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Structural Aspects of Cluster Root Development and their Possible Significance for Nutrient Acquisition in *Grevillea robusta* (Proteaceae)

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Light microscopy (LM), scanning (SEM) and transmission electron microscopy (TEM) were used to study structure and function of cluster roots in *Grevillea robusta*. These roots were developed during growth of *G. robusta* seedlings in modified Hoagland's solution lacking phosphate. Cluster rootlets formed root hairs, basipetally, only after completing their determinate development. The rootlet hairs branched in two ways and some had apical swelling. Rootlets with hairs produced two different forms of exudate, one fibrous and the other globular in nature. The fibrous material appeared to be synthesised in the cortical cells. It is released by exocytosis from the epidermis. Rootlet hairs produced only fibrous exudate. They attached firmly to pieces of vermiculite. The significance of cluster roots is discussed within the context of patchy soil resources.

Key words: *Grevillea robusta*, Silky oak, Proteaceae, cluster roots, morphology, mucilage, nutrient acquisition, root exudates.

INTRODUCTION

Grevillea robusta A. Cunn. ex R. Br. (silky oak or silver oak) is a native tree of Australia, but is grown in South and Central America, the Southwest Pacific, Malaysia, India, Madagascar and Africa (Harwood, 1989). It is widely intercropped in agroforestry in Eastern Africa, providing straight poles for construction, shade and firewood (Harwood, 1989). It is also reported to compete less with crops for nutrients than do woody species with nitrogen fixing symbioses (Ong, 1994). *Grevillea robusta* is a member of the family Proteaceae, almost all members of which have cluster roots (for references see Lamont, 1982; Dinkelaker, Romheld and Marschner, 1995). These are dense clusters of rootlets of determinate development, arising endogenously from the pericycle of lateral roots opposite protoxylem poles (Purnell, 1960). They were first called 'proteoid roots' as it was thought that they only occurred in the Proteaceae (Purnell, 1960), but they have since been found in a number of legumes and in members of the Casuarinaceae, Myricaceae and Moraceae (Lamont, 1972*a*; Trinick, 1977; Bowen, 1981; Diem *et al*., 1981; Gardner, Parberry and Barber, 1981; Louis, Racette and Torrey, 1990, 1991; Rosenfield, Reed and Kent, 1990; Clements, White and Buirchill, 1993; Crocker and Schwintzer, 1993). Low phosphate levels have been shown to encourage cluster root formation (Trinick, 1977; Gardner, Parberry and Barber, 1982; Walker and Pate, 1986; Marschner, Romheld and Cakmak, 1987; Louis *et al*., 1991). Furthermore, Gardner *et al*. (1982) found that the production of reducing agents, hydrogen ions and chelating agents were inversely related to the phosphorus status of the plant.

Cluster roots show increased uptake (per unit area) of ^{32}P and ^{86}Rb compared to ordinary roots (Malajczuk and Bowen, 1974). The uptake rates of P are 2–13 times higher in cluster roots compared to ordinary roots on a dry weight basis (Lamont, 1982 and references therein). Smith and Jooste (1986) found no such increase, but Dinkelaker *et al*. (1995) explain that such contradictory results are to be expected in view of the restricted longevity of these structures. Vorster and Jooste (1986) have shown that cluster roots have a more effective absorption mechanism for P than have normal roots based on Lineweaver–Burke kinetic analysis at both low (0·02–0·1 mM KH_2PO_4) and high (1·0–50 mM KH_2PO_4) concentration ranges. It is no surprise, then, that interest is growing in these structures, offering as they do an alternative means of improving the P status in plants of importance to agroforestry and agriculture alike.

On excavating roots of *Grevillea robusta* in the field in Machakos, Kenya, regions of cluster roots were encased in soil. This observation has previously been made on cluster roots of *Lupinus albus* L. (Moraghan, 1991). Encased roots have been noted in *Oryzopsis hymenoides* (Wullstein and Pratt, 1981), maize (St. Aubin, Canny and McCully, 1986; Watt, McCully and Canny, 1994) and other grasses (references in Vermeer and McCully, 1982 and McCully, 1987). Sprent (1975) reported enhanced adherence to soybean roots under conditions of water stress. However in these cases it was the primary and lateral roots that were encased. It has been found that the soil is easily lost upon washing the *Grevillea* roots. In the case of plants grown in more coarse material such as vermiculite, these larger particles become much more tightly bound to the cluster roots, remaining after stringent washing. The objectives of this study were (*a*) to develop a reliable experimental method for growing *Grevillea robusta* that minimized

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disturbance but maximized cluster root formation, (*b*) to examine the development of cluster roots and (*c*) to examine particle binding by cluster roots of *Grevillea robusta*.

