific fungus. Other members of the Monotropoideae also appear to be associated with very specific fungi (Bidartondo and Bruns 2001, 2002, 2005; Taylor et al. 2002; Bidartondo 2005; Leake 2005).

The first description of seed germination and early seedling development for *P. californicus* was made recently by Bidartondo and Bruns (2005); the fungal species *Tricholoma myomyces* was identified as associating with developing seedlings as well as with adult plants. Structural details of seeds and early embryos were not included.

The objectives of this study were to document the structure and storage reserves of seeds, determine the development of embryos and roots of *P. californicus* collected from seed packets buried in soil, and to determine the colonization pattern of indigenous soil fungi.

Materials and methods

Seeds of *P. californicus* were placed in seed packets made of 50-μm mesh screen cloth. Three hundred and four packets were buried at Perkins Creek (Lane County, Oregon, USA) for up to 681 days, and 200 packets were buried at Eel Creek (Coos County, Oregon, USA) for up to 603 days; all were placed near in situ Monotropoideae plants. *P. californicus* had very low germination at Perkins Creek (seedlings from only two seed packets appeared mycorrhizal) and no germination at Eel Creek (Bidartondo and Bruns 2005). For this study, packets that were removed from the soil at the end of the experiment were emptied of their contents and a subsample that included approximately 20 seeds and young mycorrhizal embryos were fixed in 2% glutaraldehyde in 0.05 M NaCacodylate buffer. Samples were then dehydrated in a graded ethanol series and embedded in LR White resin (London Resin Company) using a standard protocol (Ruzin 1999).

Light microscopy and histochemistry

Sections 1–1.5 μm thick of all fixed samples were cut with glass knives, heat-fixed onto glass slides, and stained with 0.05% toluidine blue O in 1% sodium borate for regular light microscopy. Sections were also stained with 1% aniline blue black in 7% acetic acid for protein. Controls were treated with a saturated solution of Proteinase K (Fermentas) in 0.1% 4-2-hydroxyethyl-1-piperazineethane-sulfonic acid (HEPES) buffer (pH 4.7) for several hours before staining. Sections were stained with acriflavin hydrochloride for polysaccharides (Culling 1974) and viewed under blue light on a Leitz epifluorescence microscope. Controls were incubated in 10% trichloracetic acid (TCA) for 6 h at 4°C.

Transmission electron microscopy

Thin sections (<0.1 μm) of LR White-embedded material were cut on a Reichert OM-U3 microtome, picked up on copper grids, stained with uranyl acetate and lead citrate, and viewed at 100 kV on a Philips CM10 transmission electron microscope interfaced with a digital imaging system.

