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Update on Root Biology

Proteoid Roots. Physiology and Development

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Nearly 100 years ago, Engler described the unusual root morphology of plants in the family Proteaceae growing in the Leipzig Botanic Gardens. They had extensively branched roots covered with long, densely grouped absorption hairs. It wasn't until 1960 that Purnell coined the term "proteoid root" to describe a root with "dense clusters of rootlets of limited growth." She examined 44 species from 10 genera in the family Proteaceae and observed proteoid roots in all but the more primitive genus *Persoonia*. Subsequent surveys have documented proteoid roots in 27 genera of Proteaceae (Dinkelaker et al., 1995). Proteaceae are a major component of the Mediterranean flora of southwestern Australia and South Africa, where nutrient-impoverished soils support an amazing diversity of plant species. Purnell (1960) suggested that proteoid roots were involved in nutrient uptake because they had proliferated in a layer of blood and bone manure placed in an otherwise nutrient-poor sand.

Proteoid roots have now been reported in 28 species from the Betulaceae, Casuarinaceae, Eleagnaceae, Leguminosae, Moraceae, and Myricaceae families, all of which can symbiotically fix atmospheric N_2, apart from *Ficus benjamina* and members of the Proteaceae. Clusters of swollen, short, lateral roots occur in species of the Cyperaceae and Restionaceae, and their overall morphology is similar to proteoid roots; however, to date nothing is known of their physiology (Dinkelaker et al., 1995).

All species with proteoid roots can grow in soils with poorly available nutrients, and most do not form mycorrhizal symbioses (for review, see Skene, 1998). Proteoid roots mobilize mineral P that is bound to metal cations such as Fe, Al, and Ca, extract P from organic layers in soil, obtain Fe and Mn from alkaline soils, and take up organic forms of N (Dinkelaker et al., 1995). Of the species that form proteoid roots, white lupin stands out as the only one currently used in agriculture and the one that has been the most intensively studied. Genes underlying the developmental and biochemical features of proteoid roots are being sought with the possibility of transforming non-proteoid plants.

After describing the morphology of proteoid roots, we will discuss how they are produced in response to P or Fe supply and how they enable nutrient (principally P) uptake through an increase in surface area and exudation of nutrient-solubilizing compounds. This requires an altered metabolism that is synchronized to root development. There appears to be a tightly regulated sequence of events that triggers the initiation of clusters, limits rootlet growth, alters metabolism, and subsequently activates and deactivates exudate transport mechanisms. While signals mediating proteoid root development are as yet unknown, there are striking structural similarities to root proliferation induced by auxins.

PROTEOID ROOT MORPHOLOGY

We define a proteoid root as an entire root from any species that forms one or more clusters along its length. A cluster has closely spaced lateral roots (rootlets) of limited growth (Fig. 1). The terms proteoid root, cluster root, and root cluster have all been used in the literature to describe either the proteoid root axis or clusters of rootlets, and caution should be taken when comparing studies because the definitions of these terms vary.

Along a proteoid root, discrete clusters of closely spaced rootlets develop. The rootlets emerge in contiguous rows from the cortex and grow to reach a similar length. Meristems of the rootlets develop from the pericycle, similar to non-proteoid roots. Within a few days, meristems stop dividing and differentiate (i.e. the rootlets are determinate) (Fig. 2, compare A and B). Once rootlets reach their final length, they have a stele that extends to within a few cells of the tip (Fig. 2B) and root hairs extending to the tip (Fig. 2C). Determinacy in lateral roots also occurs in non-proteoid plants such as maize and is not unique to proteoid roots (McCully, 1999).

Clusters can form singly, as is commonly observed in *Lupinus albus* (Figs. 1 and 3, B and C; Lamont, 1972), or become complex, with a root within a cluster becoming the axis for another cluster. Compound, mat-like structures, are seen in *Banksia* spp. colonizing litter layers at the soil surface (Fig. 3A; Skene, 1998). Proliferation of rootlets in a cluster presents a massive increase in root surface area for contact with soil. For example, a mature *Hakea obliqua* proteoid root cluster has a surface area (excluding root hairs) 25 times greater than that of an equivalent mass of axial root (Dell et al., 1980).

