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Pathways of assimilation and transfer of fixed nitrogen in coralloid roots of cycad-*Nostoc* symbioses

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Abstract. Freshly detached coralloid roots of several cycad species were found to bleed spontaneously from xylem, permitting identification of products of nitrogen transfer from symbiotic organ to host. Structural features relevant to the export of fixed N were described for *Macrozamia riedlei* (Fisch. ex Gaud.) Gardn. the principal species studied. Citrulline (Cit), glutamine (Gln) and glutamic acid (Glu), the latter usually in a lesser amount, were the principal translocated solutes in *Macrozamia* (5 spp.), *Encephalartos* (4 spp.) and *Lepidozamia* (1 sp.), while Gln and a smaller amount of Glu, but no Cit were present in xylem sap of *Bowenia* (1 sp.) and *Cycas* (2 spp.). Time-course studies of ^{15}N enrichment of the different tissue zones and the xylem sap of $^{15}N_2$-pulse-fed coralloid roots of *M. riedlei* showed earlier ^{15}N incorporation into Gln than into Cit, and a subsequent net decline in the ^{15}N of Gln of the coralloid-root tissues, whereas Cit labeling continued to increase in inner cortex and stele and in the xylem sap. Hydrolysis of the ^{15}N-labeled Cit and Gln consistently demonstrated much more intense labeling of the respective carbamyl and amide groups than of the other N-atoms. Coralloid roots of *M. riedlei* pulse-fed $^{14}CO_2$ in darkness showed ^{14}C labeling of aspartic acid (Asp) and Cit in all tissue zones and of Cit of xylem bleeding sap. Lateral roots and uninfected apogeotropic roots of *M. riedlei* and *M. moorei* also incorporated $^{14}CO_2$ into Cit. The ^{14}C of Cit was restricted to the carbamyl-C. Comparable $^{15}N_2$ and CO_2-feeding studies on coralloid roots of *Cycas revoluta* showed Gln to be the dominant product of N_2 fixation, with Asp and alanine as other major ^{14}C-labeled amino compounds, but a total absence of Cit in labeled or unlabeled form.

Key words: Carbon dioxide fixation – Citrulline – Coralloid roots – Cycads (nitrogen fixation) – Nitrogen fixation – Nitrogen transport – *Nostoc*

Introduction

In comparison with the legume-*Rhizobium* symbiosis, for which pathways of incorporation of fixed N into nodules and transport of fixation products to the host have been widely studied, little is known of the N metabolism of symbioses between cycads and cyanobacteria. $^{15}N_2$ studies have shown N transfer from cyanobacteria-containing coralloid root to the host (Bergersen et al. 1965; Renaut et al. 1975), but although it has been suggested that organic fixation products additional to or instead of ammonia might be transferred to the host tissues (Pate 1976; Lindblad and Bergman 1986; Perraju et al. 1986; Lindblad et al. 1987), the metabolic routes of N assimilation have not been defined.

The present study was prompted by the discovery that freshly detached coralloid roots of *M. riedlei* bleed spontaneously from the xylem, in a manner similar to that reported (Pate et al. 1969; Peoples et al. 1986) for detached legume nodules. Combining this technique with pulse $^{15}N_2$ and $^{14}CO_2$ feeding of coralloid roots, it was possible to follow time courses of labeling of fixation products in the cyanobacterial zone, the cortex and the stele of the coralloid roots and in the xylem fluid that was carrying fixed N from the symbiotic organs to the host cycad. Data on other genera and species of cycads were also obtained to determine

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Abbreviations: Ala = alanine; Asp = aspartic acid; Cit = citrulline; Gln = glutamine; Glu = glutamic acid; Orn = ornithine

whether their translocated N solutes differed from those of *M. riedlei*.

Materials and methods

Plant materials. The principal cycad investigated was *M. riedlei* (Fisch. ex Gaud.) Gardn., a species abundant in the coastal *Banksia* woodlands in and adjacent to the Perth Metropolitan Area, W.A., and cultured as potted plants at the University of Western Australia. Other cycads investigated were five other species of *Macrozamia*, one of *Lepidozamia* and one of *Bowenia* (all endemic to Australia), four species of *Encephalartos* (native to Southern Africa) and three species of *Cycas* (two, *C. angulata* and *C. armstrongii*, native to Australia and one, *C. revoluta*, to South Asia). Further details relating to species, types of material and sites of collection are given in Table 1. Coralloid roots of each species were obtained by removing soil or potting medium from around the base of the shoot, taking care to keep damage to the parent plant at a minimum.

Table 1. Species and sites of collection of cycads studied

Zamiaceae

Macrozamia riedlei (Fisch. ex Gaud.) Gardn.
Native populations in SW Australia: (A) coastal *Banksia* woodland, Perth; (B) heathland near Eneabba; (C) jarrah (*Eucalyptus marginata*) forest, Williams; (D) jarrah forest, Mt. Barker; (E) coastal heath, Esperance. Glasshouse-cultured seedlings, Botany Department, University of Western Australia

M. communis (L.) Johnson. Native population, eucalypt forest, Batemans Bay, N.S.W., Australia

M. lucida (L.) Johnson. Potted plant, Kings Park Botanic Garden, Perth, W.A., Australia

M. miquelii (F. Muell.) A.DC. Potted plant, Kings Park Botanic Garden, Australia

M. moorei F. Muell. Potted plant, Kings Park Botanic Garden, Australia

M. pauli-guilielmi W. Hill et F. Muell. Potted plant, Kings Park Botanic Garden, Australia

Lepidozamia peroffskyana Regel. Potted plant, Australian National Botanic Garden, Canberra A.C.T., Australia

Encephalartos altensteinii Lehm. Potted glasshouse plant, Royal Botanic Garden, Kew, UK

E. gratus Prain. Potted glasshouse plant, Royal Botanic Garden, Kew, UK

E. natalensis Dyer et Verdoorn. Potted glasshouse plant, Naples Botanic Garden, Italy

E. transvenosus Stapf et B. Davy. Potted glasshouse plant, Naples Botanic Garden

Boweniceae

Bowenia spectabilis Hook ex Hook. Potted glasshouse plant, Botany Department, University of Western Australia

Cycadaceae

Cycas angulata Brown. Potted glasshouse plant, Royal Botanic Garden, Kew, UK

C. revoluta Thunb. (I) Outdoor plant, Kings Park Botanic Garden, Australia; (II) Potted glasshouse plant, Royal Botanic Garden, Kew, UK

C. armstrongii Miq. Natural populations, Darwin, N.T., Australia

Structural study of coralloid roots. Hand-sectioned fresh material of *M. riedlei* was observed and photographed using a Wild (Heerbrugg, Switzerland) M650 stereomicroscope. High-resolution light microscopy was conducted on tissue fixed in glutaraldehyde and embedded in glycol methacrylate (see Pate et al. 1986).