Results

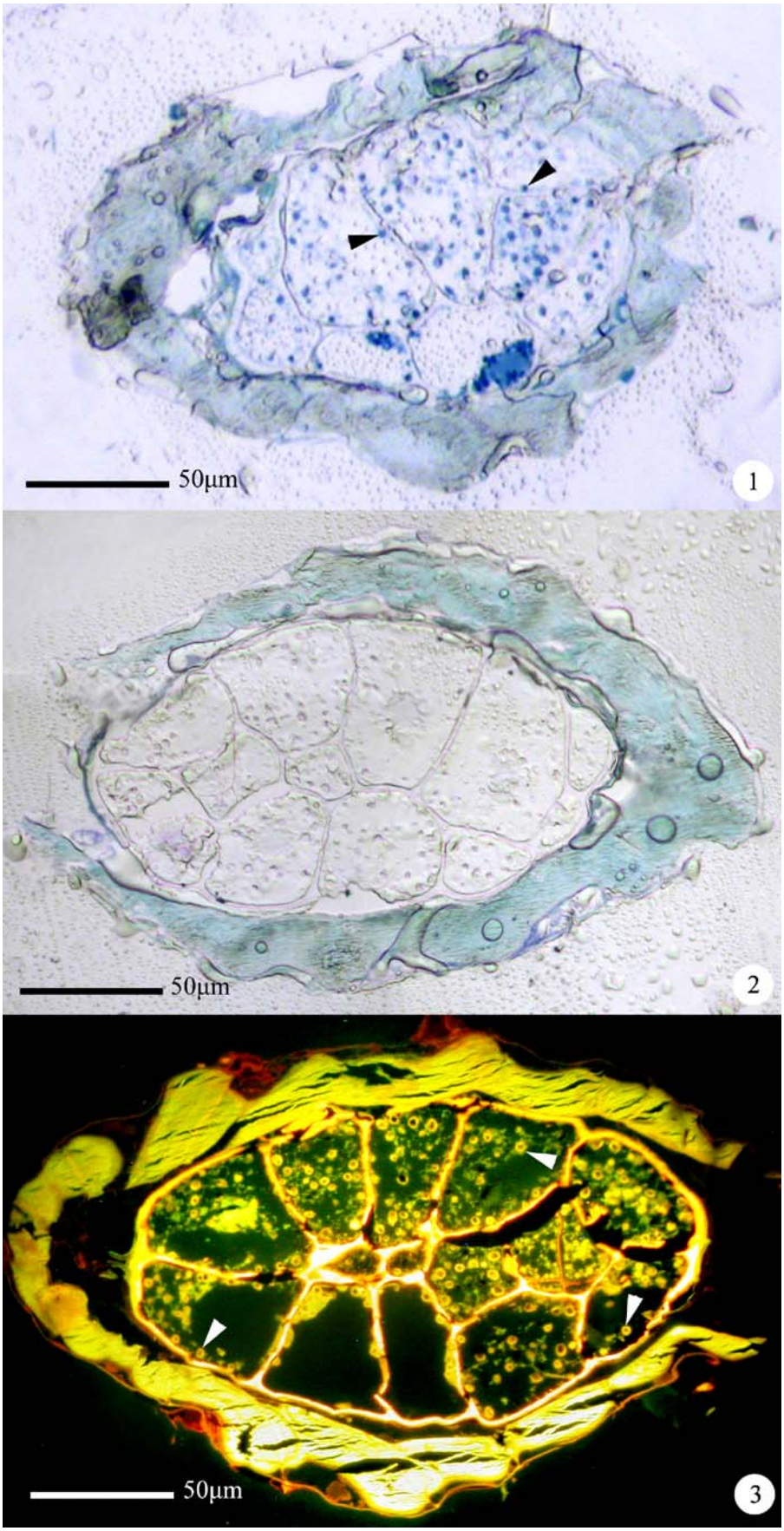
Light microscopy and histochemistry

Nongerminated seeds were small (~100 μm) with a testa of thick-walled cells. Seeds lacked an endosperm, and the simple embryo consisted of a few large parenchyma cells without the differentiation of a shoot and root meristem (Figs. 1, 2, and 3). Sections of nongerminated seeds had a positive staining reaction for protein (Fig. 1), confirmed by the lack of staining after treatment with Proteinase K (Fig. 2). Staining with acriflavin hydrochloride revealed the presence of starch grains as an additional storage reserve (Fig. 3). Control sections treated with TCA did not stain for starch (not shown).

In the earliest developmental stage of the embryo, as judged by the retention of the seed coat, fungal hyphae were present on the surface of the testa and between the testa and embryo (Fig. 4). This stage was followed by embryo elongation showing a defined meristematic region and a well-developed mantle (Figs. 5, 6, and 7). Mantle morphology indicated that at least two morphotypes were present (Figs. 5, 6, and 7): one with a thick-walled, heavily pigmented mantle similar to that of the Ascomycete, *Cenococcum geophilum* (Figs. 5 and 6) and the other with a mantle with thinner, less pigmented walls (Fig. 7). Early root development revealed extensive mantle formation, at times with more than seven layers of fungal hyphae (Fig. 8). A paraepidermal Hartig net developed between radial epidermal cell walls, and in addition, fungal pegs penetrated the outer tangential walls of epidermal cells (Figs. 9a,b and 10).