There is considerable variation in proteoid root morphology between species. The number of rows of rootlets in a cluster depends on the vascular structure of the proteoid root, as rows develop from each xylem pole. *Hakea* spp. can

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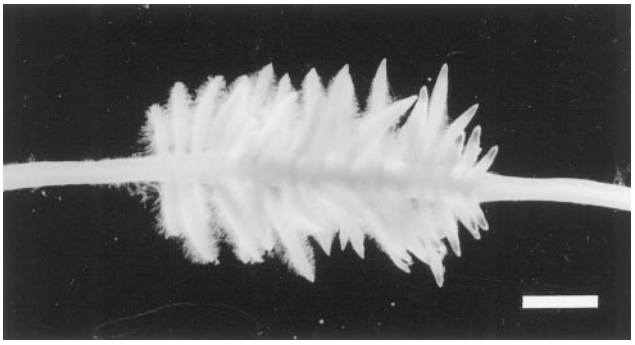

Figure 1. Cluster of rootlets on a proteoid root of *L. albus* grown in hydroponics in the absence of P. Scale bar = 3 mm.

have up to six xylem poles, and three to seven longitudinal rows of rootlets form with 280 to 1,000 rootlets cm^{-1} root axis (Lamont, 1972). *L. albus* has two xylem poles and two to four rows of rootlets form with 10 to 45 rootlets cm^{-1} (Dinkelaker et al., 1989; Johnson et al., 1996a). Rootlet final length can range from 1 to 30 mm, depending on the species, and the length of a cluster along a proteoid root axis also varies between species (Dinkelaker et al., 1995).

Environment can alter proteoid root development within a species. Clusters are generally rare or absent when plants are supplied with abundant P (Fig. 3D, see "Proteoid Root Development Depends on Nutrients"). In *L. albus*, the number of rootlets per length of axis decreases with increasing P in nutrient solution (Johnson et al., 1996a; Keerthisinghe et al., 1998). In *H. obliqua* (Dell et al., 1980), *Grevillea robusta* (Skene, 1998), and *L. albus* (Fig. 3, B and C), rootlet length is shorter when plants are grown in hydroponics compared with when they are grown in vermiculite or soil. Rootlet length is also shortened when *L. albus* is grown with elevated (700 $\mu L\ L^{-1}$) versus ambient (350 $\mu L\ L^{-1}$) atmospheric CO_2 (Watt and Evans, 1999). Root hair development is also influenced by the environment, being absent when *H. obliqua* (Dell et al., 1980) or *G. robusta* (Skene, 1998) plants were grown in hydroponics, but present when grown in soil.

PROTEOID ROOT DEVELOPMENT DEPENDS ON NUTRIENTS

P nutrition has been clearly implicated in the elaboration of proteoid roots. Clusters are most prominent when the P supply is restricted, and in most species, the formation of proteoid roots declines as P availability to roots increases (Dinkelaker et al., 1995; Keerthisinghe et al., 1998). Cluster number can be reduced by foliar application of P to *L. albus* and *Myrica cerifera* (Dinkelaker et al., 1995), which shows that the internal P concentration can influence cluster formation. In *Banksia ericifolia*, P addition had to exceed that detrimental to plant growth before proteoid root formation was reduced (Dinkelaker et al., 1995). In many species, proteoid roots will form in P concentrations commonly found in agricultural soils (10 μM).

Iron deficiency has been found to promote formation of proteoid roots in *Lupinus consentinii*, *F. benjamina* (Dinkelaker et al., 1995), and *Casuarina glauca* (Arahou and Diem, 1997) but not in *B. ericifolia*, *L. albus*, *M. cerifera*, or *Alnus incana. L. consentinii* is unique so far in producing proteoid roots in response to either P or Fe deficiency. For all other proteoid-root-forming species, those that respond to P stress do not produce proteoid roots under Fe stress and vice versa, at least for the conditions under which they have been grown.