Collection of xylem bleeding sap from detached coralloid roots. Samples of xylem sap were obtained from the 14 species studied by placing cut 1–2-cm apical portions of coralloid roots with their meristems downwards in small vials (2 ml) with the lower quarter of the segment immersed in distilled water, i.e. essentially as in the procedure of Pate et al. (1969) for detached legume nodules. Drops of fluid exuding from cut xylem of the broken ends of the coralloid root segments were collected at intervals using 1-μl microcapillaries. The sap samples were frozen and stored in liquid N_2 (local collections in Australia) or lyophilized (samples from abroad) prior to analysis. At least three separate xylem-sap samples were collected from all species studied except *C. armstrongii*, for which it proved impossible to collect xylem exudate.

$^{15}N_2$ feeding of coralloid roots. (I) *Macrozamia riedlei* (population A, Table 1). Six 35-ml incubation vessels, each containing 13 g fresh weight (FW) of coralloid root segments were flushed for 5 min with a gas mixture of 80% Ar:20% O_2 (v/v), and the atmosphere in the vessels then replaced with 80% $^{15}N_2$ (98 atom % excess, A%X): 20% O_2 (v/v). Three of the vessels were treated in darkness at room temperature for 15, 45 or 90 min. The entire segments of coralloid roots of the 15- and 45-min exposures were immediately frozen in liquid N_2. Of the 90-min treatment, half the material was frozen similarly while the remainder was separated into three distinct tissue components, namely cyanobacterium-free outer cortex, cyanobacterial zone and cyanobacteria-free inner cortex + stele (see Figs. 1, 2). This separation was accomplished by splitting open each segment of the root through the middle of the cyanobacterial zone and carefully scraping out the cyanobacteria from the exposed cortical surfaces. Microscopic examination of the scrapings and cleaned surfaces of host tissue showed that virtually all cyanobacteria had been removed by the scraping process together with the gelatinous intercellular matrix in which they were originally located in the coralloid root. A few broken remains of host cortex cells were also present in the scrapings, so the sample was likely to be contaminated to a small extent with solutes and cytoplasm of host origin. A further two vessels with coralloid-root pieces were supplied $^{15}N_2$ for 90 min, followed by a chase of 90 min or 210 min in air before harvesting as in the 90-min treatment. A final vessel was exposed to $^{15}N_2$ for 30 min and, after exposure to air, the root segments were set up for sequential collection of xylem sap over the subsequent 3.5 h.

(II) *Cycas revoluta* (plant I, Table 1). A single large (5 g FW) cluster of coralloid root was incubated for 15 h in darkness at room temperature in an atmosphere of 80% $^{15}N_2$ (55 A%X) 20% O_2 (v/v); the material was stored in liquid nitrogen until analysis.

$^{14}CO_2$ feeding. The capacity of intact coralloid roots of *M. riedlei* to fix CO_2 in darkness was tested by incubating 5–10-g samples in darkness at room temperature in 35-ml serum vials

containing air into which 7.4 MBq of $^{14}CO_2$ was liberated from a small vial using approx. 0.5 ml 1 N lactic acid applied with a syringe through the serum cap. The tissue was exposed to $^{14}CO_2$ for 10 min, followed by collection of xylem sap and harvesting. The fed coralloid roots were then separated into three tissue zones essentially as in the $^{15}N_2$-feeding study. Further feeding studies, all involving a 1-h exposure to $^{14}CO_2$, were undertaken on 0.5–1.0-g (FW) samples of uninfected apogeotropic roots of *M. riedlei* (population A, Table 1) coralloid roots and uninfected apogeotropic roots of *M. moorei*, and coralloid roots of *C. revoluta*. In all cases the tissue samples were extracted with 80% ethanol for analysis of ^{14}C-labeled solutes.

Assay of nitrogenous solutes in coralloid root tissue and xylem sap. Whole coralloid roots or separate zones of such roots were homogenized in 80% (v/v) ethanol (1 g FW per 10 ml), and the insoluble material discarded. The extracts were evaporated to dryness and partitioned between water and petroleum ether (BP 60° C). The aqueous extract was then analyzed for amino compounds using a Varian 5560 high-pressure liquid chromatographic system (HPLC; Varian Instrument Group, Palo Alto, Cal., USA), incorporating a Li-form ion-exchange column (Varian Micropak; 4 mm diameter, 150 mm length), lithium-based buffers, and a ninhydrin post-column detection system (Varian UV-200 spectrophotometer; reading at 570 nm). Tissue extracts or xylem sap from the $^{14}CO_2$-feeding experiments were assayed for labeled amino compounds by passing the eluate from the HPLC column directly into a Packard Trace 7140 (Packard Instrument Co., Downers Grove, Ill., USA) flow-through liquid scintillation spectrometer equipped with a 600-µl flow cell. Identity of ^{14}C-labeled peaks was determined by co-chromatography with authentic ^{14}C-labeled compounds (Amersham, North Ryde, N.S.W., Australia). Citrulline was collected by HPLC separation of compounds in tissue extracts or xylem-sap samples and the ^{14}C in the carbamyl carbon assayed separately from that of the ornithine (Orn) moiety, following hydrolysis under N_2 for 72 h in 6 N HCL at 100° C.

