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Morphological and functional stasis in mycorrhizal root nodules as exhibited by a Triassic conifer

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Mycorrhizal root nodules occur in the conifer families Araucariaceae, Podocarpaceae, and Sciadopityaceae. Although the fossil record of these families can be traced back into the early Mesozoic, the oldest fossil evidence of root nodules previously came from the Cretaceous. Here we report on cellularly preserved root nodules of the early conifer *Notophytum* from Middle Triassic permineralized peat of Antarctica. These fossil root nodules contain fungal arbuscules, hyphal coils, and vesicles in their cortex. Numerous glomoid-type spores are found in the peat matrix surrounding the nodules. This discovery indicates that mutualistic associations between conifer root nodules and arbuscular mycorrhizal fungi date back to at least the early Mesozoic, the period during which most of the modern conifer families first appeared. *Notophytum* root nodules predate the next known appearance of this association by 100 million years, indicating that this specialized form of mycorrhizal symbiosis has ancient origins.

Antarctica | Glomeromycota

Arbuscular mycorrhizae (AM) occur in the majority of extant land plants and can be traced back to the Early Devonian, more than 400 million years ago (1). The presence of the fungi (members of the Glomeromycota) within the root facilitates the uptake of nutrients and water, and may also have a protective effect against soil pathogens (2, 3). In most cases the AM fungi occur in the root cortex of the host. However, some plants develop specialized root nodules that are colonized by the fungi. Such mycorrhizal nodules have been documented in angiosperms belonging to the Casuarinaceae (4), Fabaceae (5, 6), and Sapindaceae (7), as well as in three extant conifer families: the Araucariaceae, Podocarpaceae s.l. (i.e., including *Phyllocladus*), and Sciadopityaceae (8). Conifer mycorrhizal nodules occur as small spherical structures produced in rows on small lateral roots (Fig. 1A). Based on the regular arrangement and anatomy, they were originally interpreted as modified short roots, but differences, such as the lack of a root cap and the presence of a continuous endodermis around the vascular tissues, could indicate that they represent in fact a unique structure (9, 10). After colonization, the fungi remain in the cortex of the nodules and do not spread to the rest of the root system (Fig. 1A, *Inset*). As a result, each nodule can be viewed as a small colony, in some cases containing several different lineages of AM fungi (10). The exact benefits of this association are still unclear, but there is evidence that it increases phosphate uptake (11) and might also enhance nitrogen uptake (12).

Despite their conspicuous morphology, roots with mycorrhizal nodules are exceedingly rare as fossils. Previous evidence consists of Early Cretaceous compression/impression specimens of roots that are associated with conifer wood and leaves; these fossils bear nodules morphologically similar to the extant ones (13–15), but without anatomy preserved, it is not possible to make a detailed comparison with modern mycorrhizal nodules. Here, we report the occurrence of mycorrhizal nodules on early Middle Triassic (~240 Ma) anatomically preserved roots of the putatively podocarpaceous conifer *Notophytum krauselii* Meyer-

Berthaud and Taylor from the Fremouw Formation of the Central Transantarctic Mountains, Antarctica, which represent the oldest evidence of mycorrhizal nodules. The exceptional preservation of both the host roots and the fungus allows us to document in detail this complex fossil plant–fungal interaction and compare it with its extant equivalents. This discovery suggests that the particular mutualistic association found today between podocarpaceous conifers and AM fungi was already well established during the early Mesozoic.

Description

Morphology and Anatomy. Mycorrhizal root nodules are dispersed throughout silicified peat from Fremouw Peak in association with *N. krauselii* stems, leaves, and roots (Fig. 1B), *Parasciadopitys aequata* Yao, Taylor, and Taylor ovulate cones (all conifers), *Petriellaea triangulata* Taylor, Del Fueyo, and Taylor seeds (a seed fern), and *Spaciodum collinsonii* (Osborn and Taylor) Schwendemann, Taylor, Taylor, Krings, and Osborn (sphenophyte) stems and strobili. Both young and mature nodules have been found in differing states of preservation. The best preserved specimens are completely preserved cellularly (Fig. 1D), and others yield mostly morphological data (Fig. 1C). Each mature nodule is ~1.1-mm long in longitudinal section and 0.8-mm wide in cross section at their greatest thickness. Nodules typically occur in groups of two formed at opposite ends of the root protoxylem poles. While grinding through specimens in longitudinal section, the changes in nodule dimension suggest that they have the shape of a prolate spheroid. In mature specimens, the base of each nodule is deeply embedded within the cortex of the root. Mycorrhizal root nodules have not been discovered attached to roots with a diameter greater than 0.5 mm.

