Journal of Experimental Botany, Vol. 22, No. 70, pp. 163–8, February 1971

Scanning Electron Microscopy of Plant Roots

P. J. DART

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire.

Received 28 April 1970

ABSTRACT

A glycol methacrylate infiltration and polymerization technique was used to prepare clover roots inoculated with *Rhizobium* for scanning reflection electron microscopy. Root hairs and epidermal cells were coated with many bacteria; some bacteria seemed to be embedded in the wall surface. Root hair tips were often smooth but some older root hair surfaces showed a fibrillar meshwork pattern. Small granules *c.* 0·18 μm diameter were present on the root hair and epidermal cell walls. The root cap, some root hairs, and some epidermal cells were covered by an amorphous film thought to be the mucigel.

INTRODUCTION

Striking pictures of the surface of plant pollen grains, seeds, diatoms, and some small algae, have been obtained by scanning reflection electron microscopy (e.g. Echlin, 1968*a*, *b*; Echlin and Chapman, 1968; Heslop-Harrison, 1968; Heywood, 1969), but softer plant tissues are more difficult to prepare satisfactorily for surface examination. Protozoan cilia have been prepared by freeze-drying and critical-point drying techniques (Boyde and Barber, 1969; Horridge and Tamm, 1969), suggesting that less rigid plant structures than pollen may be made amenable for scanning microscopy by similar methods. Bacteria have been shown on the surface of soybean stipules (Leben, 1969) and a low-resolution scanning picture of *Rhizobium* on air-dried *Trifolium repens* roots was published by Grey (1967).

Phase-contrast microscopy of living roots, and light and transmission electron microscopy of fixed roots have shown that they are covered in parts by layers of bacteria, and an amorphous film, the mucigel (e.g. Jenny and Grossenbacher, 1963; Dart and Mercer, 1964; Head, 1964; Samtsevich, 1968; Bowen and Rovira, 1968). The preparative techniques now described were devised for scanning electron microscopy of root surfaces as part of a study of the root-hair infection of legume plants by the nodule-forming bacterium *Rhizobium*.

MATERIALS AND METHODS

Plants of *Trifolium subterraneum* L. and *T. glomeratum* L. were grown in 150 × 18 mm glass tubes with cotton-wool plugs on N-free agar slopes (Jensen, 1942), or on slide assemblies in dilute N-free agar medium (Fåhraeus, 1957). The cultures were inoculated with *Rhizobium trifolii* but were usually otherwise sterile. Plants were fixed *in situ* for a minimum of 5 h, usually overnight, at ice-bath temperature with cold 3 per cent acrolein in tap water, or 1 per

WITH FIVE PLATES IN THE TEXT

cent glutaraldehyde in 0·025 M Na-K phosphate buffer pH 6·8. The fixative was then replaced by 0·025 M Na-K phosphate buffer, pH 6·8. Stoppered tubes could be stored successfully at 3 °C as enough fixative remained to maintain sterility.

Suitable areas were chosen by light microscopy—the roots stained when necessary with dilute toluidene blue (Sidman, Mottla, and Feder, 1961) and mounted in phosphate buffer. Several methods of processing from this stage were tried, including glycerol infiltration (Greene, 1967) and freeze drying. For the latter, roots were quenched in copper boats in isopentane at dry-ice temperatures, and then transferred to a flask, cooled by dry ice, attached to a rotary vacuum pump. P_2O_5 traps were incorporated in the vacuum line and the roots were left under a pumped vacuum for at least 24 h. Roots were then quickly attached to a stub and stored over desiccant until shadowed.