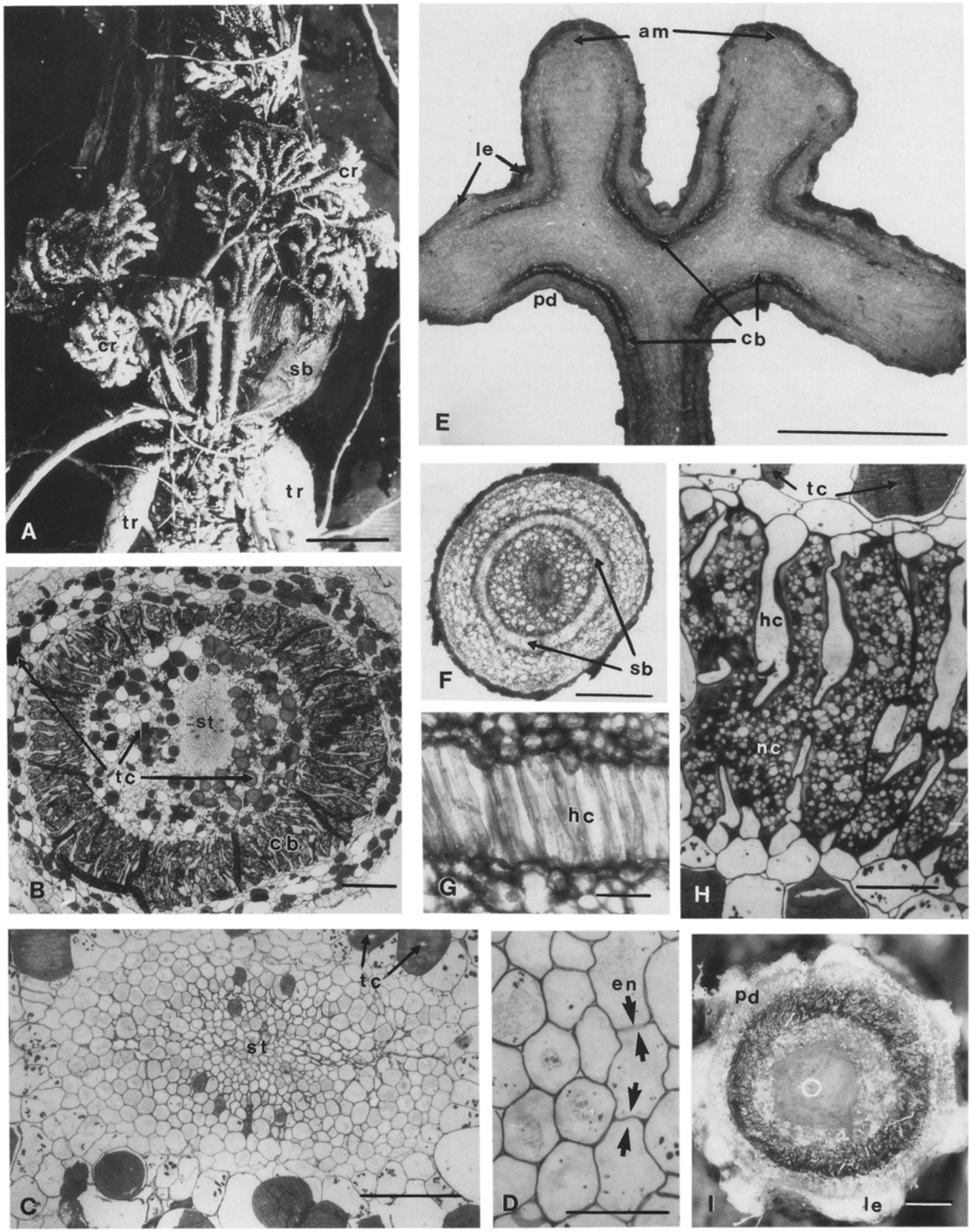
Bulk amounts of amino compounds for ^{15}N analysis were obtained by separation of xylem-sap samples or tissue extracts on a large-capacity (9 mm diameter, 560 mm length) cation-exchange column (Beckman 118C Amino Acid Analyzer; Beckman Instruments, Berkeley, Cal., USA) operating in the physiological fluids mode with lithium-based buffers. Sub-samples (10 µl) of each 2-ml fraction of eluate were assayed for amino acids by HPLC, and fractions thus identified as containing pure samples of an amino compound under study were combined to obtain sufficient total N for mass spectrometry. The combined fractions were subjected to Kjeldahl digestion and distillation (see Bremner 1965), and the recovered ammonia converted to N_2 for mass-spectral measurement following hypobromite oxidation (Porter and O'Deen 1977). Subsamples of the [^{15}N]glutamine (Gln)- and [^{15}N]citrulline (Cit)-containing fractions from extracts of whole coralloid root were hydrolysed at 105° C for 3 h in 1 N HCl and 72 h in 6 N HCl, respectively, and the resulting amide-N and amino-N of Gln and the carbamyl-N and Orn-atoms-N of cit were then assayed separately for ^{15}N. ^{15}N analyses were performed using a triple ion collector, dual-inlet mass-spectrometer (Sira 9; VG Isogas, Middlewich, UK), following techniques described by Ofori et al. (1987). Tests for the ureides allantoin and allantoic acid in tissue extracts and xylem sap were made using the colorimetric assay of Trijbels and Vogels (1966).

Determinations of total N in coralloid-root dry matter and in the ethanolic extracts of whole coralloid roots or specific tissue zones of coralloid roots were performed by standard Kjeldahl digestion followed by steam distillation of the ammonia from the extracts and liberation of the recovered ammonia against standard HCl.

Results

Anatomy of the coralloid root. Gross morphology and ontogeny of coralloid roots of *M. riedlei*, the species mainly studied, have been detailed previously (Pate 1976), including descriptions of the apogeotropic roots through which infection by *Nostoc* is presumed to occur, and from the base of which each new set of N_2-fixing coralloid roots originates. A typical cluster of symbiotic organs of a plant approx. 20 years old is shown in Fig. 1 A. The species has prominent contractile roots which progressively pull the stem and laterally placed coralloid roots as much as 50 cm below ground in the sandy habitats where the species is mainly found (Pate 1976). Transverse sections through the mature region of an apical segment of a coralloid root show the well-marked cyanobacterial zone in the mid cortex, and the central stele (Fig. 1 B), bounded by a distinct endodermis with Casparian thickenings (Fig. 1 D). Prominent lenticels on the coralloid-root surface are likely to be principal sites of gaseous diffusion into and out of the coralloid root. Longitudinal sections show the dichotomous branching and apical growth of the coralloid root, and typical swelling of the gelatinous material which occurs within the cyanobacterial zone upon imbibition of water released at the cut surface of the coralloid root (Fig. 1 E). When thick (0.1–0.5 mm) hand sections of fresh coralloid root are rinsed repeatedly in water the cyanobacteria become dislodged and the radial configuration of the highly distinctive columnar host cells spanning the cyanobacterial zone is exposed (Fig. 1 F, G). From this evidence, and that of high-resolution light microscopy (Fig. 1 H), it is concluded that the cyanobacteria of this species are of intercellular location.