The conditions that result in proteoid root formation between species becomes increasingly complex as more species are described. In *Lupinus*, eight species produce proteoid roots while four do not (Clements et al., 1993). In *Casuarina*, four species produce proteoid roots in response to P deficiency, while *C. glauca* produces them only in response to Fe deficiency. In *Alnus*, *Alnus rubra* produces proteoid roots in the absence of P, *A. incana* produces them

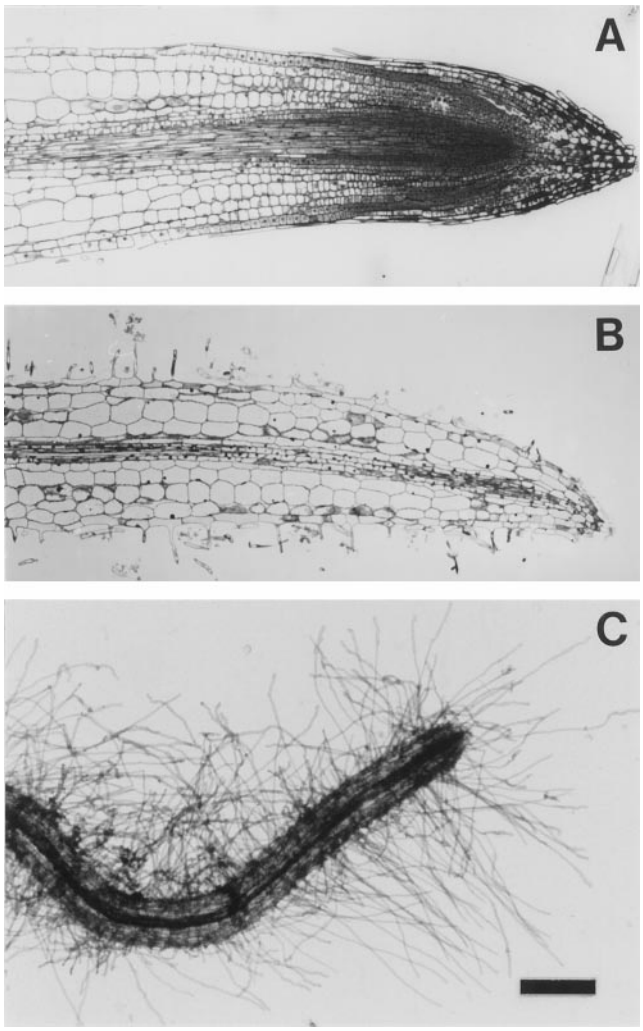

Figure 2. Longitudinal sections of a normal root (A) or proteoid rootlets (B) from *L. albus* and a whole mount of a proteoid rootlet of *Banksia serrata* (C), all shown to the same scale (bar = 0.15 mm). A, Non-proteoid root with a dense, active meristem, indicative of continuing growth, and root hair development back from the tip. B and C, Proteoid rootlets with no apical meristem, root hairs surrounding the tip, the stele extending to within a few cells of the tip, and a sloughed-off root cap.