For methacrylate infiltration root pieces in 1–2 ml of phosphate buffer were gradually infiltrated at room temperature by adding drops of purified glycol methacrylate (GMA) containing 5 per cent polyethylene glycol 400 and 0·14 per cent w/v 2, 2′-azobis (2-methyl proprionitrile) as accelerator, until the concentration of GMA was approximately 10 per cent (Feder and O'Brien, 1968). Infiltration then proceeded by successive transfers through solutions of GMA in water with 10 per cent increase of GMA concentration, during 6–8 h, and left at least overnight in 100 per cent GMA mixture. Alternatively, roots were transferred from buffer to 2-methoxyethanol in an ice bath for 6–8 h, then transferred to ethanol at −20 °C for 8–24 h, followed by *n*-propanol 8–24 h at −20 °C and then infiltrated at room temperature as above through a GMA series in *n*-propanol (Feder and O'Brien, 1968). Root pieces were polymerized as follows: roots were placed directly from the methacrylate solution on to a bright aluminium surface which was *c.* 80 mm from a high-pressure 80 W, Hg ultra-violet light source with the glass jacket removed. Lamp and roots were enclosed in a plastic bag filled with nitrogen, and left at least 4 h for polymerization. Polymerization at 60 °C in N_2 was also used but needed care to prevent methacrylate evaporation from the specimens. Roots were fixed to the stubs with Durofix, nail varnish, a 50 per cent solution of collodion in amyl acetate, or a chloroform solution of cellulose tape glue. Preparations were coated under vacuum with a 20–40 nm thick film of Pd-Au while being rotated and moved at a continually varying angle to the electrode. Specimens were examined in a Cambridge Scientific Instruments Stereoscan scanning electron microscope, operating at accelerating voltages of 10–20V. Photographs were taken on Ilford HP3 35-mm film.

RESULTS

Roots were difficult to prepare for scanning microscopy and some tissue collapsed with all techniques tried. However, GMA infiltration and polymerization, either following dehydration or through direct replacement of water by GMA, usually gave good preparations that suffered little beam damage. All photographs except Plates 2C and 6 were prepared by GMA infiltration and polymerization. Freeze drying (Plate 2C) gave more collapse, but it should be possible to overcome this using more sophisticated techniques; beam damage was minimal. Glycerol infiltration, and GMA infiltration without polymerization (Plate 5A–C), resulted in root hairs collapsing during evacuation before coating. Beam damage was common; large bubbles formed in the coating, probably because the infiltration medium evaporated, and the coating cracked readily.

Some root hairs and epidermal cells were prolifically coated with bacteria, which were attached strongly enough to withstand the effect of several solution changes during preparation. Some of the bacteria are attached 'end on' to the root hairs rather than lying flat on the surface (e.g. Plate 2A). This can be seen with light microscopy of living material. A thin film covering the bacteria was sometimes apparent (Plates 2A and 4B) and some bacteria were joined by a fibre (Plate 4). Differences in bacterial shape can be readily resolved; the small rod-shaped bacteria in Plate 4 (e.g. single arrow) are probably not *Rhizobium*.

Root hairs were sometimes covered by a film of material (Plate 5E) or by more particulate material (Plates 1A and 3A, B). The hairs were usually smooth at the tip, but surface structure was apparent on the older parts (Plates 1B, 2B, C and 3A, C). An interlacing fibrillar meshwork is suggested. Freeze drying usually made root hairs collapse, but even collapsed hairs showed fibrillar structure (Plate 2C). Small granules (*c.* 0·18 μm diameter) are common on the surface with all preparations and their prevalence differed in different plant species (Plates 2A and 5E).

Inoculation by *Rhizobium* induces branching and curling of some root hairs on the appropriate legume root, and it is these hairs that become infected. Plate 5D shows a branched hair with a constriction differentiated behind the branching. Small hollows occur on other root hairs (Plates 2A and 5E)but it is uncertain whether this is real structure or artifact. Light microscopy of living fresh material shows the collapse of some hairs.

Epidermal cells (Plate 4) were contoured rather than flat. The fibrillar structure present in parts of the root hairs was not obvious, although the surface was not smooth (Plate 4B) and contained many small granules (Plates 5B, C). Near the root tip the junction between files of cells in the epidermis was very obvious (Plate 5B). The cell surface layer (root cuticle?) was not always complete at the junction between cells (Plate 4A). Bacteria were abundant on the epidermis and sometimes seemed to be embedded in the surface layer (Plate 4B).