Transmission electron microscopy

Electron microscopy showed fungal pegs similar to those described in *Monotropa* spp., with a single penetration point in the outer tangential epidermal cell wall originating from hyphae in the inner mantle (Fig. 11). Dolipore septa (Fig. 12), typical of Basidiomycete fungi, were present in mantle hyphae of the morphotype in which fungal pegs



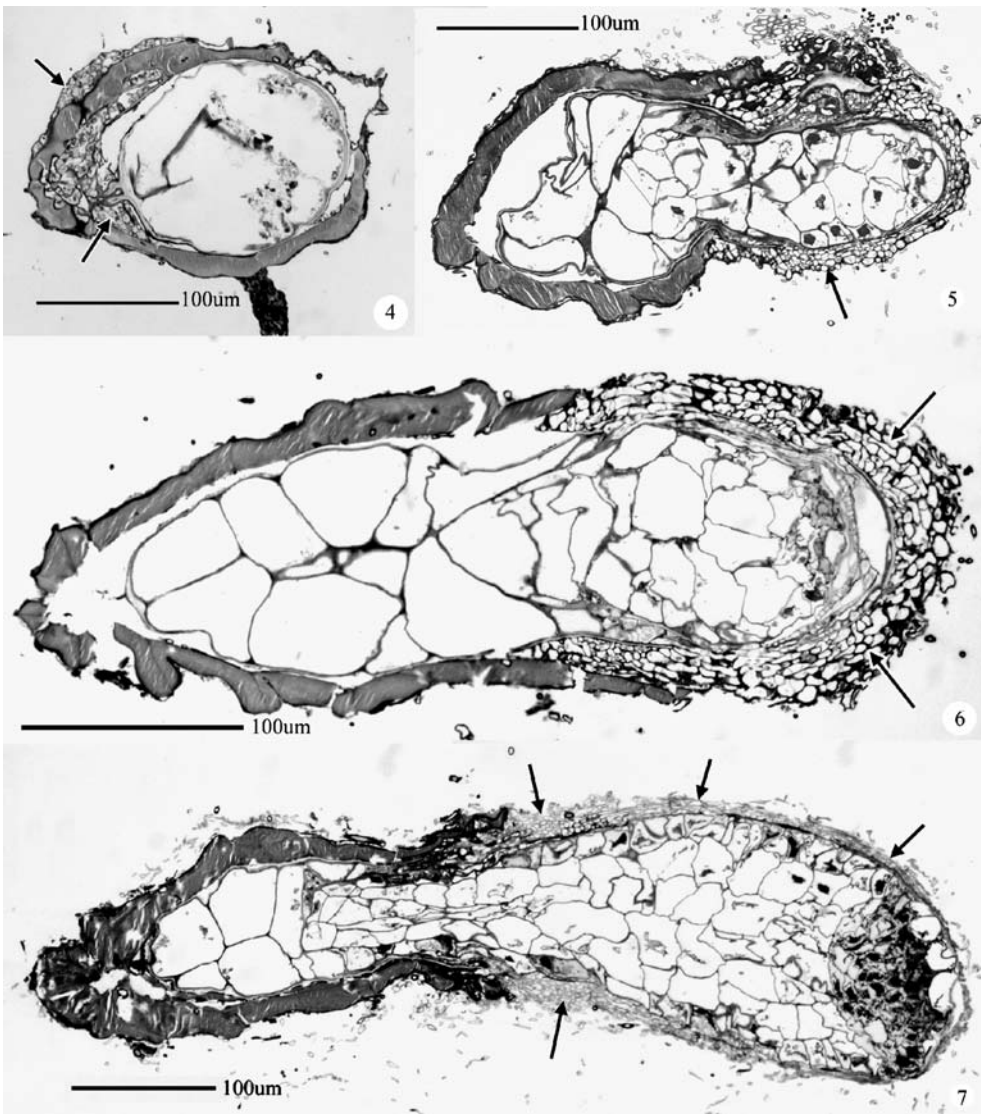
Figs. 1, 2 and 3 Resin-embedded sections of nongerminated *Pityopus californicus* seeds. **Fig. 1** Section stained with aniline blue black showing protein bodies (*arrowheads*) in large parenchyma cells of the embryo. **Fig. 2** Control section treated with Proteinase K before staining with aniline blue black, demonstrating the absence of staining. **Fig. 3** Fluorescence microscopy of section stained with acriflavin hydrochloride showing starch grains (*arrowheads*) in embryo cells