Bleeding from detached coralloid roots. Profuse bleeding was observed from the cut surface of coralloid roots whose apical ends had been immersed in water. Exudation commenced exclusively from patches of mature xylem elements, and the resulting sap spread eventually up to and beyond the endodermis (Fig. 1 H). From 0.1 to 2.5 µl of xylem sap were collected per 1 g of coralloid root. There were no consistent differences in bleeding rate between species, but in *M. riedlei* young, new season's segments of coralloid root tended to bleed more promptly and at a faster rate than did coralloid roots collected in mid-summer when extension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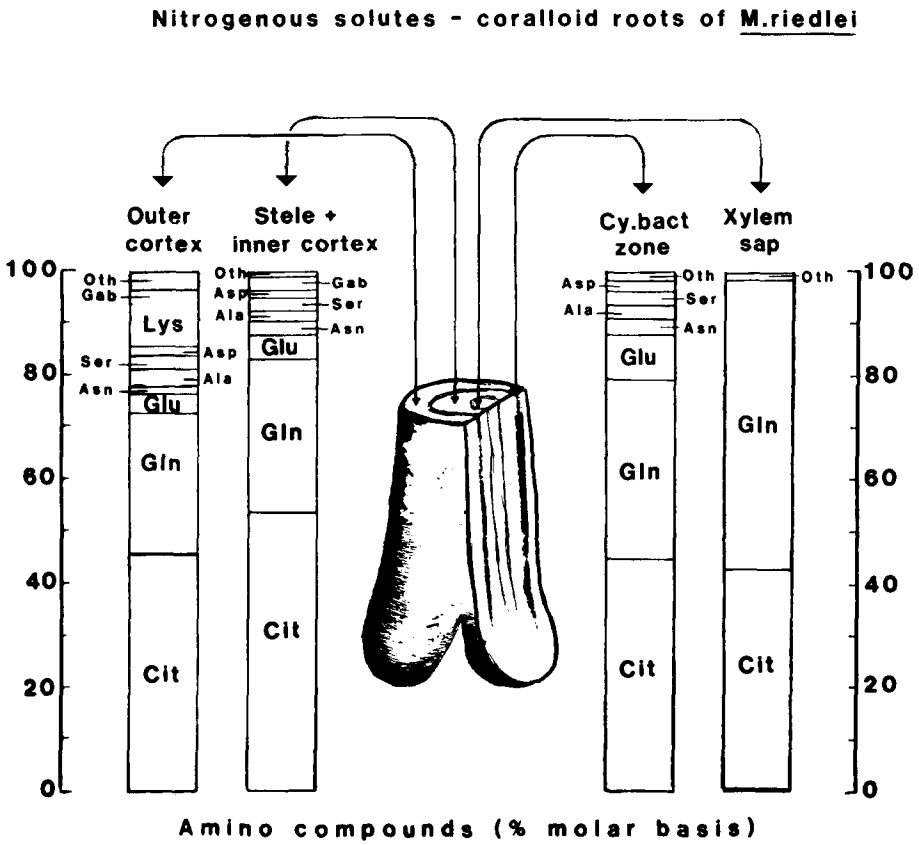

Fig. 2. Amino-compound composition of xylem bleeding-sap and tissue zones of N_2-fixing coralloid roots of *Macrozamia riedlei*. Note dominance of Cit and Gln in all compartments of the system, but especially in xylem exudate. Ala = alanine, Asn = asparagine, Asp = aspartic acid, Cit = citrulline, Gab = γ-amino butyric acid, Gln = glutamine, Glu = glutamic acid, Lys = lysine, Ser = Serine. "Others" (Oth) refer to a number of minor constituents including arginine, lysine, ornithine, tyrosine, phenylalanine, histidine, threonine and serine

Fig. 1 A–I. Morphological and anatomical features of the coralloid roots of *Macrozamia riedlei*. **A** Coralloid-root clusters (*cr*) on an approx. 20-year-old plant, showing the apogeotropic character of symbiotic organs, contractile tap roots (*tr*), and swollen stem base (*sb*). Stem-root junction of specimen was buried 40 cm below ground. Bar marker 5 cm; ×0.28. **B–D** Light micrographs of transverse sections of glycol-methacrylate-embedded mature region of a coralloid root. *st*, Stele; *cb*, cyanobacterial zone in mid-cortex. Note tannin-filled cells (*tc*) in outer and inner cortex and stress shrinkage in cyanobacterial zone of **B** because of ineffective penetration of resin into gelatinous matrix. The diarch nature of the stele is shown in **C**; the endodermis (*en*) with its well-defined casparian strips (*arrows*) shown in **D**. Bar markers and magnifications: **B** 0.2 mm, ×50; **C** 0.1 mm, ×180; **D** 25 µm, ×760. **E** Fresh longitudinal hand section of part of coralloid-root cluster showing cyanobacterial zone (*cb*), apical meristems (*am*) periderm (*pd*), lenticels (*le*) and dichotomous branching of the cluster. Bar marker 5 mm, ×6. **F–G** Thin transverse hand sections after repeated washing in water to dislodge *Nostoc* cells and thereby expose columnar host cells (*hc*) of the cyanobacterial zone (*sb*). Bar markers and magnifications: **F** 1 mm, ×13, **G** 0.1 mm, ×100. **H** Detail of cyanobacterial zone of glycol-methacrylate-embedded material showing intercellular *Nostoc* cells (*nc*) in the mucilaginous matrix between the columnar host cells (*hc*). *tc*, Tannin-filled cells. Bar marker 50 cm, ×290. **I** Surface view of cut proximal end of portion of coralloid root placed with its intact apical end resting on wet filter paper. Note profuse bleeding from the steele (xylem), and presence of large lenticels (*le*) facilitating gaseous exchange through the outer periderm (*pd*). Bar marker 0.5 mm, ×16

growth of the symbiotic organs was at its seasonal minimum.

Nitrogenous solutes of coralloid roots and their xylem sap. The dry matter of coralloid roots of *M. riedlei* contained 1.2–1.5% N and approx. 10–20% of this was extractable in 80% ethanol and identifiable as amino compounds. Cit and Gln were the two most abundant free amino compounds of all zones of the coralloid root of this species (Fig. 2), together comprising from 74 to 84% (molar basis) of the total amino compounds recovered in an extract. Minor amino compounds of the tissue zones of the coralloid root were glutamate (Glu), asparagine (Asn), alanine (Ala), serine (Ser), aspartate (Asp), lysine (Lys), γ-amino butyric acid (Gab) and a range of trace constituents. In the xylem sap, 95% of the N consisted of Cit and Gln (Fig. 2). Neither allantoin nor allantoic acid were detected in the tissues or xylem sap of the coralloid roots. Xylem sap collected at a range of sites throughout the distribution range of *M. riedlei* showed considerable variation in concentration and composition between sites (see Table 2), but all samples contained Cit and Gln as the principal constituents. However, the ratio between these two compounds varied from 0.85 to 4.5.