The root tip and root cap region were covered with an amorphous layer (of mucilage?) obscuring the individual root cap cells (Plate 5A).

DISCUSSION

The scanning electron microscope with its great depth of field shows how extensively the space adjacent to the root epidermis is covered by root hairs, many of which are branched and bent as a reaction to *Rhizobium* inoculation.

The root tip, and sometimes the root hairs and root epidermis, are covered by an amorphous layer, which has been called the mucigel (Jenny and Grossenbacher, 1963) and was first reported by Roberts in 1916. It is seen in light-microscope examinations of roots (Dart and Mercer, 1964; Head, 1964; Samtsevich, 1968; Dart, Nutman, and Roughley, unpublished observations) and usually excludes bacteria from the root tip (Samtsevich, 1968) but can enclose many bacteria in the root hair zone (Jenny and Grossenbacher, 1963; Dart and Mercer, 1964). It contains polysaccharide (Rovira, 1962), thought to be hemicelluloses and pectins (Samtsevich, 1968), and stains a metachromatic red with toluidene-blue. Root hairs also exude globules of material (Head, 1964) and sometimes burst, releasing a large part of their cytoplasm and these materials may be deposited on or contribute to the nature of the root hair surface.

It is possible that the film covering some of the root hairs (e.g. Plate 5E) is an artefact produced by residual surface methacrylate not removed during the preparation. Roots were carefully surface dried on filter-paper prior to polymerization and methacrylate drains from roots not surface dried on to the aluminium support during polymerization.

The root hair surface is rather smooth at the tip but the older part of the hair

sometimes shows a fibrillar meshwork. The fibrillar surface of unextracted clover root hairs inoculated with *Rhizobium* (Dart and Mercer, 1964) was also clearly shown by replica techniques and transmission electron microscopy; *Rhizobium* is thought to be involved in this exposure of fibrillar structure.

The fibrils may be the cellulose fibrils of the primary wall layer, prominent in thin sections of root hair walls (Newcomb and Bonnett, 1965). The meshwork pattern in root-hair walls has been clearly demonstrated by transmission electron microscopy of shadowed preparations of hairs from which the non-cellulosic substances have been chemically extracted (e.g. Frey-Wyssling and Mühlethaler, 1949; Houwink and Roelofsen, 1954; Belford, Myers, and Preston, 1958; Dawes and Bowler, 1959); and also from unextracted root hairs of *Sinapis alba* (Belford and Preston, 1961).

The fibre joining some bacteria (Plate 4) resembles the tube-like structure found in sectioned material of *Rhizobium*-inoculated clover roots (see Fig. 11, Dart and Mercer, 1964). Tube-like structures of similar size sometimes join *Bacteroides* cells (Bladen, 1963) and are possibly conjugation bridges.

The surface structures found (e.g. fibrillar mesh in Plates 1–2 and the tubes joining bacteria in Plate 4) are not thought to be artefacts produced by the methacrylate since glycol methacrylate itself polymerizes to an isomorphic surface which has no structure when examined by scanning reflection electron microscopy. (Dart and Drake unpublished observations.)

The number of bacteria, their close contact, and tenacious adherance to the root surface is striking. A similar layer of bacteria on the root surface can be seen in thin sections of clover roots (Dart and Mercer, 1964). Such a population of bacteria may affect the physiology of the root. Rhizosphere bacteria compete with the root for some soil nutrients, and also degrade other soil compounds to products more readily available for uptake by the plant (Nicholas, 1965). Bacteria and plant root metabolites also interact. For example, tryptophan secreted by clover roots is used by *Rhizobium* to produce indol-3yl-acetic acid (Kefford, Brockwell, and Zwar, 1960), which is thought to play a role in the root hair curling and branching associated with the development of the infection thread that leads to nodule formation (Thimann, 1936). The scanning electron microscope is potentially a very valuable tool for examining the interaction of micro-organisms and plant roots, especially interactions with pathogens.