MATERIALS AND METHODS

Grevillea robusta seedlings (Provenance Loitokitok, Kenya Forestry Seed Centre) were grown in Vermiperl (medium grade from William Sinclair Hort. Ltd., Firth Rd., Lincoln, UK)/Silvaperl (graded horticultural perlite from Silvaperl Ltd., Albion Works, Ropery Rd., Gainsborough, Lincs., UK) (3:1), in a greenhouse with a photoperiod of 12 h and a quantum irradiance between 200 and 400 μmol m^{-2} s^{-1}. Day/night temperatures were 25/15 °C, respectively. Plants were watered once every 2 d with tap water. After 8 weeks, cotyledons had died and were removed.

Cluster root production for light and transmission electron microscopy

Following 8 weeks growth on vermiculite, seedlings of *Grevillea robusta* were transferred to an aerated (350 ml min^{-1}) modified Hoagland's solution (Louis *et al.*, 1990) with (control) or without phosphate (Louis *et al.*, 1990). After 8 weeks, plants were examined for the presence of cluster roots. Plants were moved to Hoagland's solution after cotyledons were shed based on an observation by Broadhurst (cited in Purnell, 1960) that in *Hakea pugioniformis*, it was after this event that cluster rootlets could form. It should be noted, however, that proteoid roots do form in *Hakea prostrata* with green cotyledons showing no tendency to abscind (Lamont, 1972*b*).

Light microscopy

Cluster roots were placed in 4% glutaraldehyde in phosphate buffer (1:1 KH_2PO_4 (50 mM):K_2HPO_4 (50 mM), pH 6·8) at 2 °C for 24 h. Specimens were rinsed twice in buffer for 1 h, then embedded in 1% agar. Blocks were trimmed in desired orientation, before being taken through an ethanol series (30, 40, 50, 60, 70, 80 and 90%, made up in distilled water), followed by two rinses in 100% ethanol, 30 min at each stage. Blocks were placed in L.R. White resin 1:1 (London Resin Company) (v/v) in absolute alcohol, vacuum infiltrated and placed on a rotary wheel at 2 °C for 24 h, before being transferred to 100% L.R. White resin, changed every 2 d, for 6 d. They were then put in gelatine capsules which were filled with resin, and left to polymerize at 60 °C for 24 h. They were sliced at 1 µm thickness using a Reichert (OM U3) microtome with a glass knife, and stained with 33 mM toluidine blue in 162 mM sodium tetraborate before viewing, using an Olympus BH2 microscope.

Transmission electron microscopy

Specimens were prepared as for light microscopy with the following differences in protocol. Following glutaraldehyde and the buffer washes, cluster roots were postfixed in 0·2% OsO_4 (w/v) in buffer for 24 h. After two rinses in distilled water (two times 30 min), they were agar embedded. Sections were sliced at 90 nm thickness and placed on pioloform and pyroxylin copper grids (50–200 mesh). The sections were allowed to dry for a short time before they were stained with uranyl acetate for 30 min, followed by lead citrate for 10 min, at room temperature. They were then viewed and photographed using a JEOL 1200EX transmission electron microscope.

Scanning electron microscopy

Cluster roots with associated particles were removed and placed in 4% glutaraldehyde in phosphate buffer at 2 °C for 24 h. They were not vacuum-infiltrated as this led to disturbance of the attached particles of vermiculite. Specimens were washed twice in buffer, then postfixed in 0·2% OsO_4 in phosphate buffer for 24 h. OsO_4 was used to assist in the maintenance of structure and improvement in earthing during coating. Following two 30 min rinses in distilled water, the roots were given two 30 min rinses in 100% ethanol. Next, the cluster roots were transferred to 50% freon 113 in 100% ethanol for 15 min and put through a series of Freon solutions (60, 70, 80, 90 and 100% freon) each for 15 min. Great care was taken to minimize disturbance of the cluster root. The cluster roots were transferred to a critical point drying apparatus (Polaron Equipment, Watford, Herts., UK). Following drying, specimens were mounted on aluminium stubs, using double sided adhesive pads (Agar Scientific, Stansted, Essex, UK). Care was taken to ensure good contact between specimen and pad to assist coating. Stubs were placed in a desiccator until ready for coating.

Specimens were coated with gold-palladium in a Polaron E 5100 sputter coater. Voltage was set at 2·5 kV and the current to 15 mA. Coating using Au/Pd was carried out for 2 min. The mounted roots were then viewed using a JEOL JSM-35 scanning electron microscope.

RESULTS

Cluster root production

Plants grown in Hoagland's solution without phosphate had an average of 18·2 cluster roots per plant whereas no cluster roots were found in plants grown in full Hoagland's solution (Fig. 1A).