Table 2. Amino-compound composition (in $\mu mol \cdot ml^{-1}$) of xylem bleeding sap of coralloid roots of *Macrozamia riedlei* collected (May–July) in native habitat at different populations in S.W. Australia[a]

Amino compound	Sites				
	A	B	C	D	E
Glutamine	99.7	36.1	25.5	1.9	45.3
Citrulline	105.0	30.8	116.0	8.1	106.3
Glutamic acid	0.5	0.6	1.2		0.7
Aspartic acid	0.2	0.5	1.0		
Serine	0.09	0.4	0.2		
Others[b]	1.1	0.2	1.0		

[a] Sites span almost the full distribution range of *M. riedlei*; see Table 1 for locations

[b] Includes asparagine, lysine, threonine, tyrosine and a range of other trace constituents

Analyses of xylem sap from coralloid roots of species other than *M. riedlei* yielded the data summarized in Fig. 3. All xylem-sap samples contained extremely low levels of ammonia (less than 0.5% of the total sap), indicating it was not involved to any appreciable extent in transfer of N from the coralloid root to the rest of the host. From 34 to 76% of the translocated amino compounds (molar basis) in the species of *Macrozamia, Encephalartos* and *Lepidozamia* that were studied consisted of Cit, and in all of these cases, except *M. lucida*, Gln was the only other major constituent. Coralloid-root xylem sap of *Bowenia spectabilis* (Boweniaceae) lacked Cit and 92% of its amino fraction consisted of Gln. The two *Cycas* species (Cycadaceae) studied also proved negative for Cit while showing 60–96% of their xylem amino-compounds as Gln. Ethanolic extracts of the coralloid roots of *B. spectablis* and *C. revoluta* from which the xylem-sap samples had been collected also contained large amounts of Gln but no detectable Cit, and Cit was absent from ethanolic extracts of *C. angulata* and *C. armstrongii* (data not shown). Additionally coralloid-root tissue of these four species contained large amounts of Ala, Gab, Ser, Asp and a number of trace amino compounds not detectable in xylem sap.

Products of $^{15}N_2$ fixation in coralloid roots and xylem sap. A 3.5-h chase in air reduced the recoveries of ^{15}N in amino-compounds in the tissue zones of *M. riedlei* coralloid roots which had been given a 1.5-h pulse of ^{15}N (Fig. 4). This reduction applied particularly to ^{15}N in Gln and, to a lesser extent, in Cit of the cyanobacterial zone, and is consistent with net export of fixed N from this zone to other parts of the root. The stele and the inner cortex, by contrast, showed sharply increasing amounts of ^{15}N recovered as Cit and a small decline in ^{15}N of Gln. These two compounds comprised 80% or more of the ^{15}N recovered. Glutamic acid was also labeled in all extracts, and trace amounts of ^{15}N were recovered in Asn, Asp and Ala (collectively recorded as "others" in Fig. 4). Ammonia was present in only trace amounts in the ethanolic extracts of all zones of the coralloid roots, so study of its ^{15}N enrichment was not possible.

Xylem sap of coralloid roots fed with $^{15}N_2$ (Fig. 5) showed measurable ^{15}N in Gln within 1 h after the end of $^{15}N_2$-feeding period. ^{15}N enrichment of this compound reached a maximum after a further 1 h. The time course of Cit labeling was consistently 1 h or more behind that of Gln, whether expressed on a specific-activity basis or in terms of total ^{15}N recovered. When bleeding ceased, 3.5 h after the end of $^{15}N_2$ feeding, the amounts of ^{15}N present in Cit slightly exceeded those in Gln.

Data provided in Table 3 are values for A%X of ^{15}N in major amino acids recovered from whole coralloid-root segments harvested during and after the 1.5-h pulse of $^{15}N_2$. Asparagine, Ala and the amide-N of Gln tended to be more heavily enriched early in the time course than other amino compounds. Asparagine incorporated only small amounts of ^{15}N and, being a relatively minor component of coralloid-root tissue and xylem sap, appeared to be little involved in N_2 assimilation. Citrulline also showed relatively low ^{15}N enrichment, but, in view of its large pool size in tissue and xylem sap (Fig. 2), still accounted for a large proportion of the N fixed during the time course. Glutamine at all times showed much higher levels of ^{15}N incorporation into its amide-N than its Glu-N. Citrulline, by contrast, showed more uniform ^{15}N labeling between its carbamyl- and Orn-N atoms (see Table 3).

Coralloid root tissue of *C. revoluta* fed with $^{15}N_2$ showed high ^{15}N enrichment in Gln, Gab and Ala and much lower enrichments in all other compounds including Glu (Table 4). Citrulline was absent in the extracts, and an unidentified compound, present in large amounts in terms of total N (see Table 3) was not detectable labeled. Percentage distribution of ^{15}N (Table 3) showed approx. 95% in Gln, 3% in Ala, 1% in Gab and the remainder distributed fairly equally among a range of minor constituents. Only trace amounts of free ammonia were present in the extracts, precluding study of the ^{15}N enrichment of this compound.