The root surface is thus not structurally uniform; root hairs are relatively smooth at tip, but fibrillar in older parts, coated with small granules, bacteria, and larger particulate material, and sometimes with a mucigel film.

ACKNOWLEDGEMENTS

I am grateful to the staff of the Cambridge Instruments Ltd., Stereoscan Applications Division, for coating and photographing this material and to Dr. C. Greene for discussions on preparative techniques.

LITERATURE CITED

BELFORD, D. S., MYERS, A., and PRESTON, R. D., 1958. Spatial and temporal variation of microfibrillar organization in plant cell walls. *Nature, Lond.* **181,** 1251–3.

—— and PRESTON, R. D., 1961. The structure and growth of root hairs. *J. exp. Bot.* **12,** 157–68.

BLADEN, H. F., 1963. Demonstration of an unusual ultrastructure found in *Bacteroides*. *J. Bact.* **85,** 250–3.

BOWEN, G. D., and ROVIRA, A. D., 1968. The influence of micro-organisms on growth and metabolism of plant roots. In *Root growth*, ed. Whittington, J. Butterworths, London. Pp. 170–99.

BOYDE, A., and BARBER, V. C., 1969. Freeze-drying methods for the scanning electron-microscopical study of the protozoan *Spriostomum ambiguum* and the statocyst of the cephalopod mollusc *Loligo vulgaris*. *J. Cell. Sci.* **4,** 223–40.

DART, P. J., and MERCER, F. V., 1964. The legume rhizosphere. *Arch. Mikrobiol.* **47,** 344–78.

DAWES, C. J., and BOWLER, E., 1959. Light and electron microscope studies of the cell wall structure of the root hairs of *Raphanus sativus*. *Am. J. Bot.* **46,** 561–5.

ECHLIN, P., 1968*a*. The use of the scanning reflection microscope in the study of plant and microbial material. *J. R. microsc. Soc.* **88,** 407–18.

—— 1968*b*. Pollen. *Scient. Am.*, **218,** 81–90.

—— and CHAPMAN, B., 1968. Scanning reflection electron microscopy of plant surfaces. *4th Europ. Reg. Conf. Electron Microscopy*, **2,** 49–50.

FÅHRAEUS, G., 1957. The infection of clover root hairs by nodule bacteria, studied by a simple glass slide technique. *J. gen. Microbiol.* **16,** 374–81.

FEDER, N., and O'BRIEN, T. P., 1968. Plant microtechnique: some principles and new methods. *Am. J. Bot.* **55,** 123–42.

FREY-WYSSLING, A., and MÜHLETHALER, K., 1949. Über den Feinbau der Zellwand von Wurzelhaaren. *Mikroscopie*, **4,** 257–66.

GREENE, G., 1967. Preparation of nematodes for examination under Stereoscan electron microscope. *Nematologica*, **13,** 279–82.

GREY, T. R. G., 1967. Stereoscan electron microscopy of soil micro-organisms. *Science, N.Y.* **155,** 1668–70.

HEAD, G. C., 1964. A study of 'exudation' from the root hairs of apple roots by time-lapse ciné-photomicrography. *Ann. Bot.* **28,** 495–8.

HESLOP-HARRISON, J., 1968. Pollen wall development. *Science, N.Y.* **161,** 230–7.

HEYWOOD, V. H., 1969. Scanning electron microscopy in the study of plant materials. *Micron.* **1,** 1–14.

HORRIDGE, G. A., and TAMM, S. L., 1969. Critical-point drying for scanning electron micro-scopic study of ciliary motion. *Science, N.Y.* **163,** 817–18.

HOUWINK, A. L., and ROELOFSEN, P. A., 1954. Fibrillar architecture of growing plant cell walls. *Acta. bot. neerl.* **3,** 385–95.