occurred (Fig. 11). Wall deposits typical of those seen in transfer cells were evident in the plant cytoplasm around fungal pegs (Fig. 13); occasionally, the fungal pegs tapered in width to a narrow tip inside an epidermal cell (Fig. 13). Electron-dense bands were present in the wall of each fungal peg, and an occlusion was present within the tip (Fig. 14). Remnants of cytoplasm occurred adjacent to the open tip of the fungal peg (Fig. 14). Micrographs of sections of developing embryos colonized by the *Cenococcum*-like fungus showed that a multi-layered mantle had formed, but fungal pegs were absent (Fig. 15). Mantle hyphae of this morphotype had numerous septa (Fig. 16) with Woronin bodies adjacent to the septal pore (Fig. 17), typical of Ascomycete fungi.

Discussion

Seeds of *P. californicus* are typical of many myco-heterotrophic species (Leake 1994) in that they are very small, lack an endosperm, and possess a simple embryo without the differentiation of a shoot and root meristem. Protein and starch are the main reserve substances deposited within enlarged embryo cells. In most myco-heterotrophs, starch is present in immature seeds but tends to disappear by the time of seed maturation (Leake 1994); this does not seem to be the case in *P. californicus*, but additional seeds need to be examined to confirm this. Protein is a common storage compound in seeds of many myco-heterotrophic species (Leake 1994; Peterson et al. 1998). Both protein and starch disappeared from embryo cells during early embryo growth after germination.

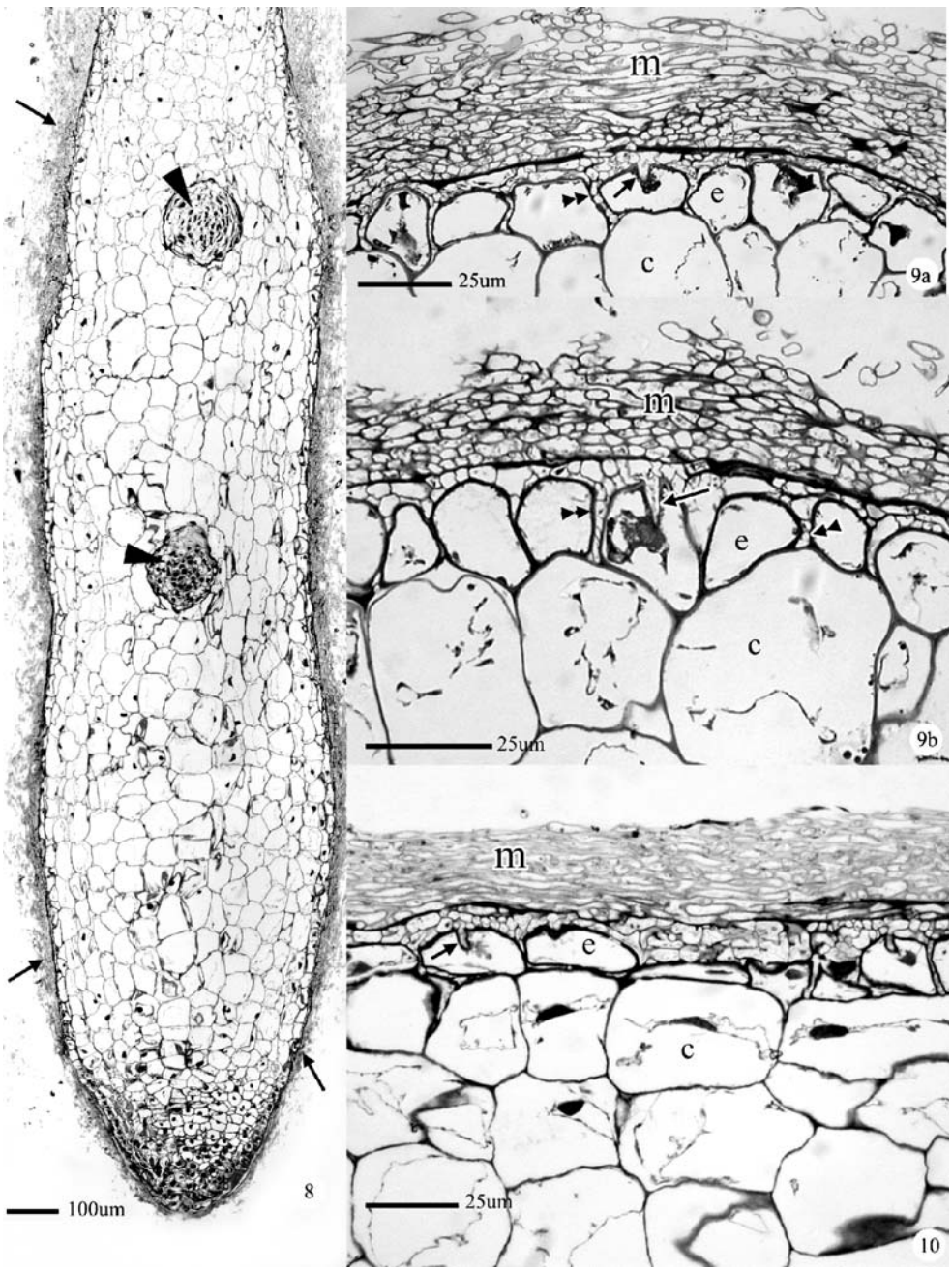
Although overall seed germination was low in seed packets, those examined suggest that fungal colonization is