External morphology

Cluster roots developed throughout the root system and in later stages occurred in nodal form (i.e. at a given frequency along a root) along lateral roots (Fig. 1A). Cluster rootlets emerging from lateral roots initially lacked hairs but, later, had a dense covering of hairs (Fig. 1A, B). Rootlet hair development would appear to begin following or nearing the completion of rootlet growth. They developed basipetally from the tip (Fig. 1A) and at maturity, extend back to about one third of the length of the rootlet (Fig. 1B). This differs from observations made by Lamont,

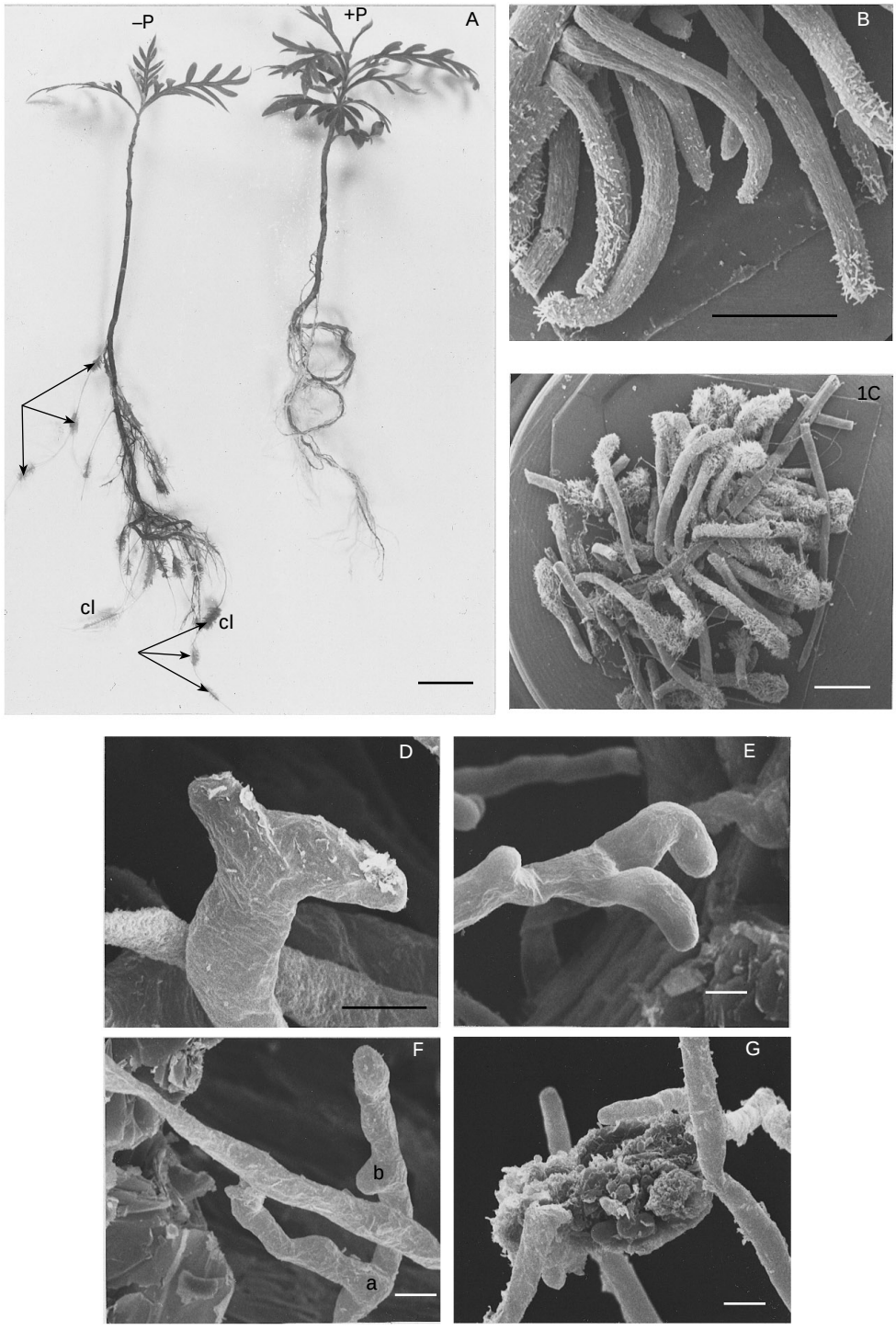

FIG. 1. External structure of cluster roots in *G. robusta*. A, Eighteen-week-old seedlings of *G. robusta* grown in −P and +P Hoagland's solutions. Cluster roots (cl) can be seen to occur only on those seedlings grown under −P conditions. Also, nodal, multiple occurrence of cluster roots along individual lateral roots can be seen (arrows). Bar = 20 mm. B and C, SEM. Development of hairs on cluster roots. B, Young cluster roots with naked rootlets and emerging hairs. Bar = 500 μm. C, Mature cluster root with hairs growing basipetally. Hairs do not develop at the base of rootlets. Bar = 500 μm. D and E, SEM. 'Stigmatic branching' in rootlet hairs on mature cluster rootlets. D, Early stage of stigmatic branching. Note that behind the two lobes is a swollen subtending region of the hair. Bar = 10 μm. E, Later stage of stigmatic branching with both branches growing in a similar fashion. Bar = 10 μm. F, SEM. Axial branching (a) on a rootlet hair growing from a mature rootlet. Also visible is a bud (b), presumably the beginning of another axial branch. Bar = 10 μm. G, Root hairs binding to small particles of vermiculite. Bar = 10 μm.