JENNY, H., and GROSSENBACHER, K., 1963. Root soil boundaries as seen in the electron microscope. *Proc. Soil Sci. Soc. Am.* **27,** 273–7.

JENSEN, H. L., 1942. Nitrogen fixation in leguminous plants. II. *Proc. Linn. Soc. N.S.W.* **67,** 205–12.

KEFFORD, N. P., BROCKWELL, J., and ZWAR, J. A., 1960. The symbiotic synthesis of auxin by legumes and nodule bacteria and its role in nodule development. *Aust. J. biol. Sci.* **13,** 456–67.

LEBEN, N. C., 1969. Colonization of soybean buds by bacteria: observations with the scanning electron microscope. *Can. J. Microbiol.* **15,** 319–20.

NEWCOMB, E. H., and BONNETT, H. T., 1965. Cytoplasmic microtubule and wall micro-fibril orientation in root hairs of radish. *J. Cell Biol.* **27,** 575–89.

NICHOLAS, D. J. D., 1965. Influence of the rhizosphere on the mineral nutrition of the plant. In the *Ecology of soil-borne pathogens*, ed. Baker, K. F., and Snyder, W. C. Univ. Calif. Press, Berkeley. Pp. 210–17.

ROBERTS, E. A., 1916. The epidermal cells of roots. *Bot. Gaz.* **62,** 488–506.

ROVIRA, A. D., 1962. Plant-root exudates in relation to the rhizosphere microflora. *Soils Fertil.* **25,** 167–72.

SAMTSEVICH, S. A., 1968. Gel-like excretions of plant roots and their influence upon soil and rhizosphere microflora. Symp. U.S.S.R. Academy of Sciences, *Methods of productivity studies in root systems and rhizosphere organisms*, 200–4.

SIDMAN, R. L., MOTTLA, P. A., and FEDER, N., 1961. Improved polyester wax embedding for histology. *Stain Technol.* **36,** 279–84.

THIMANN, K. V., 1936. On the physiology of the formation of nodules on legume roots. *Proc. natn. Acad. Sci. U.S.A.* **22,** 511–14.

EXPLANATION OF PLATES

PLATES 1–2B, 3, 4, 5D, E are polymerized GMA preparations of *T. glomeratum* roots.