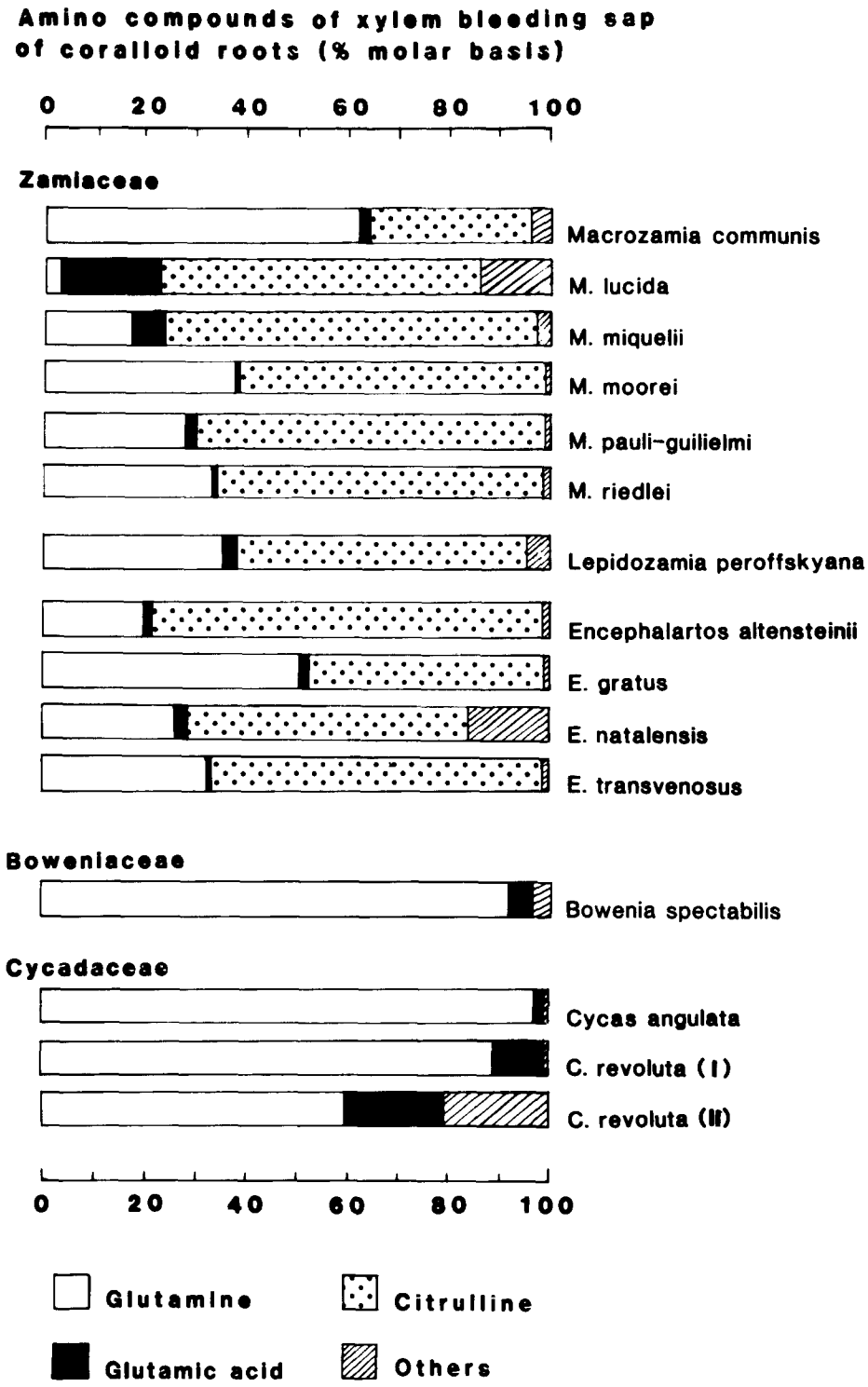

Fig. 3. Nitrogenous solutes of xylem bleeding sap of coralloid roots of a range of cycad species and genera. Note the preponderance of citrulline in members of the Zamiaceae but absence of this compound in *Cycas* and *Bowenia*. See Table 1 for sources of plant material used for sap collection. The data for *Macrozamia riedlei* record mean composition of sap sampled at a number of habitats throughout the range of the species in S.W. Australia (see Table 2)

Hydrolysis of the [^{15}N]Gln showed 56% of the ^{15}N in the amide group, versus 44% in the Glu-N of the molecule (see Table 3).

Dark carbon-dioxide fixation by coralloid and-apogeotropic roots of cycads. Feeding $^{14}CO_2$ to coralloid roots of *M. riedlei* resulted in incorporation mainly into two nitrogenous molecules, Asp and Cit, and to a much lesser extent in Glu and Ala. The time courses of ^{14}C enrichment of these are shown in Fig. 6. Citrulline was the dominant labelled compound throughout the time course in

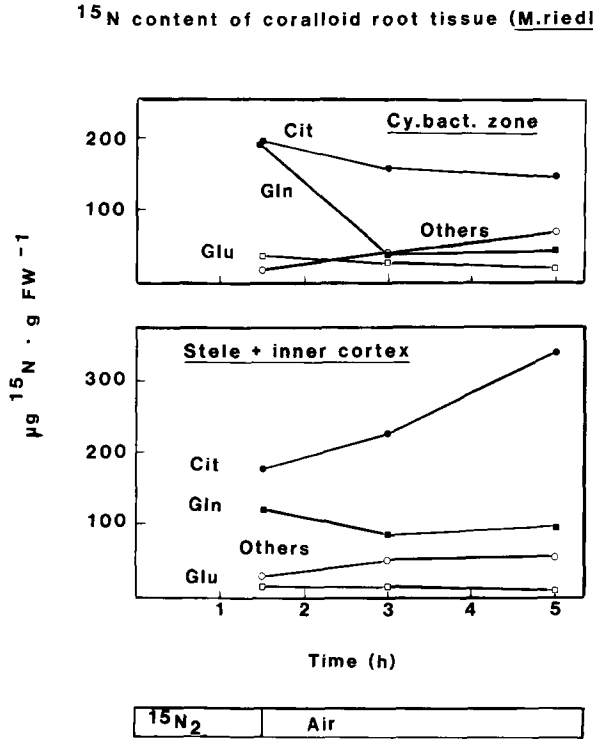

Fig. 4. Distribution of ^{15}N in cyanobacterial zone and stele + inner cortex of coralloid roots of *Macrozamia riedlei* following a 90-min feeding of $^{15}N_2$. Labeling marked as "others" was recovered in alanine, γ-amino butyric acid, serine and glycine. The tissue samples from the cyanobacterial zone were too small (50–90 mg FW) to study labeling of minor constituents

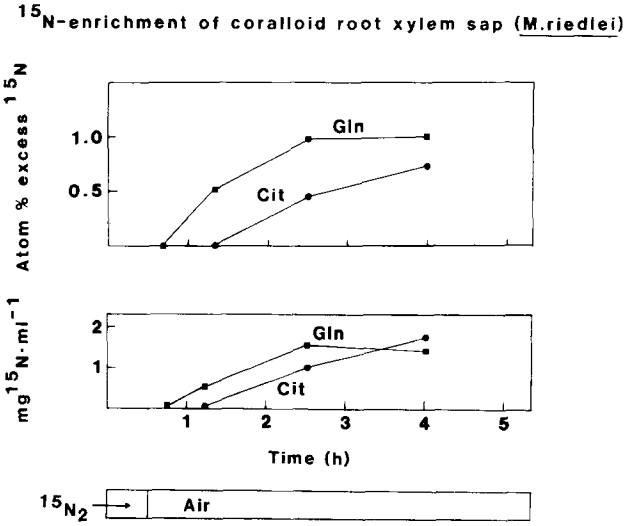

Fig. 5. Changes with time in ^{15}N enrichment and total ^{15}N content of the two major solutes of xylem bleeding sap from coralloid roots of *Macrozamia riedlei* after a 30-min exposure to $^{15}N_2$. These two compounds comprised more than 95% of the total ^{15}N recovered from the sap

Table 3. ^{15}N enrichments of nitrogenous solutes of whole coralloid-root tissue of *Macrozamia riedlei* during and after 1.5 h pulse feeding of $^{15}N_2$[a]. All values are A%X ^{15}N

Compound	Time of harvest of corraloid root (h)			
	0.25	0.75	1.5	3.0
Aspartic acid	0.63	0.78	0.53	0.12
Asparagine	0.00	0.02	0.01	0.00
Glutamic acid	0.00	0.36	0.16	0.07
Glutamine (amide-N)	0.27	1.98	1.50	0.56
Glutamine (amino-N)	0.00	0.11	0.15	0.09
Glycine	0.00	0.18	0.36	0.18
Alanine	0.00	0.64	0.27	0.14
Citrulline (carbamyl-N)	0.01	0.02	0.07	0.03
Citrulline (ornithine-N atoms)	0.00	0.01	0.02	0.02

[a] See Figs. 4 and 5 for complementary information on labeling of different tissue zones and xylem sap of similarly fed coralloid roots

stele + inner cortex and to a lesser extent in the cyanobacterial zone, while the outer cortex generally showed more equal ^{14}C distribution between the two compounds. When expressed as specific activities (Bq·ng C^{-1}) (top half of Fig. 7) the pool of Asp in the outer cortex was found to be more intensely labeled than that of other tissue zones, and the peak in ^{14}C labeling of this compound in the stele + inner cortex occurred 20 min later than the other tissue zones.