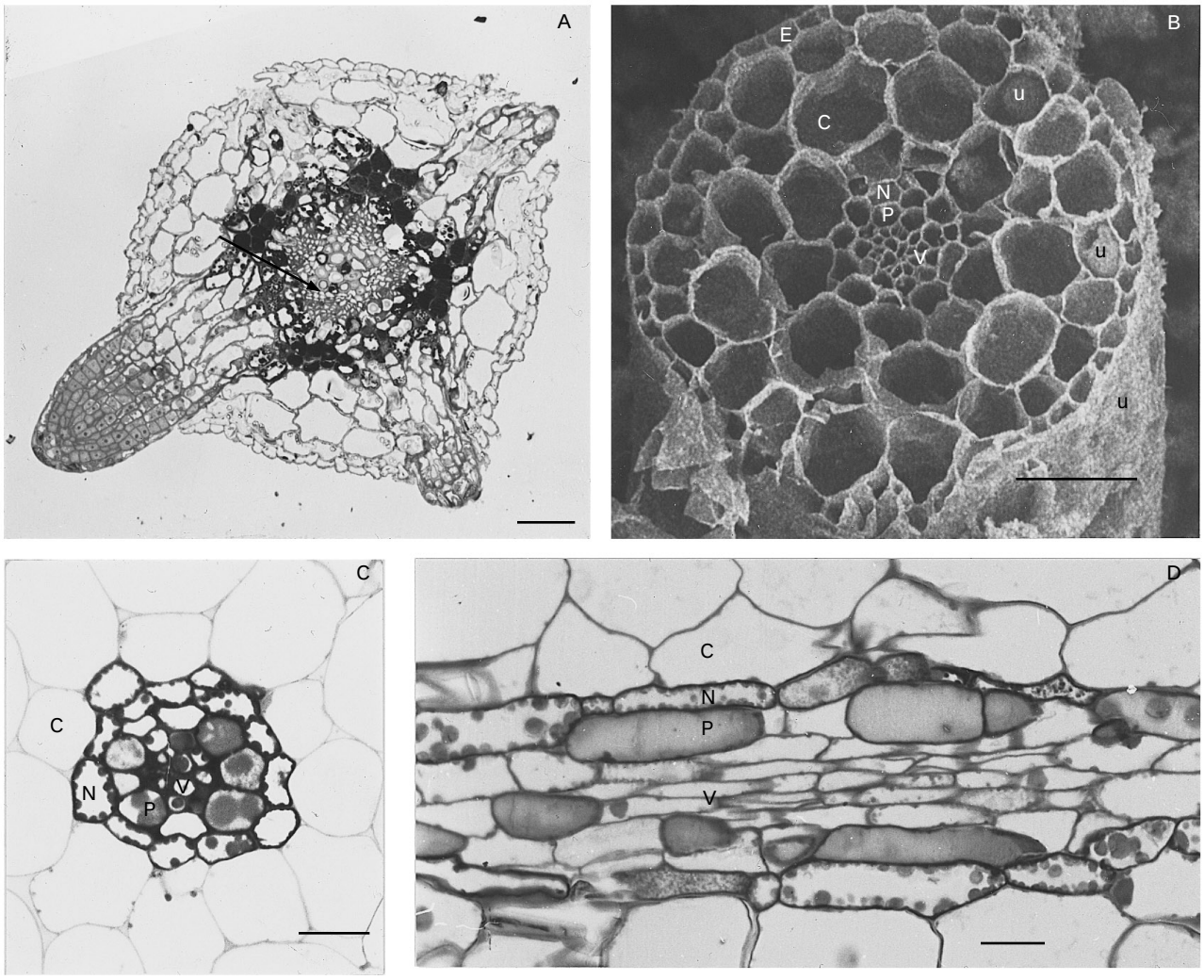

FIG. 2. Internal structure of cluster rootlets in *G. robusta*. A, LM. T.S. of a young cluster root with rootlets growing from pericycle of a lateral root, opposite the protoxylem poles (marked with an arrow). This section had been postfixed in 0·2% OsO_4 prior to agar embedding. Bar = 100 μm. B, SEM. T.S. of cluster rootlet. There is evidence of material within cortical cells (u) resembling the exudate covering the surface of the rootlet. Bar = 50 μm. C, LM. T.S. of cluster root stele and endodermis. Note characteristic globular bodies in endodermis and the dense pericycle cells. Xylem and phloem are unorganized. Bar = 15 μm. D. LM. L.S. of cluster rootlet. C, Cortex; E, Epidermis; N, Endodermis; P, Pericycle; V, xylem and phloem. Bar = 15 μm.

Brown and Mitchell (1984) on *Leucadendron laureolum*, where cluster rootlet tips were hairless. This difference may be explained by the fact that root caps would appear to be absent in *G. robusta* seedlings grown in water culture. Rootlet hairs were about 7 µm in diameter and up to 500 µm in length. *Grevillea* hairs displayed at least three distinct morphological variants. There was a 'stigmatic branching' occurring at the tip of the hair, so called because of the resemblance to the stigma of a flower (Fig. 1D, E). There was an 'axial branching', occurring along the axis of the rootlet hair (Fig. 1F). Finally, there was an 'apical swelling' at the tip of root hairs (not shown). All three 'variants' were seen to occur in the space of a few tens of micrometres.

There was evidence of attachment between rootlet hairs and particles of vermiculite (Fig. 1G). Further evidence comes from the fact that broken root hairs have been seen, where part of the root hair remains attached to the particle, indicating attachment between rootlet hair and particle.

Internal structure

Since, to our knowledge, this is the first detailed microscopic examination of the structure of cluster roots in *Grevillea robusta*, a basic histological study was first carried out. It was noted that cluster rootlets arise endogenously from the pericycle (Fig. 2A), confirming observations by Purnell (1960). Cluster rootlets had an epidermis, cortical cells, an endodermis, a pericycle and an unorganized vascular system (Fig. 2B, C, D) which connected directly with the root vascular system, as noted by Purnell (1960). They were approx. 160–230 µm in diameter. Rootlets had an apical meristem (Fig. 3B). Mature rootlets had no meristematic regions and all cells were vacuolate.