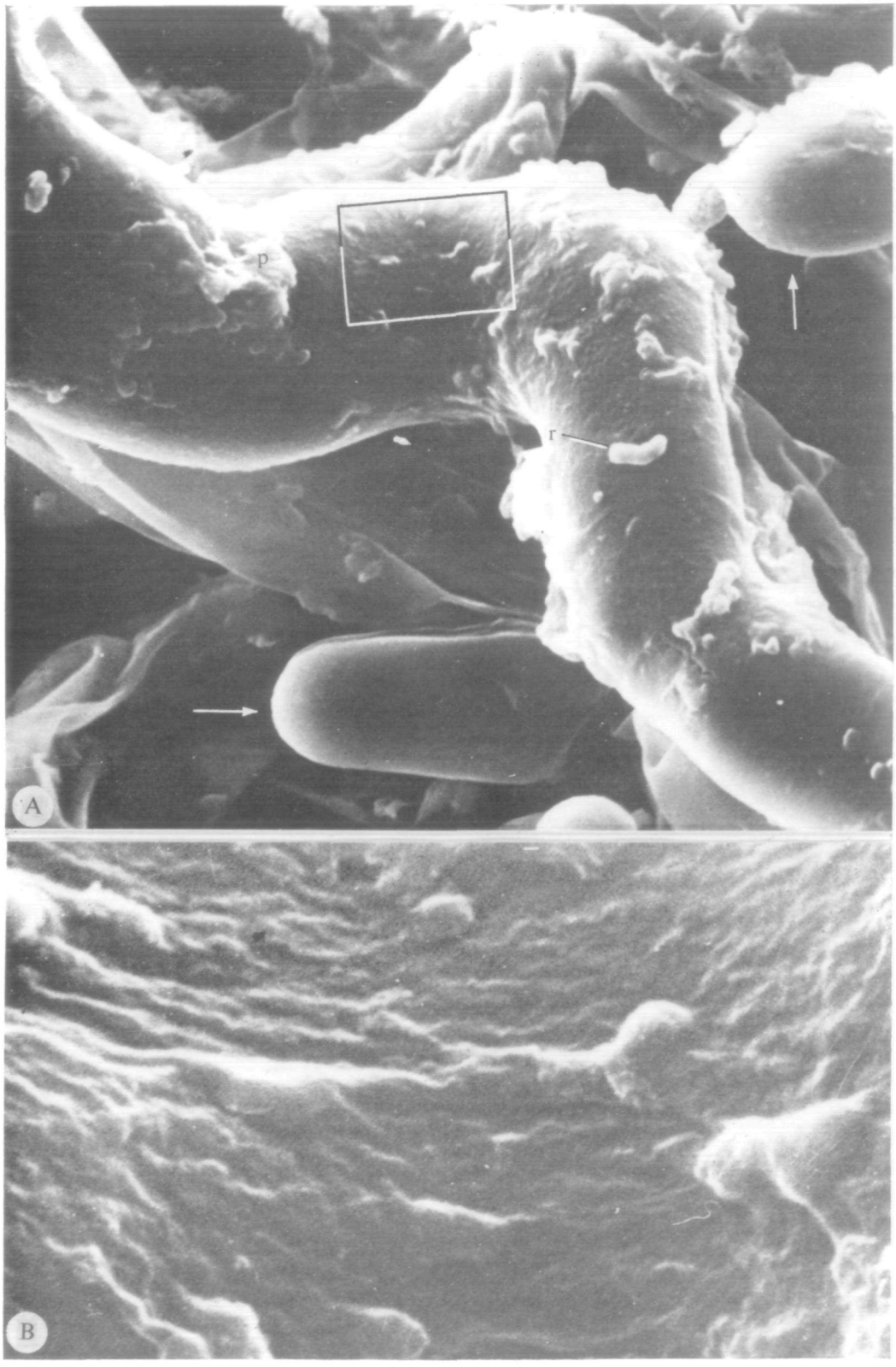
PLATE 1A. Root hairs, with *Rhizobium* cells (r) on the surface, as well as other particulate matter (p). The two root hair tips (arrows) appear to be free of rhizobia. ×2100. PLATE 1B. Enlargement of the part of the root hair outlined in PLATE 1A. Coccoid bacteria are attached to the uneven hair surface. ×5300.

PLATE 2A, B. Root hairs back from the tip and epidermis with attached *Rhizobium* cells, some of which appear covered by a film continuous with the hair surface. Some rhizobia (e.g. arrows) are attached 'end on' to the root. Small granules (g) are also prominent on the hairs. PLATE 2B. Microfibrillar pattern on the hair surface. 2A × 3100; 2B × 4900. PLATE 2C. Collapsed *T. glomeratum* root hair, frozen dried preparation with fibrils on the surface. r-*Rhizobium* cells. ×4600.