Root nodules of *Notophytum* show different ranges of preservation throughout the peat matrix. The ontogenetically younger specimens typically contain well-preserved cells and fungal hyphae (Fig. 1D), although more mature specimens only display cellular preservation in the peripheral layers of the cortex (Fig. 1C). Nodules lacking cellular detail are filled with opaque materials of uncertain origin (Fig. 1E). At higher magnification, the material appears to be a dense aggregation of small globules. Both young and mature nodules contain evidence of mycorrhizal fungi. Cells of young nodules contain arbuscules (Fig. 1F), and intracellular hyphal coils (Fig. 1G) can be found in the outer cortex of mature root nodules. Hyphae of these specimens measure < 3.5 μm in thickness. Spherical vesicles (Fig. 1H) measuring 23 μm in diameter also occur within the outer cortex of the mature nodules.

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The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the University of Kansas Paleobotanical Collections Database, <http://paleobotany.bio.ku.edu/PaleoCollections.htm> (accession nos. 23015–23110, 26339, 26343–26364, and 24090–24101).

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the presence of AM in a Triassic plant, even in the absence of data on physiological interaction. The anatomy of the host roots is consistent with that seen in the Triassic conifer *N. krauselii*, which is thought to be an early member of the Podocarpaceae (19–21). The root nodules of *N. krauselii* share several similarities with those in extant podocarps (e.g., the nodules in both commonly contain fungal hyphae aggregated into arbuscules and hyphal coils as well as vesicles in the outer cortex) (4, 10, 22–26). Moreover, the development of the nodules in both extant podocarps (10) and the fossils starts from the same region of the root (i.e., in the pericycle near a protoxylem pole). This finding suggests that the root nodules of *Notophytum* and extant podocarps are likely homologous structures. In most extant podocarp species, the nodules develop opposite the protoxylem poles (25). Although the nodules in both extant podocarps and the fossil arise opposite protoxylem poles, in *Notophytum* the nodules developed outward through the cortex at an angle, but those in modern species tend to grow outward in a horizontal plane.

The presence of fungi in the cortex of the fossil nodules is particularly important for establishing the mycorrhizal nature of the association. In extant podocarps, formation of nodules is known to be a constitutive developmental trait (i.e., they can be produced in the absence of the fungi) (9, 10). As a result, the presence of root nodules alone does not suffice to establish the mutualistic nature of the fossil association. Studies of extant podocarps have shown that the fungi present in the cortex of the nodules belong to at least two genera of Glomeromycota, *Glomus* and *Archaeospora* (10). The presence of arbuscular fungi in the cortex of *Notophytum* root nodules indicates that these were indeed mycorrhizal nodules, which probably functioned like those of extant podocarps. Arbuscules are only found in the youngest nodules that have better cellular preservation. It is not surprising that older nodules may not contain arbuscules, as arbuscules are ephemeral structures that may only last a few days (27). Fungal spores have not been found attached to hyphae directly connected to the root nodules, but the spores occur in the presence of *Notophytum* nodules at a greater rate than elsewhere in the peat matrix. Fungal hyphae are more delicate than the cell walls of plants and extensive mycelia are unlikely to be preserved. Nevertheless, fungal spores with attached hyphae and mycelial mats can be found nearby the *Notophytum* root nodules. These fungal spores share several characters with extant glomoid spores, including size, shape, and hyphal connection (28). These characteristics add support to our interpretation that the symbiont associated with the fossil nodules was a member of the Glomeromycota, as is the case in extant mycorrhizal root nodules.