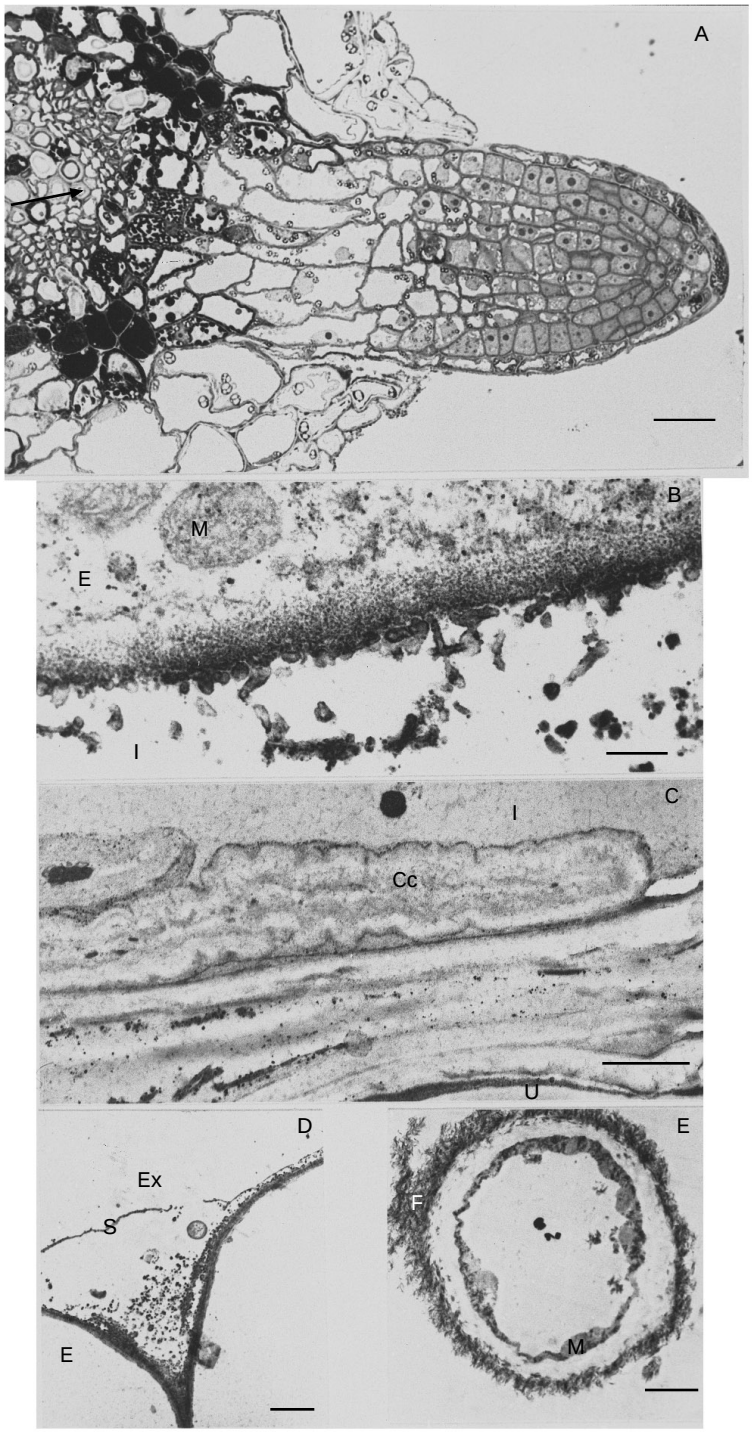

FIG. 3. Exudation in young cluster rootlets of *G. robusta*. A, LM. T.S. of lateral root showing young, emergent cluster rootlet with apical meristem. Arrow indicates protoxylem pole. This section was postfixed in OsO_4 prior to agar embedding. Bar = 50 μm. B, TEM. T.S. of epidermis of pre-emergent cluster rootlet (i.e. a rootlet that has not yet broken the surface of the lateral root), showing exudate. E, epidermal cell of cluster rootlet; I, intercellular space in the lateral root cortex; M, mitochondrion. Bar = 200 nm. C, TEM. T.S. showing collapsed cortical cells around the pre-emergent cluster root epidermis, shown in Fig. 3B. Possibly the exudate shown in Fig. 3B acts to lyse the cortical cells, thus allowing the cluster rootlet to grow with minimum disturbance to the lateral root. Cc, collapsed cortical cell of lateral root; I, intercellular space between cortical cells and pre-emergent cluster rootlet; U, uncollapsed cortical cell. Bar = 1 μm. D, TEM. Epidermal layer of young cluster rootlet. E, Epidermal cell; S, smooth exudate; Ex, external environment. Bar = 1 μm. E, TEM. T.S. of rootlet hair growing from mature rootlet. Packed with mitochondria (M), it is surrounded in fibrous exudate (F). Bar = 1 μm.