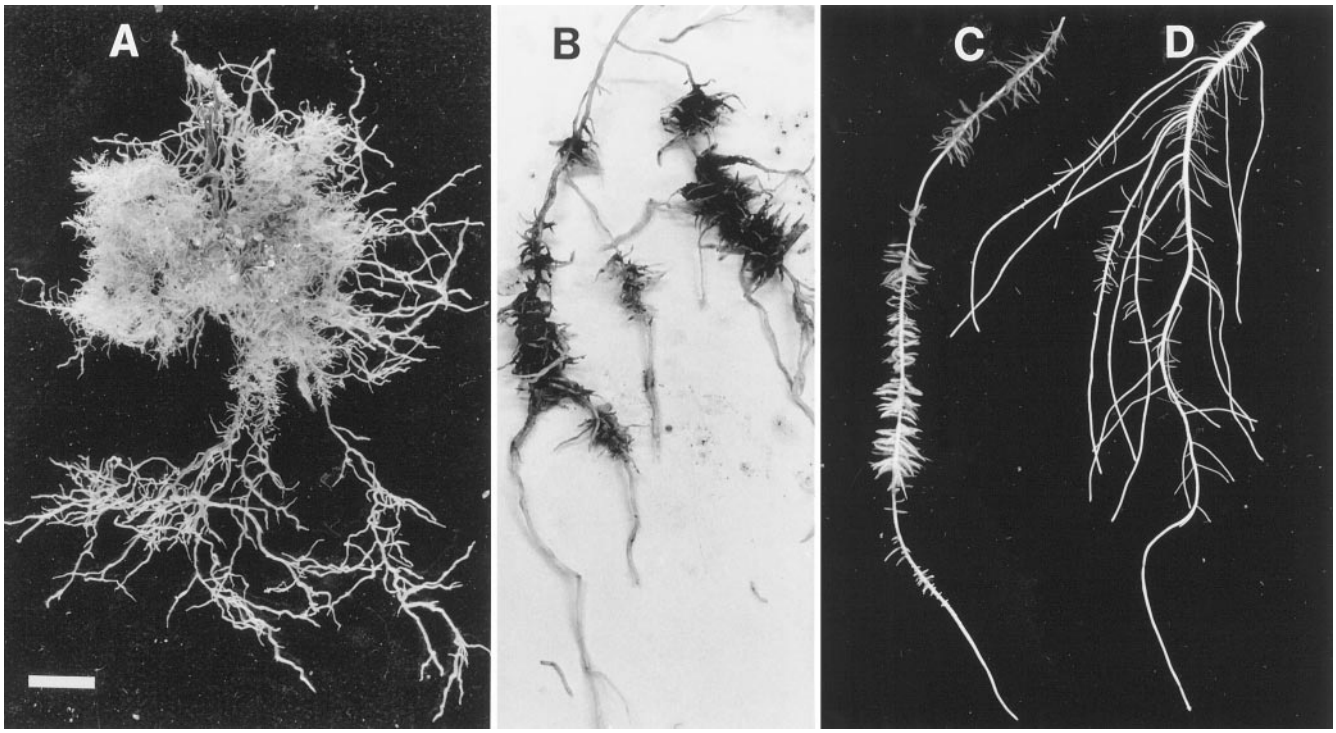

Figure 3. Proteoid roots from *B. serrata* (A) and *L. albus* (B–D) grown in soil (A and B) or hydroponics without P (C) or with 0.5 mM P (D). Scale bar = 1 cm.

in the presence of P, and *Alnus viridis* does not produce them at all (Hurd and Schwintzer, 1996). This diversity of response, together with the increasing number of families in which proteoid roots have been observed, suggests that proteoid roots have evolved independently. The common overall morphology, however, points to a similar combination of signals inducing a cluster. These signals may be triggered by different thresholds in plant nutrient status in the various species.

Proteoid roots tend to form where nutrients are likely to become available. In the field, *Banksia prionotes* proteoid roots form a dense mat in the organic matter layer on the surface of sandy soils, where they take up greater amounts of P, N, and micronutrients than the deeper, non-proteoid roots (Jeschke and Pate, 1995). Proteoid roots can be induced to form in artificial layers of organic matter placed deep in a low-P sandy soil and not elsewhere in the low-P soil (Lamont et al., 1984). That is, they form in response to the presence of organic matter and do not proliferate randomly through the soil profile. Lamont (1973) attempted to distinguish between root proliferation occurring due to the presence of inorganic N in the organic matter versus organic matter without inorganic N. He concluded that while N stimulated non-proteoid root growth, it did not increase cluster formation. Cluster formation in the organic matter layer could, however, be suppressed by the addition of P fertilizer to the soil (Lamont et al., 1984). Thus, proteoid root development depends on an internal control within the plant, but can also respond to a local presence of organic matter adjacent to the root.

EXUDATION BY PROTEOID ROOTS MOBILIZES BOUND NUTRIENTS

Coupled with the proliferation of surface area, proteoid root clusters chemically modify the surrounding soil by exuding compounds. These compounds include carboxylate organic anions, acid phosphatases, phenolics, mucilages, and water, and they facilitate the mobilization of nutrients from soil.

Export of organic anions occurs in several non-proteoid species in response to P limitations and exposure to the toxic element Al^{3+} (Jones, 1998). Rates of export from proteoid roots, however, are among the highest found in plants (see tables II and III of Jones, 1998). Organic anions, especially citrate, mobilize P by chelating soil minerals such as Fe, Al, and Ca, all of which bind P (Jones, 1998). Gardner et al. (1982, 1983) were the first to show that proteoid roots of *L. albus* could mobilize precipitates of P, Fe, Al, and Mn, and that they secreted citrate, which acted as a chelating agent. Dinkelaker et al. (1989) grew *L. albus* in a calcareous soil in which phosphate was bound to Ca and unavailable in soil solution. After 90 d of growth, plants were harvested, and white precipitates of calcium citrate were found in the rhizosphere of proteoid root clusters. Citrate is the major organic anion exported by *L. albus*, but malate and succinate are also exported (Johnson et al., 1996a).