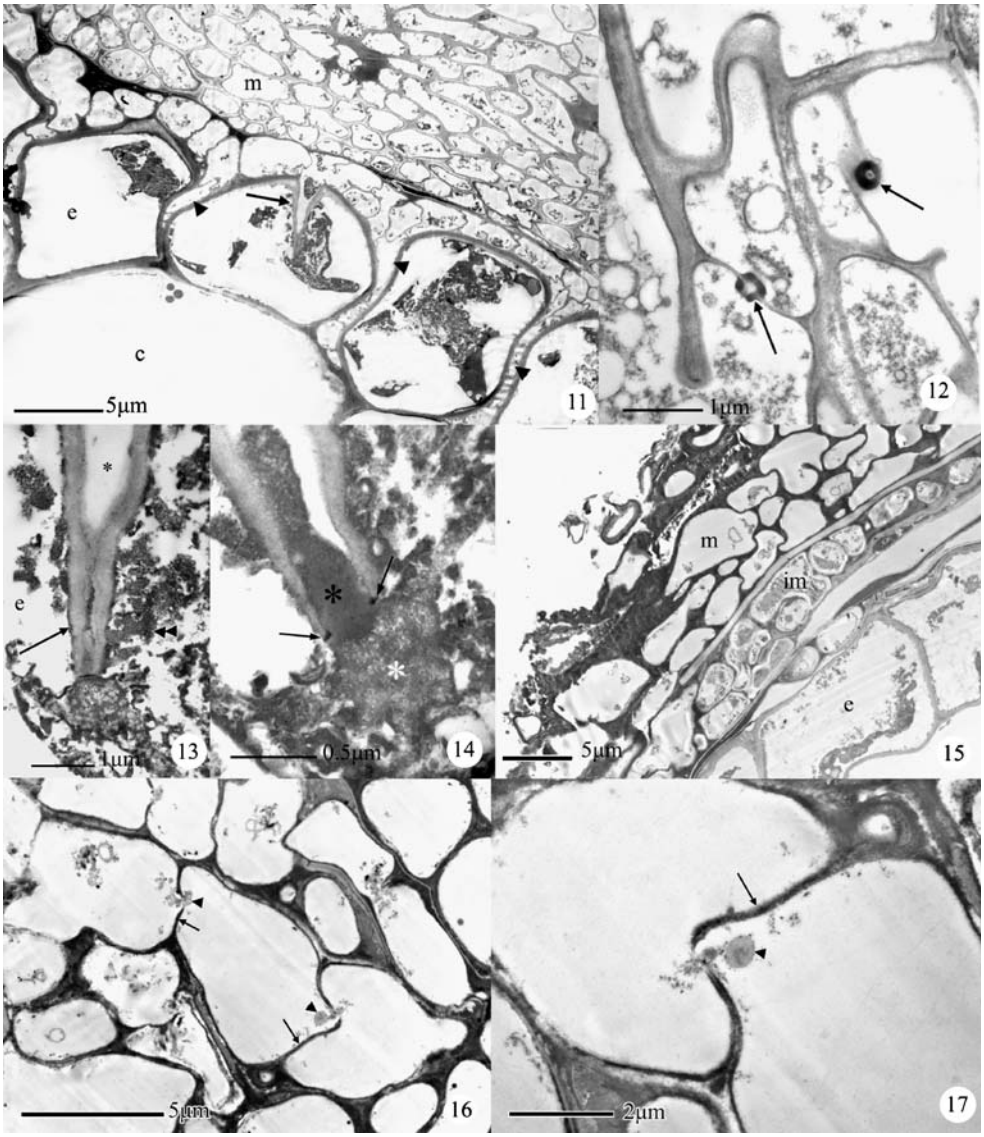
Figs. 4, 5, 6 and 7 Resin-embedded developing embryos of *Pityopus californicus* stained with Toluidine Blue O. **Fig. 4** Young embryo showing early fungal mantle development (*arrows*) on and beneath the testa. **Fig. 5** Developing embryo showing multilayered mantle development (*arrows*) on surface of embryo. The fungal hyphae are large and well defined, similar to *Cenococcum*. Protein and starch are no longer present in embryo cells. **Fig. 6** Developing embryo showing mantle (*arrows*) and a small region of meristematic tissue at apex of a developing root. Mantle hyphae are wide and well defined and appear to have laid down dark interhyphal material, similar to *Cenococcum.* **Fig. 7** Developing embryo showing meristem at apex of developing root and two different fungi colonizing the root (*arrows*); closer to the seed coat, the fungal hyphae are well defined and darker (especially on the top), whereas further along the root, the hyphae have thinner walls, and the mantle is thin. No obvious intercellular hyphae (Hartig net) can be detected in this root