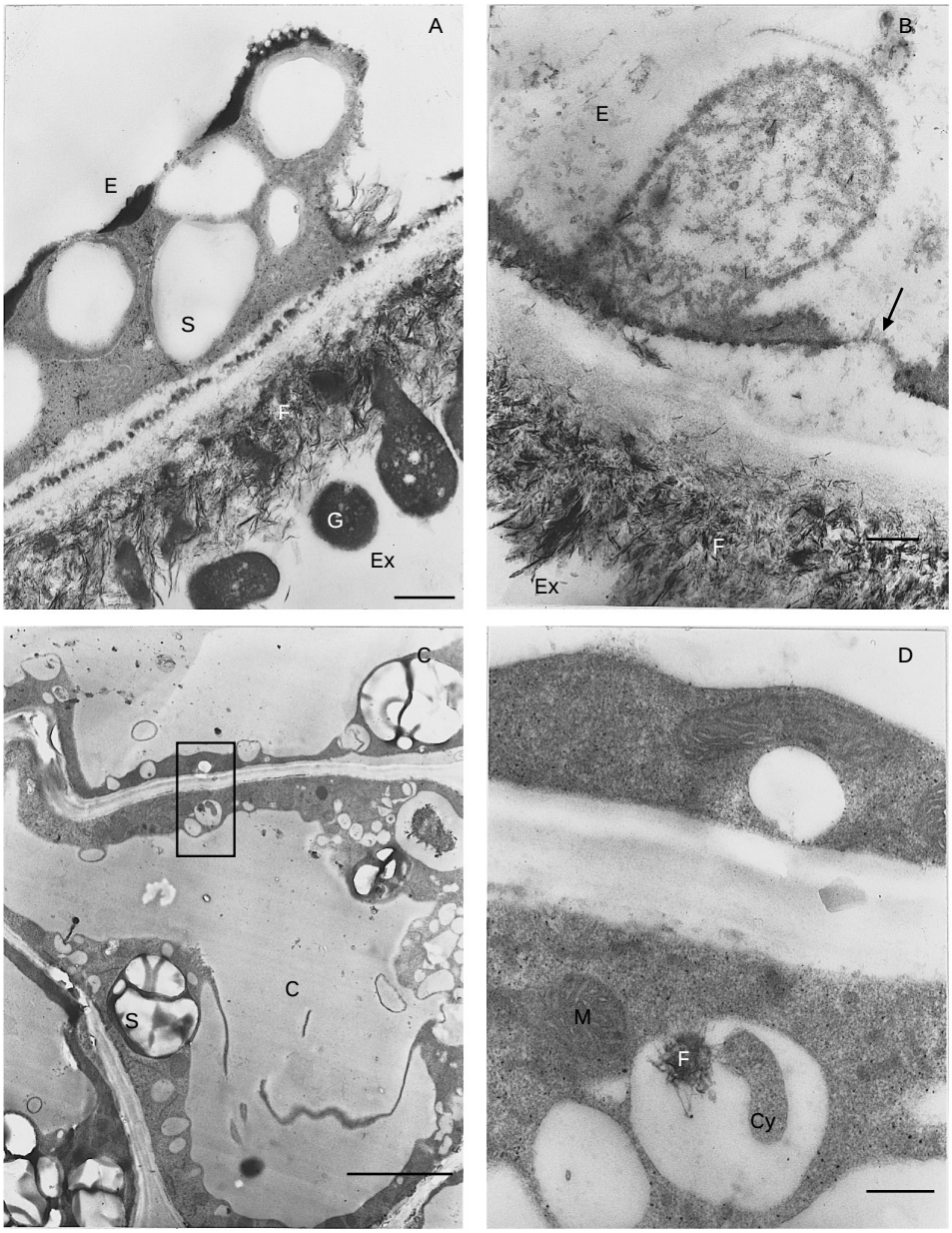

FIG. 4. Exudation in mature cluster rootlets of *G. robusta*. A, Two forms of exudate, one fibrous and the other globular, at the surface of a mature cluster rootlet. Bar = 500 nm. B, TEM. Evidence of exocytosis at the boundary between the external environment and the cluster rootlet. The arrow indicates a later stage in exocytosis. Bar = 200 nm. C and D, TEM. Synthesis of exudate. C, Cortical cell with vesicles, starch grains and mitochondria. Bar = 2 μm. D, Enlargement of part of Fig. 4C, showing vesicle containing material that resembles the fibrous exudate found at the surface. Also, part of the cytoplasm appears within the vesicle. This is probably due to infolding of the cytoplasm. Bar = 200 nm. C, Cortical cell; Cy, infolded cytoplasm; E, epidermal cell; Ex, external environment; F, fibrous exudate; G, globular exudate; M, mitochondrion; S, starch.

Functional histology

There was evidence that the epidermal layer of pre-emergent cluster rootlets secreted material (Fig. 3B). Lateral root cortical cells adjacent to these regions of secretion appeared collapsed (Fig. 3C). Material (marked *u* in Fig. 2B) in the cortical cells of cluster rootlets resembled the mucilage on the surface of the rootlet. Young, growing rootlets produced a smooth form of exudate (Fig. 3D). Rootlet hairs exuded only fibrous material (Fig. 3E). A second, globular type of exudate (Fig. 4A), was exuded from mature rootlets, but not from the rootlet hairs. Evidence of exocytosis from the epidermis was noted (Fig. 4B) and material identical to the fibrous exudate was found in vesicles within the cortex of the rootlet (Fig. 4C, D). The presence of starch grains (Fig. 4A, C) and mitochondria with many cristae (Fig. 4D) was indicative of high metabolic activity (Gunning and Steer, 1986).

DISCUSSION

Although their production has been linked, spatially, to nutrient-rich zones in soils (Purnell, 1960), when grown in aerated Hoagland's solution lacking P, cluster root production occurs throughout the root system and at fixed distances apart (Fig. 1A). Plants grown in vermiculite also produced cluster roots at fixed distances apart along