Proteaceae also exude organic anions. About one-half of the organic anions recovered from the proteoid root layer of a mature stand of *Banksia integrifolia* was citrate with lesser amounts of malate and aconitate (Grierson, 1992).

For *Hakea undulata*, malate and fumarate were the main constituents of exudates, while citrate was a minor one (Dinkelaker et al., 1997). The rhizosphere around root clusters of *Banksia*, *Hakea*, *Lupinus*, and *Ficus* becomes acidic, and it is thought that protons are exuded via a plasma membrane ATPase, along with organic anions (Dinkelaker et al., 1995). Alternative accompanying cations such as K have not yet been reported.

Proteoid roots of *L. albus* (Adams and Pate, 1992), *H. undulata* (Dinkelaker et al., 1997), and *Casuarina cunninghamiana* (cited in Skene, 1998) exude acid phosphatases, enzymes that hydrolyze organic forms of P. A novel acid phosphatase is specifically induced in and exuded from proteoid roots of *L. albus* under P deficiency (Gilbert et al., 1999). Citrate and acid phosphatase exuded by *L. albus* roots both diffuse outward into the soil. The amount of P removed up to 2 mm away from the root surface correlated strongly with the profiles of both acid phosphatase and citrate (Li et al., 1997). Uptake of P released by acid phosphatases is probably improved by the presence of citrate, which can chelate metals that would otherwise compete for that released P (Braum and Helmke, 1995). It is therefore beneficial for phosphatases to be exuded into the rhizosphere in synchrony with organic anions.

Root cap cells produce anionic mucopolysaccharides (Dell et al., 1980), which can also chelate metals in soils, releasing P (Nagarajah et al., 1970). Since there is a high density of proteoid rootlets within a volume of soil (e.g. in *Hakea*, 95 root tips per cubic millimeter of soil), root cap mucilage likely represents an important exudate. Root cap mucilage also contributes to the binding of soil particles to improve soil-root contact and minimize the distance that nutrients must diffuse to reach the root surface (Watt et al., 1994). Skene et al. (1996) suggest that material from *G. robusta* rootlets was released by exocytosis from epidermal cells, and that a different type of exudation occurred from the rootlet hairs that adhered them to soil particles.

Water has recently been added to the suite of exudates found in soil surrounding proteoid roots. Pate and Dawson (1999), working with *B. prionotes* during early summer in the sand plains of western Australia, placed bags around intact proteoid roots near the soil surface. Water was exported from these proteoid roots during the night and taken up during the day. The ^{2}H isotopic signature in exported water indicated that it had come from a significant depth, as has been shown for "hydraulic lift" in a range of other plant species. Hydration of the proteoid rhizosphere would facilitate diffusion of nutrients mobilized by other exudates and perhaps increase the longevity of rootlets in an otherwise dry environment (Pate and Dawson, 1999) and enable soil binding by mucilages.

PROTEOID ROOTS ENHANCE NUTRIENT UPTAKE

Species that form proteoid roots can acquire more P from soils low in available P compared with non-proteoid species. Studies using soils labeled with ^{32}P show that *L. albus* can extract P bound to clay surfaces that is unavailable to crop species that do not form proteoid roots (Fig. 4; Braum and Helmke, 1995; Hocking et al., 1998). When the solution from a clay soil around proteoid roots was extracted and analyzed, it had higher levels of P, Fe, and Al compared with a solution from the bulk soil. As a consequence of exudation, Mn uptake is also increased by the formation of proteoid roots in *L. albus* (Braum and Helmke, 1995). The solubilization of bound nutrients also makes them available to other species whose roots may be growing among the proteoid roots. For example, wheat intercropped with *L. albus* was able to capture two times more P and more N and Mn than when grown in monoculture (Dinkelaker et al., 1995). Soybean intercropped with *L. albus* had increased Cu, Fe, and Zn concentrations compared with in monoculture; however, it did not have an increased P concentration (Braum and Helmke, 1995).