Element analysis of the opaque matter within the mycorrhizal root nodules shows the presence of calcium throughout, whereas only trace amounts of calcium were found in any nearby plant tissues that were examined. Although the preservation of these older root nodules does not provide visual evidence of mycorrhizal fungi, the presence of calcium in only the root nodules offers some chemical evidence that fungi were once there. In extant plants, mycorrhizal fungi often contain a large amount of calcium oxalate, which is used to break down soil into usable minerals (4, 29).

The presence of mycorrhizal nodules on the roots of a Triassic conifer results in a significant extension of the temporal range of this type of association. Before this work, the fossil record of mycorrhizal nodules consisted of specimens without anatomical detail (compression/impressions or molds) from the Otway Basin in Australia (13), Alexander Island in Antarctica (14), and possibly the Rajmahal Basin in India (15), all of Early Cretaceous age (Table 1). The recent specimens from Antarctica thus push back the earliest evidence of mycorrhizal nodules on conifer roots by ~100 Myr before previously described specimens. The anatomical preservation of the roots allows for a better identification of the affinities of the nodule-producing plant. In the case of the Cretaceous specimens, the affinities of the parent plants have been hypothesized based on the morphology of the root systems and affinities of other remains found in association with the roots. The Australian specimens are associated with probable taxodiaceous foliage (13) and the Antarctic ones with podocarpaceous wood (14). The Indian roots are found with an assemblage that contains remains of both Araucariaceae and Podocarpaceae (15).

Previous paleobotanical investigations have led to the emergence of a whole-plant concept for *N. krauselii* based on anatomical connections, as well as morphological, anatomical, and epidermal similarities among dispersed organs. This species is now one of the best known conifers from the Triassic of the Southern Hemisphere: it was a forest tree with trunks at least 20 cm in diameter (19), stems and roots had podocarpaceous-type wood (19), and the plant bore broad, multiveined leaves whose anatomy is also consistent with placement in the Podocarpaceae (20). The permineralized leaves probably represent the same biological taxon as compressed *Heidiphyllum* leaves (20). Large *Notophytum* roots were capable of producing sucker shoots (30), a trait that in extant conifers is restricted to the Araucariaceae and Podocarpaceae s.l. Associated reproductive structures consist of a seed cone (*Parasciadopitys/Telemachus*) and an anatomically preserved embryo (21, 31). The existence of young *Notophytum* roots bearing nodules thus enhances our knowledge of the anatomy of this plant and demonstrates that the production

Table 1. Evidence of mycorrhizal nodules in fossil conifers

Age	Locality	Preservation	Nodule	Affinities of the roots	Evidence of fungi in nodules
Middle Triassic (245–230 Ma)	Antarctica	Permineralization	Prolate spheroid nodules 1.1 × 0.8 mm, usually in groups of two opposite root protoxylem poles	Podocarpaceous	+
Early Cretaceous (145–100 Ma)	Australia	Compression/impressions	—	Associated with possible taxodiaceous foliage	—
	Antarctica	Compression/impressions	Two opposite rows of spherical nodules ≤ 2 mm wide on ultimate rootlets	Associated with podocarpaceous wood	—
	India	Molds	Numerous spherical-oval nodules, 1 × 1 mm to 2.5 × 2 mm	Associated with remains of Podocarpaceae and Araucariaceae	—

Fossil evidence of mycorrhizal nodules in fossil conifers, including the recent specimens described in this article (boldface) and previous reports (10–12). (+/–) indicates the presence or absence of fungi in the nodules, respectively.

of root nodules in podocarps goes back at least to the origin of the group in the Triassic, coinciding with the diversification of the modern conifers.

Among the extant gymnosperms, mycorrhizal root nodules can be found in all members of the Podocarpaceae, Araucariaceae, and Sciadopityaceae (8). These three families are occasionally grouped in phylogenetic analyses of conifers (e.g., refs. 32 and 33). As *Notophytum* contains many features of the Podocarpaceae and is one of the earliest known fossils of the group (34), it is possible that mycorrhizal root nodules are a defining feature of these groups and arose early in their evolution. The only extant conifer group that frequently appears more basal than the Podocarpaceae-Araucariaceae-Sciadopityaceae group in phylogenetic analysis is the Pinaceae (33). No mycorrhizal root nodules have ever been reported for this family, but members do contain roots with specialized mycorrhizal symbionts. The ectomycorrhizal fungi of the Pinaceae (35) and the endomycorrhizae of cordaite roots (36) suggest that mycorrhizal associations were important in the evolution of early conifer groups. The cordaites are a group of extinct fossil plants thought to be involved in the evolution of the conifers (34). Nearly all of the extant nodulating conifers, as well as the mycorrhizal nodule-forming angiosperm *Gymnostoma* (4), occur in tropical to subtropical ecosystems in exposed mafic, rocky habitats containing little phosphorus, which are occasionally inundated with water (e.g., 22). These nodulating plants are commonly stabilizing and pioneering species for these habitats.