any given lateral root. Other species appear to have a similar spacing of cluster roots (see photographs in Vorster and Jooste, 1986; Dinkelaker, Romheld and Marschner, 1989; Racette, Louis and Torrey, 1990). This would indicate that their production is unrelated to patches, since patches are extremely unlikely to be dispersed at fixed distances apart in vermiculite nor do they exist in stirred Hoagland's solution. Development appears to occur under some sort of preset pattern. These observations provide evidence that internal cues can control development of cluster root formation. How, then, do we explain the greater occurrence of cluster roots in nutrient-rich zones as observed by Broadhurst (in Purnell, 1960)? We would suggest that this could be explained by returning to the roles of the various components of the root system. Lateral roots are involved with exploration and export. Cluster roots cannot occur where lateral roots do not occur. It has been shown that many plant species display a proclivity to proliferate root systems in nutrient rich patches (e.g. Passioura and Wetselaar, 1972; Drew and Saker, 1975, 1978). Root systems of many proteaceous species are dimorphic, consisting of deep growing sinker roots and superficial lateral roots (Laycock and Wood, 1963; Lamont *et al*., 1984; Low and Lamont, 1990; Pate and Jeschke, 1993). Thus, if lateral roots occur at a much higher level in a particular region of the soil, then, provided that phosphate levels within the plant are below a threshold level, there will be a concomitant production of cluster roots. This can be seen in proteaceous species, where cluster roots are found near the soil surface *in situ* (Jeffrey, 1967; Lamont, 1983; Low and Lamont, 1990). In an environment without nutrient-rich patches, such as a stirred, minus P Hoagland's solution, lateral roots will be more evenly distributed, as will cluster root development. This explanation therefore puts emphasis on cluster root initiation being triggered by internal plant nutrition as opposed to external conditions, and thus the patch response would be within the domain of lateral root development, not cluster root development.