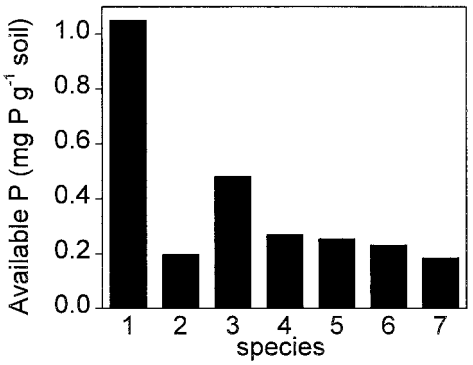

Figure 4. Bound P accessed by a range of species grown in a P binding, oxisol soil. 1, *L. albus*; 2, *Lupinus angustifolius*; 3, *Cajanus cajun*; 4, *Helianthus annuus*; 5, *Triticum aestivum*; 6, *Brassica napus*; 7, *Glycine max* (from Hocking et al., 1998).

DEVELOPMENT AND PHYSIOLOGY LINKED TO CITRATE EXUDATION

Efflux of citrate is linked to proteoid root development, a modified organic anion metabolism, and a regulated membrane transport process. Keerthisinghe et al. (1998) enclosed different sections of proteoid roots of *L. albus* growing in hydroponics and quantified the rates of exudation. They found the greatest rates of 2 nmol of citrate m^{-1} root axis s^{-1} from young rootlets between 1 and 3 cm behind the axis root tip, and low rates elsewhere along the root. Using the same technique, Watt and Evans (1999) followed the development of a cluster and showed that citrate exudation began the day after rootlets had reached their final length, 4 d after emergence (Fig. 5). Growth under elevated CO_2 resulted in both the rootlet final length being reached and efflux starting 1 d earlier than ambient CO_2 controls. Determinacy limits the length rootlets attain and may synchronize the growth and exudation of a cohort of rootlets to maximize the concentration of exudates and therefore maximize nutrient solubilization in the rhizosphere.

Organic anion efflux from a proteoid root cluster appears limited to a few days. Dinkelaker et al. (1997) grew *H. undulata* in boxes of soil with a detachable window to gain access to proteoid roots. Malate recovered from soil solution near clusters increased to 0.9 mM over 2 d before

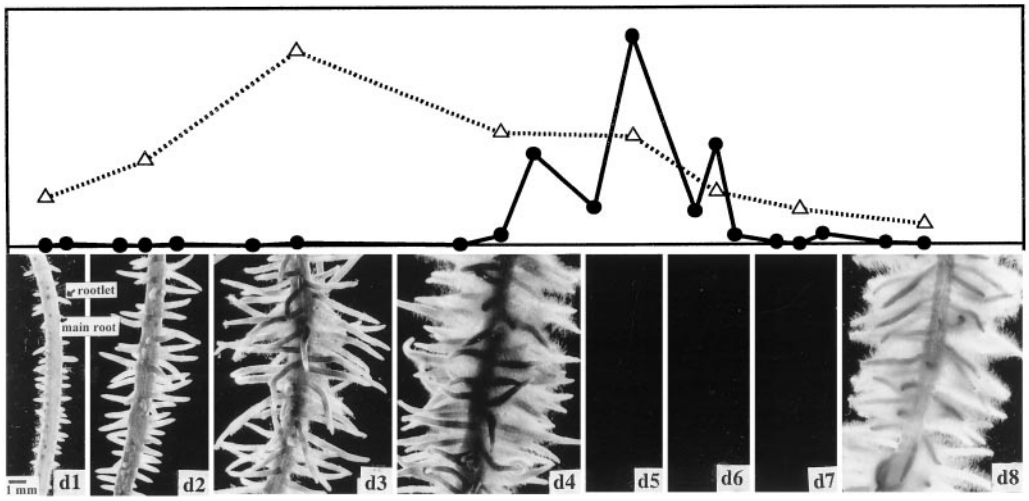

Figure 5. Developmental time course of proteoid rootlet growth, PEPC activity (△, 0–1.6 μmol NADH m^{-1} root axis min^{-1}), and citrate efflux (●, 0–0.11 μmol m^{-1} root axis min^{-1}) (Watt and Evans, 1999). Note that the time scale is not linear but is scaled to fit the size of the micrographs. PEPC peaks as rootlets reach their final length (d3) and no longer have meristems. Citrate efflux starts (d4) 1 d after PEPC activity peaks and rootlets have stopped growing, and continues for 3 d with peaks during the photoperiods.