Mycorrhizal root nodules are not the only type of root nodules found in plants. Actinorhizal and *Rhizobium* nodules also occur in angiosperms (37, 38); both types contain nitrogen-fixing microorganisms. Although mycorrhizal root nodules were once thought to be involved in nitrogen fixation (39), those ideas have since fallen out of favor (40). Actinorhizal and *Rhizobium* nodules differ mainly in their anatomy and development (37). Genes known to be involved in symbiotic relationships between plants and microorganisms are found in plants with all three nodule types (37) and some plant species contain both mycorrhizal and actinorhizal nodules in the same root system (4–6). These genes have been shown to be expressed during nodule formation and maintenance. Although several studies have examined the relationship between actinorhizal plants and *Rhizobium* nodule plants, the role of mycorrhizal nodules has been largely overlooked. Recent discoveries have shown that some legume mutants that are incapable of forming nodules are also defective with respect to their arbuscular mycorrhizal development. This finding has led to the hypothesis that the root nodule associations in angiosperms have evolved by recruiting genes from arbuscular mycorrhizal symbiosis (41, 42). It may be that all of these different

nodules represent convergent evolution toward a common symbiotic relationship, but the presence of nodules in these fossil organisms should call for a closer inspection into the genetics of modern mycorrhizal root nodules in an attempt to trace the origins of this ancient mycorrhizal association.

Conclusion

The presence of mycorrhizal nodules on the roots of a Triassic fossil conifer illustrates the importance of mycorrhizal interactions in the early conifers. This fossil represents the earliest known interaction between a microorganism and a root nodule; the special mycorrhizal type exhibited here appears during the diversification of the conifers. Many of the genes involved in angiosperm bacterial nodule interactions are shared with arbuscular mycorrhizae interactions. Formation of mycorrhizal root nodules likely represents an early evolutionary experiment into more advanced mutualistic interactions.

Material and Methods

Mycorrhizal root nodules of *Notophytum* are preserved in permineralized peat collected from Fremouw Peak in the Queen Alexandra Range of the Transantarctic Mountains (84°17'41" S, 164°21'48" E) (43). The peat is dated early Middle Triassic based on the palynomorph assemblage in the peat and vertebrate fossils from nearby (44, 45). Peat blocks were sectioned and the polished surface etched with 49% hydrofluoric acid for 1 to 5 min. Cellulose acetate peels (46) were made from the prepared surface, with some peels subsequently mounted on slides using Eukitt mounting medium. Slides are housed in the Paleobotany Division of the Natural History Museum and Biodiversity Institute, University of Kansas, Lawrence, under accession numbers 23015 to 23110, 26339, 26343 to 26364, and 24090 to 24101. Peels and slides of the nodules were made from blocks 11277 B₂side botr, 11277 B₃side top, 13790 C₂side, 16207 G, and 10206 E.

For light microscopy, all specimens were photographed using a Leica DC500 digital camera attachment on a Leica DM 5000B compound microscope and a Leica MZ 16 dissecting microscope. Digital images were processed using Adobe Photoshop CS2 Version 9.0 (Adobe Systems Incorporated). High magnification (> 640×) images were taken under oil immersion.

For elemental analysis, energy-dispersive X-ray spectroscopy was used. Acetate peels were mounted on stubs and sputter coated with gold. Specimens were then examined with a Leo 1550 Scanning Electron Microscope at 20 kV. Scanning electron microscope stubs are housed in the Paleobotany Division of the Natural History Museum and Biodiversity Institute, University of Kansas, Lawrence, under accession numbers AS(1)11.

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