It is unknown why rootlet hairs only develop after the cluster rootlet stops growing. Certainly, earlier hair development would increase the frictional coefficient of the elongating rootlet. Developmentally, the rootlet cells may be incapable of rootlet hair development until cell division at the rootlet tip has ceased.

Hairs growing from cluster rootlets would appear to adhere to large (up to 3 mm) particles by a process involving a substance as yet unidentified (Fig. 1G). Branching of root hairs of the Proteaceae is well known (see Purnell, 1960) but this, to the authors' knowledge, is the first detailed examination of cluster rootlet hair morphology. Branching of root hairs in *G. robusta* bears a striking resemblance to branching in unicellular rhizoids of hepatics, as reported by Pocock and Duckett (1985). Pocock and Duckett (1985) suggest that branching may facilitate adherence or may play a role in water relations. Their study confirmed that branching was associated with contact with the substratum. These observations may also apply to branching in rootlet hairs of *G. robusta*.

It was wondered whether or not axial branching was merely a later stage of stigmatic branching, where one branch continues as the main axis, while the other becomes the side branch. However, small 'buds' were seen back from the tip of hairs (Fig. 1F) that would appear to be the beginning of axial branching, thus indicating that not all branching occurs as a result of branching at the tip of hairs. Apical swelling may be an early stage of stigmatic branching. The role of root hairs in nutrient uptake has recently been questioned (Wen and Schnable, 1994) and their role in structural aspects of plant biology has received more interest (e.g. Watt *et al*., 1994). The branching patterns in the hairs of the cluster roots in *Grevillea robusta* could be involved in either particle binding, small scale exploration (*of* a patch rather than *for* a patch), resource exploitation, water relations or resource export. They could be involved in a combination of some or all of these things. Certainly, cluster roots that have not yet developed hairs do not bind soil particles.

This study has shown that mature rootlets release two forms of exudate. Gardner, Barber and Parberry (1982) were the first to suggest that cluster root function was more likely to be related to secretion than to phosphorus uptake. Since then, it has been shown that cluster roots release organic acids and phenolic compounds (reviewed in Dinkelaker *et al*., 1995). Dell, Kuo and Thomson (1980) reported mucilage along with 'finger like projections' at the surface of cluster rootcaps of *Hakea obliqua*. These 'projections' would appear to be identical to the globular exudate shown in Fig. 4A. However, our micrographs were taken from epidermal cells, not from root cap cells. Also of interest is the observation that fibrous material is visible in cortical cells (Figs 2B and 4C, D). The cluster rootlet primordium also appears to exude material (Fig. 3B). Bonfante and Peretto (1993) suggest that pectinolytic enzymes may be involved in 'controlled cell separation' in lateral root outgrowth in *Allium porrum*. A similar event may occur in cluster root development. Further work is needed to investigate this.

In conclusion, minus P Hoagland's solution provides a suitable method of production of cluster roots on *Grevillea robusta*. The development of cluster roots leads to a structure capable of nutrient-rich patch exploitation. We would suggest that the development of these organs is not a direct response to a resource rich patch. Rather, the internal nutrient status of the plant and the distribution of the root system would appear to have most bearing on the spatial and temporal development of cluster roots.

The next stage is to identify the different forms of exudate and to elucidate the functional significance of each of these. The observation that globular exudate was produced only by the rootlet and not by the root hair would suggest that rootlets are not merely acting as regions of root hair growth, but may play a more direct role in nutrient acquisition. It is

also hoped to elucidate the signal pathway by which the spatial and temporal contexts of cluster root development and function are defined. This is extremely important, as the plant must be tuned into the spatial and temporal variations in resource availability (Fitter, 1994).

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