declining back to 0.01 mM after another 2 d, which the authors argued was due to microbial consumption. The pH changes (acidification followed by alkalinization) and the efflux of phenolics also followed a similar, transient pattern in the cluster rhizosphere. Exudation from *L. albus* clusters is also transient, following a diurnal pulse over 2 or 3 d and then returning to trace levels (Fig. 5). The timing of P uptake during cluster development and organic anion efflux has not been reported, and the longer-term (greater that 1 week) physiology and viability of mature clusters in different environments has not been studied in detail.

Evidence from *L. albus* indicates that cluster development alone does not necessarily result in organic anion export (Newmann et al., 1999). No organic anions were recovered in leachate from intact root systems supplied with 1 mM P that had formed clusters (Johnson et al., 1996a, 1996b). Citrate efflux by proteoid roots from minus-P plants was three times greater than that from plants supplied with 1 to 10 μM P (Keerthisinghe et al., 1998). In addition to cluster formation, a modified organic anion metabolism mediated by the P supply to the plant and a transport mechanism are necessary for the efflux of organic anions.

The P stress response in *L. albus* and several non-proteoid plants commonly involves an increase in CO_2 fixation by PEP carboxylase (PEPC). PEPC is a highly regulated enzyme and has multiple roles, including organic acid synthesis and the provision of carbon skeletons for amino acids, generation of substrate for the tricarboxylic acid (TCA) cycle, and maintenance of cellular pH. PEPC mRNA levels and PEPC activity were greater in proteoid roots grown without P compared with roots grown with 1 mM P (Johnson et al., 1996a). Neumann et al. (1999) have shown that citrate concentrations in proteoid root tissues increase as proteoid rootlets mature, being roughly equivalent to the amount of citrate exuded in 1 d. Johnson et al. (1996b) radiolabeled the shoots and roots of white lupin with $^{14}CO_2$ and showed that approximately 25% of the carbon atoms in exuded citrate were fixed in proteoid roots by the enzyme PEPC. Other studies measuring in vitro PEPC activities on the same tissue used to collect exudates indicated that PEPC activity was not tightly coupled to citrate exudation (Fig. 5; Keerthisinghe et al., 1998; Watt and Evans, 1999). Respiration rates can be used as an indirect measure of flux through the TCA cycle, and the potential production of citrate in proteoid root cells. The amount of exuded citrate represents only a small percentage of the cycled citrate, assuming that all tissues contribute to citrate export (Neumann et al., 1999; Watt and Evans, 1999). The additional flux required to produce organic anions for exudation thus does not appear to be very large. Therefore, while PEPC facilitates anapleurotic functioning of the TCA cycle for accumulation of citrate and for amino acid synthesis (Jeschke and Pate, 1995) and directly contributes significant amounts of carbon to exported citrate, PEPC does not control the rate of exudation.

The mechanism by which exudation occurs is not yet known. Composition of organic anions within the tissue does not reflect that of exudates (Johnson et al., 1996b; Keerthisinghe et al., 1998), and rates of exudation do not reflect tissue concentration (Neumann et al., 1999), indicating that the mechanism has specificity and is not driven solely by a concentration gradient between the roots and the rhizosphere. Citrate may be exported via anion channels (Johnson et al., 1996b) such as those active in wheat root tips exposed to Al^{3+} (Ryan et al., 1997). Efflux could be reduced by 50% when anion channel inhibitors were applied to proteoid roots (Neumann et al., 1999), although such inhibitor studies should be interpreted with caution. It is also possible that export is mediated by packaging of citrate in vesicles and release to the rhizosphere by exocytosis.

SIGNALING FOR PROTEOID ROOT DEVELOPMENT AND METABOLISM

Two distinct developmental processes related to cell division and differentiation are evident during the development of a cluster of rootlets along a proteoid root axis. First, numerous pericycle cells are triggered to divide, develop into primordia, and emerge through the cortex. Second, these primordia are prompted to cease dividing, elongate, and differentiate into mature root tissues, resulting in determinate rootlets. Both processes are likely to be mediated through hormonal signals. At least one of the signals is systemic, since clusters develop in near synchrony on all proteoid roots of a plant grown in hydroponics (Watt and Evans, 1999). Grafting experiments show that for clusters to develop, the root stock must come from a proteoid-root-forming species (Dinkelaker et al., 1995).