required for embryo development of *P. californicus* and that it is likely critical for seedling establishment of this species. In their 2005 study, Bidartondo and Bruns identified the fungus that associates with germinating seeds of *P. californicus* as *T. myomyces*, the same fungus that forms the mycorrhizal symbiosis with mature *P. californicus* plants.

The presence of two different fungal mantle morphotypes, one with dolipore septa and the other with simple septa associated with Woronin bodies, on developing embryos as well as roots of *P. californicus* observed in this study, suggests either that both a basidiomycete and ascomycete fungal species (Strullu and Gerault 1977) may be able to induce seed germination in this species or, more likely, that it is only *T. myomyces* that induces germination, and the ascomycete simply colonizes some of the roots. As *Cenococcum* has a wide host range, it may be viewed as a promiscuous opportunist. It is likely that, during later stages of development, *T. myomyces* becomes the dominant fungal symbiont that links seedlings to an autotrophic host. Embryos colonized by the *Cenococcum*-like fungus failed to develop pegs within epidermal cells indicating that, while this fungus may be able to trigger germination of *P. californicus* seeds, it may not be developing a functional mycorrhizal association. It is possible, however, that more



Figs. 8, **9 and 10** Sections of resin-embedded developing roots of *Pityopus californicus* stained with Toluidine Blue O. **Fig. 8** Longitudinal section showing complete mantle (*arrows*) and two emerging lateral roots (*arrowheads*). A meristematic zone is evident. **Fig. 9a,b-10** Detail of colonized roots: fungal mantle (*m*), fungal pegs (*arrows*), epidermis (*e*), cortex (*c*), and Hartig net (*double arrowheads*). The mantle in all figures is well defined (more so in Fig. 9a and b) and the fungal pegs penetrate the outer tangential wall of the epidermal cells. Pegs appear variable in shape. The Hartig net is always paraepidermal