A massive progressive ^{14}C enrichment of Cit in xylem bleeding sap of coralloid roots occurred when freshly detached coralloid roots were fed with $^{14}CO_2$ (bottom half of Fig. 7). By the end of the 1-h time period the total ^{14}C recovered in Cit was 14–70 times greater per unit volume of xylem sap than was ever achieved by this compound on a tissue-water basis in the parent coralloid root. However, when expressed as specific activities (Bq·ng C^{-1}) the peak values for Cit in xylem sap, outer cortex, cyanobacterial zone and stele + inner cortex were closely similar, namely, in the sequence given, 649, 522, 584 and 653. Thus, the high ^{14}C labeling of xylem sap reflected high total amounts of Cit in this compartment, and not differentially high labeling of the Cit molecules themselves. The Gln and Asp of xylem sap were not appreciably labeled during the 1-h time period of the experiment.

Distribution of ^{14}C amongst ethanol-soluble solutes of the $^{14}CO_2$-fed coralloid roots of *M. moorei* and *C. revoluta* were compared with that of *M. riedlei*. All species showed highest percentage incorporation (37–84% of ethanol-soluble ^{14}C) into non-amino compounds, principally or-

Table 4. ^{15}N incorporation into amino-compounds of ethanol-soluble fractions of coralloid roots of *Cycas revoluta* after exposure to $^{15}N_2$[a]

Compound	Total N ($\mu g \cdot g(FW)^{-1}$)	^{15}N enrichment A%X ^{15}N	^{15}N content ($\mu g \cdot g(FW)^{-1}$)	^{15}N distribution (%)
Glutamine	8076	5.47[b]	441.8	95.3
Unknown compound	1683	N.S.[c]		
Alanine	537	2.50	13.4	2.9
Glutamic acid	275	0.08	0.2	
Aspartic acid	185	0.43	0.8	
γ-Amino butyric acid	169	2.64	4.5	1.0
Serine	151	0.56	0.8	
Isoleucine and leucine	118	0.78	0.9	
Valine	113	0.57	0.6	
Glycine	104	0.40	0.4	
Citrulline	N.D.[d]			

[a] Cluster (5 g FW) of coralloid root incubated for 15 h in darkness at room temperature in an atmosphere of $^{15}N_2$ (45%): $^{14}N_2$ (35%): O_2 (20%)
[b] Hydrolysis of the glutamine showed 56% of the ^{15}N in the amide group and 44% in the amino group
[c] N.S. = no significant enrichment
[d] N.D. = not detectable in extract

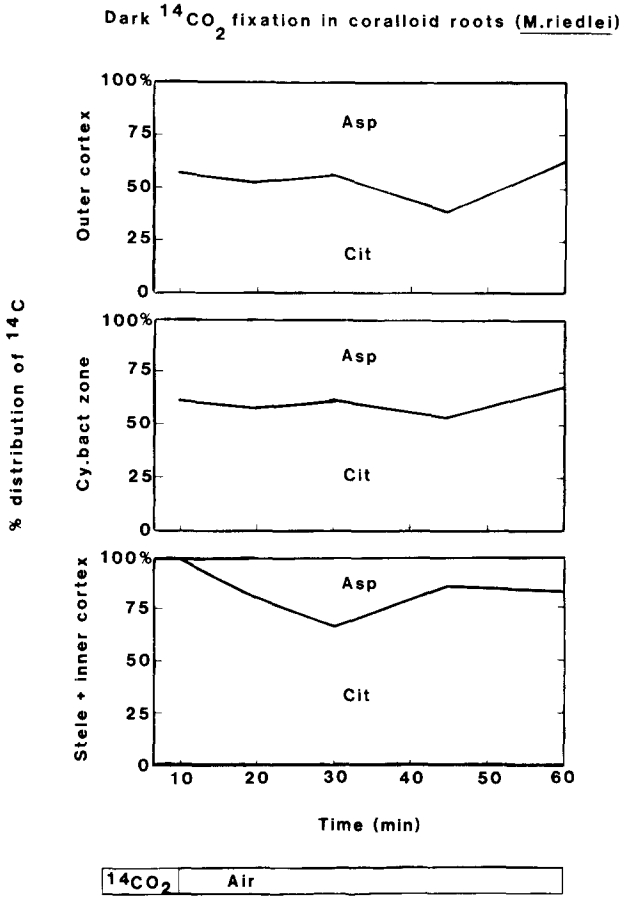

Fig. 6. Proportional labeling of Asp and Cit after applying a 10-min pulse of $^{14}CO_2$ to coralloid roots of *Macrozamia riedlei*. Note the particularly high ^{14}C recovery in Cit relative to Asp in the stele + inner cortex. Further data from this experiment in Fig. 7

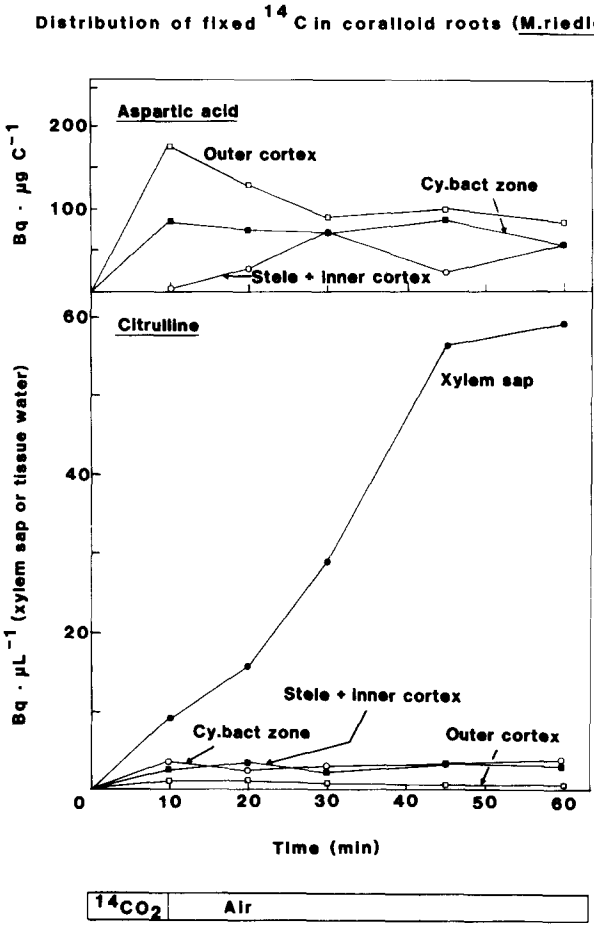

Fig. 7. Dark fixation of $^{14}CO_2$ by coralloid roots of *Macrozamia riedlei* showing ^{14}C specific activities of Asp in the tissue compartments of the nodule, and comparisons of the specific labeling of Cit per unit volume of sap or tissue water in xylem bleeding-sap or tissue zones of the coralloid roots. Other data from the study are shown in Fig. 6