Auxin is clearly implicated as one of the signals involved in cluster formation. An intense proliferation of lateral meristems along a root axis can be mimicked in non-proteoid plants such as radish by exogenous application of auxin (compare figure 2 of Laskowski et al., 1995, depicting laterals emerging from a radish root, with figure 1 of Johnson et al., 1996a, of rootlets of an *L. albus* proteoid root). Similarly, a mutant of Arabidopsis that overproduces auxin has a taproot similar to a proteoid root, with numerous, closely spaced lateral roots (Boerjan et al., 1995). When Gilbert et al. (1998) grew *L. albus* with the auxin IAA at a P level that normally suppresses proteoid root development, 30% more proteoid roots clusters developed compared with control plants without IAA. They showed that when auxin transport inhibitors were applied to the root systems, proteoid root development was drastically reduced in the treatments without P, where formation of proteoid roots was normally stimulated.

Auxin probably works in concert with other hormones such as ethylene and cytokinin during proteoid root development (Gilbert et al., 1998). Ethylene can mediate auxin signals and has been implicated in altering root morphology under conditions of limited P (Borch et al., 1999). Coralloid (short, multibranched) roots form during colonization by ectomycorrhizae and in response to Fe deficiency (Hutchinson, 1967). They could be induced to form when auxin transport inhibitors and ethylene were applied to pine roots in the absence of mycorrhizae, and could be blocked by the inhibition of ethylene synthesis (Kaska et al., 1999). Root nodules of *Sesbania rostrata* could be stimulated to switch from indeterminacy to determinacy by growth in vermiculite instead of an agar environment, or by the application of ethylene (Fernández-López et al., 1998). By analogy, ethylene may alter rootlet determinacy in proteoid roots.

Auxin (Landsberg, 1986) and ethylene (Romera and Alcántara, 1994) have been implicated in both morphological and metabolic changes associated with responses to Fe deficiency in non-proteoid plants. Some dicotyledonous plants subjected to Fe limitation show transient elevated PEPC activity, carboxylate accumulation, and proton extrusion in localized regions close to their root tips (Landsberg, 1986), similar to plants subjected to P deficiency. Working with Fe-limited bean, de Vos et al. (1986) suggested that PEPC facilitates the accumulation of citrate because ATP-dependent phosphofructokinase has been made insensitive to citrate via a rise in endogenous levels of ammonia. Ammonia levels can increase in tissues subjected to P deficiency and growth reduction (i.e. proteoid rootlet determinacy) due to conversion of accumulating nitrate and/or degradation of amino acids normally incorporated in protein (Rabe and Lovatt, 1986). Roots of P-deficient *L. albus* have a 3- to 5-fold increase in Asn concentration over plus-P roots (Johnson et al., 1996b), suggesting an altered N metabolism. Metabolic similarities between P and Fe deficiencies may be linked by levels of a common signaling molecule such as ammonia. Ammonia has been linked to both the auxin- and ethylene-responsive pathways. Interestingly, auxin transport inhibitors reduced PEPC and malate dehydrogenase activities in proteoid roots of minus-P plants compared with those grown without the inhibitors (Gilbert et al., 1997). This suggests that in addition to being involved in triggering rootlet development, auxins may be involved in altering rootlet metabolism.

PROTEOID ROOTS AS A MODEL SYSTEM

Proteoid rootlet initiation, limited meristem development, and biochemical changes associated with exudation make proteoid roots an ideal system with which to study the nutritional and hormonal signals triggering these defined developmental and biochemical events. Transport mechanisms mediating carboxylate movement across plasma and vacuolar membranes have yet to be found in any plant species. Proteoid roots are a good system with which to study such transport, since organic anion efflux can occur in an intense pulse at a predictable developmental stage. Finally, comparisons of lateral root development on non-proteoid plants growing in varying environments are difficult due to the lack of obvious patterns in their development. Proteoid roots offer the great advantage over more randomly organized roots in their regular, nearly synchronized development of clusters of lateral roots, which can be observed regardless of plant size.

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