Figs. 11, 12, 13, 14, 15, 16 and 17 Transmission electron micrographs of thin sections of colonized *Pityopus californicus* roots. **Fig. 11** Low-magnification view of fungal mantle (*m*), epidermal (*e*) cells, one with a fungal peg (*arrow*). *Arrowheads* Hartig net; *c* cortical cell. **Fig. 12** Detail of mantle hyphae showing dolipore septa (*arrows*). **Fig. 13** High-magnification view of fungal peg tip (*arrow*) in plant epidermal cell (*e*) showing wall deposits (*double arrowheads*) around fungal peg (*). **Fig. 14** Tip of a fungal peg showing electron-dense wall deposits (*arrows*), an occlusion (black asterisk) and remnants of cytoplasm (*white **). **Fig. 15** Low-magnification view showing colonization by a *Cenococcum*-like fungus with thick-walled, electron-dense outer mantle hyphae (*m*) and cytoplasmic inner mantle hyphae (*im*), epidermis (*e*). **Fig. 16** Detail of above mantle hyphae showing thin septal crosswalls (*arrows*) and occluded septal pores (*arrowheads*). **Fig. 17** Detail of septum from Fig. 16 showing thin septal crosswall (*arrow*) and Woronin body adjacent to the septal pore (*arrowhead*)

advanced root development might have revealed epidermal pegs. We also assume, from what is known about monotropoid mycorrhizas, that a peg is an absolute requirement for its functioning, but it is perhaps possible to have a functioning interface without the peg, as an Hartig net develops in these *Cenococcum*-like roots. It would be of interest to use the method of separating seeds from potential fungal symbionts by a permeable membrane such as that developed by Bruns and Read (2000) to test the effect of various fungal species on germination of *P. californicus* seeds.

The presence of a well-defined mantle on the first root initiated out of the seed also suggests that the signals required to recognize and colonize the host by the fungus are present at this very early stage of root growth. The multilayered mantle also indicates that the fungus is quite capable to "cover the energy costs" of this fungal structure, the purpose of which here is unclear.

The presence of fungal pegs that penetrate the outer tangential epidermal cell walls of *P. californicus* roots, a distinctive feature of *Monotropa uniflora* (Lutz and Sjolund 1973; Massicotte et al. 2005), *M. hypopitys* (Duddridge and Read 1982), and *Monotropastrum humile* (Matsuda and Yamada 2003) mycorrhizas, suggests that *P. californicus* may be more closely related to these species than to other species in the Monotropoideae such as *Pterospora andromedea*, in which fungal pegs penetrate the radial epidermal cell walls (Massicotte et al. 2005). The formation of fungal pegs and the appearance of transfer cell-like wall deposits encasing the peg, as described previously for *M. uniflora* (Lutz and Sjolund 1973; Massicotte et al. 2005) and *M. hypopitys* (Duddridge and Read 1982; Dexheimer and Pargney 1991; Dexheimer and Gérard 1993), suggest that nutrient exchange may occur at this site (Peterson and Massicotte 2004); however, function with respect to these

structures is still poorly understood (Bidartondo 2005). The development of these complex wall depositions is in many ways comparable to what occurs as haustoria develop in biotrophic fungal pathogen–plant interactions (e.g., Mims et al. 2001). The apparent opening of the tip of the fungal peg at a later stage in its development, with a sac-like structure surrounding the tip, corresponds with observations made on *M. uniflora* (Lutz and Sjolund 1973), *M. hypopitys* (Duddridge and Read 1982), and *M. humile* (Matsuda and Yamada 2003) and differs from haustoria of biotrophic fungal pathogens. The deposition of an electron-dense band in the fungal wall immediately behind the tip in *P. californicus* corresponds with observations on *M. uniflora* (Lutz and Sjolund 1973) and is not unlike the neck bands present in haustoria of many biotrophic pathogenic fungi (Heath 1997; Mims et al. 2001) which function to prevent apoplastic movement of solutes along the junction between fungus and host.

P. californicus mycorrhizas also have a very well-developed Hartig net in addition to the fungal pegs, and this could also participate in nutrient exchange between symbionts. The determination of the site(s) of transfer of carbon and other nutrients from fungus to epidermal cells will, however, require the use of radioactive labeling and subsequent autoradiography of rapidly frozen and freeze-substituted material, similar to the studies of ectomycorrhizas by Bücking and Heyser (2001).

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