ganic acids. Alanine, Gln, Glu, and Asp showed ^{14}C incorporation in all samples although proportional distribution of label in these compounds varied considerably between species. Citrulline was labeled in coralloid roots of *M. moorei* (17% of ^{14}C) and *M. riedlei* (32% of ^{14}C) but was not detected (labeled or unlabeled) in coralloid roots of *C. revoluta*. A particularly important finding was that apogeotropic roots of *M. riedlei* and *M. moorei* showed heavy ^{14}C labeling in Cit although they are totally devoid of cyanobacteria.

Discussion

Our data for *M. riedlei*, the main object of our studies, provide certain information on the structural and biochemical pathways for transfer of fixed N from the cyanobacterium to coralloid-root tissue, and thence by way of the xylem to the rest of the cycad. The intercellular location of the microsymbiont in the gelatinous matrix of the cyanobacterial zone of the mid-cortex of the coralloid root strongly indicates some form of apoplastic exchange between the partners, probably mediated through the highly distinctive columnar cells within this zone. This tissue might be viewed as the loading site for symplastic transfer of nitrogenous solutes through inner cortex, endodermis, and stele to the conducting elements of xylem. The presence of well-defined Casparian thickenings in the endodermis, taken together with the capacity of freshly detached coralloid roots to bleed profusely from their xylem, indicate an export system essentially similar to that proposed for legume nodules (Pate and Gunning 1972), namely, an osmotically-operated exudation of solutes following their excretion into the stelar apoplast. Unlike the situation in certain legume nodules (Pate et al. 1969), coralloid roots of *M. reidlei* do not contain pericycle transfer cells in their stelar tissue. However, transfer-cell-type ingrowths have been recorded for host tissue at the apoplastic interface with the cyanobacteria in coralloid roots of *Encephalartos altensteinii* (Grilli Caiola 1975) and *Zamia skinneri* (Lindblad et al. 1985).

Analysis of xylem bleeding sap from coralloid roots permitted comparisons of the translocation products of a number of cycads. The results so far indicate a consistent difference between the genera *Macrozamia, Lepidozamia*, and *Encephalartos* (all members of the Zamiaceae), in which all species examined were found to transport both Cit and Gln from their coralloid roots, and species of the genera *Cycas* (Cycadaceae) and *Bowenia* (Boweniaceae) in which only Gln appeared to be transported in large amount. The composition of ethanol-soluble amino-compounds in coralloid-root tissue broadly reflected these differences. Of importance is the virtual absence of free ammonium-N in xylem sap and in tissue compartments of the coralloid root. While the relative roles of endophyte and host in ammonium assimilation have not been defined, ammonia does not appear to be involved to any appreciable extent in bulk transfer of N across and out of the host tissues of the coralloid root.

Although the results of $^{14}CO_2$- and $^{15}N_2$-feeding experiments with coralloid roots of *M. reidlei* are broadly consistent with the metabolic pathways for net synthesis of Gln and Cit proposed for free-living N_2-fixing *Anabaena* by Carr (1983), they do not indicate whether all or part of the enzymatic sequences involved are confined to the endosymbiont. The demonstration of Cit synthesis from $^{14}CO_2$ by non-infected apogeotropic roots indicates clearly that carbamyl-phosphate metabolism is not specifically confined to the cyanobacteria component, and might well be shared between the symbiotic partners. The key to resolving these questions will lie in enzymatic studies of the symbiotic partners and in attempting to identify solutes of fixed N leaving the cyanobacterium for the host. More specifically, one must ascertain at what stages and locations ammonia and primary products of its assimilation are involved.

Notwithstanding these uncertainties, the labeling experiments do indicate some general features of metabolism of fixed N. The high initial labeling of the amide group of gln (showing the highest A%X of all ^{15}N-labeled compounds recovered), with subsequent ^{15}N-labeling of Cit, indicates that the primary route for assimilation of ammonia is likely to be via glutamine synthetase (GS), and that the carbamyl-P involved in Cit synthesis is probably formed subsequent to this. The extremely low level of ^{15}N in the amino-N of Gln, especially compared to that of the amino-N of Asp and Ala, indicates that if Glu synthase (GOGAT) is indeed operational in utilising Gln it is located in a compartment separate to that of GS-mediated Gln synthesis. In this regard it is interesting that substantial levels of GS activity and specific GS-immunolabeling have been found in both heterocysts and vegetative cells of the cyanobionts of *C. revoluta* and *Z. skinneri* (Lindblad and Bergman 1986). This led Lindblad and Bergman (1986) to postulate that Gln might be exported from the endophyte, either with or in place of ammonia.

Turning to the labeling studies with other cycads, the $^{14}CO_2$-feeding data on coralloid roots

and apogeotropic roots of *M. moorei*, and the presence of large quantities of Gln and Cit in the xylem sap of coralloid roots of this species, indicate a similar general metabolism to that of *M. riedlei*. However, in *C. revoluta* all labeling data indicate that symbiotic N_2 fixation in this species involves massive net synthesis of Gln, and no measurable synthesis or accumulation of Cit by either symbiotic or non-symbiotic tissues. It is not possible to identify the primary site of Gln synthesis in *Cycas* but, judging from the recent demonstrations of in-vitro functional GS (and GOGAT) in the cyanobiont of coralloid roots of *C. revoluta* (Lindblad and Bergman 1986; Lindblad et al. 1987), as well as in-vitro functional GS in the cyanobiont of *C. circinalis* (Perraju et al. 1986), one may conclude that the microsymbiont has the capacity to assimilate the ammonia produced through nitrogen fixation. The equal distribution of ^{15}N between the two N atoms of Gln in the N_2-fixing study on *Cycas* reported here is consistent with synthesis of Gln via GS-GOGAT in this system.

Finally, our demonstration of two distinct groupings of cycads, based upon the specificity in solutes utilized for assimilation and translocation of fixed N, exhibits interesting parallels with the situation in both N_2-fixing legumes and *Frankia*-based symbioses. In either case, specificities are known within genera or groups of species as to whether amides, allantoic acid and allantoin, or Cit serve as the principal translocation solutes (see reviews by Dixon and Wheeler 1986; Schubert 1986; Pate in press). It would be of value to explore the cycads more extensively to determine how closely their N metabolism matches that of other symbiotic systems.

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