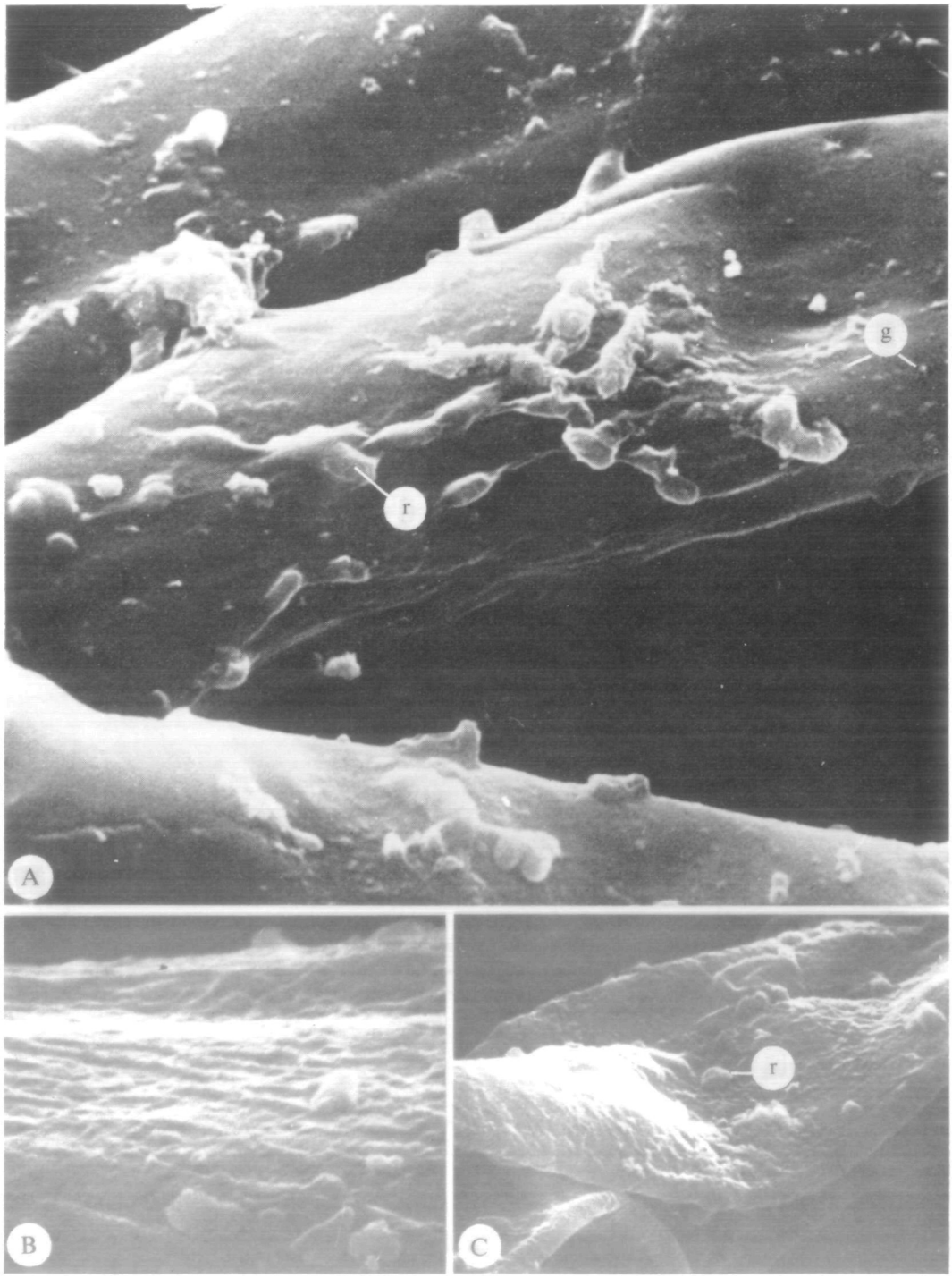
PLATE 3. *T. glomeratum* root hair with attached *Rhizobium* cells (e.g. r). The root tip is enlarged in PLATE 3B (×3200) showing other material (e.g. arrow) as well as the bacteria attached to the cell wall. The material at the base of the root hair is possibly the mucigel layer. PLATE 3C is a further enlargement (×5700) of the hair surface showing the fibrillar meshwork. 3A × 1900.

PLATE 4. Shows an epidermal surface in the root hair zone. Particulate material, small granules (g), and bacteria coat the surface; the small rod-shaped bacteria (e.g. arrow) are thought not to be *Rhizobium*. Some bacteria are attached to the surface by a film (e.g. double arrow), others appear embedded in the surface layer. Some of the bacteria are joined by a narrow tube (t). The root surface layer is discontinuous over the junction (j) between cells. PLATE 4B is an enlargement of the area outlined in PLATE 4A. 4A × 3200; 4B × 6300.

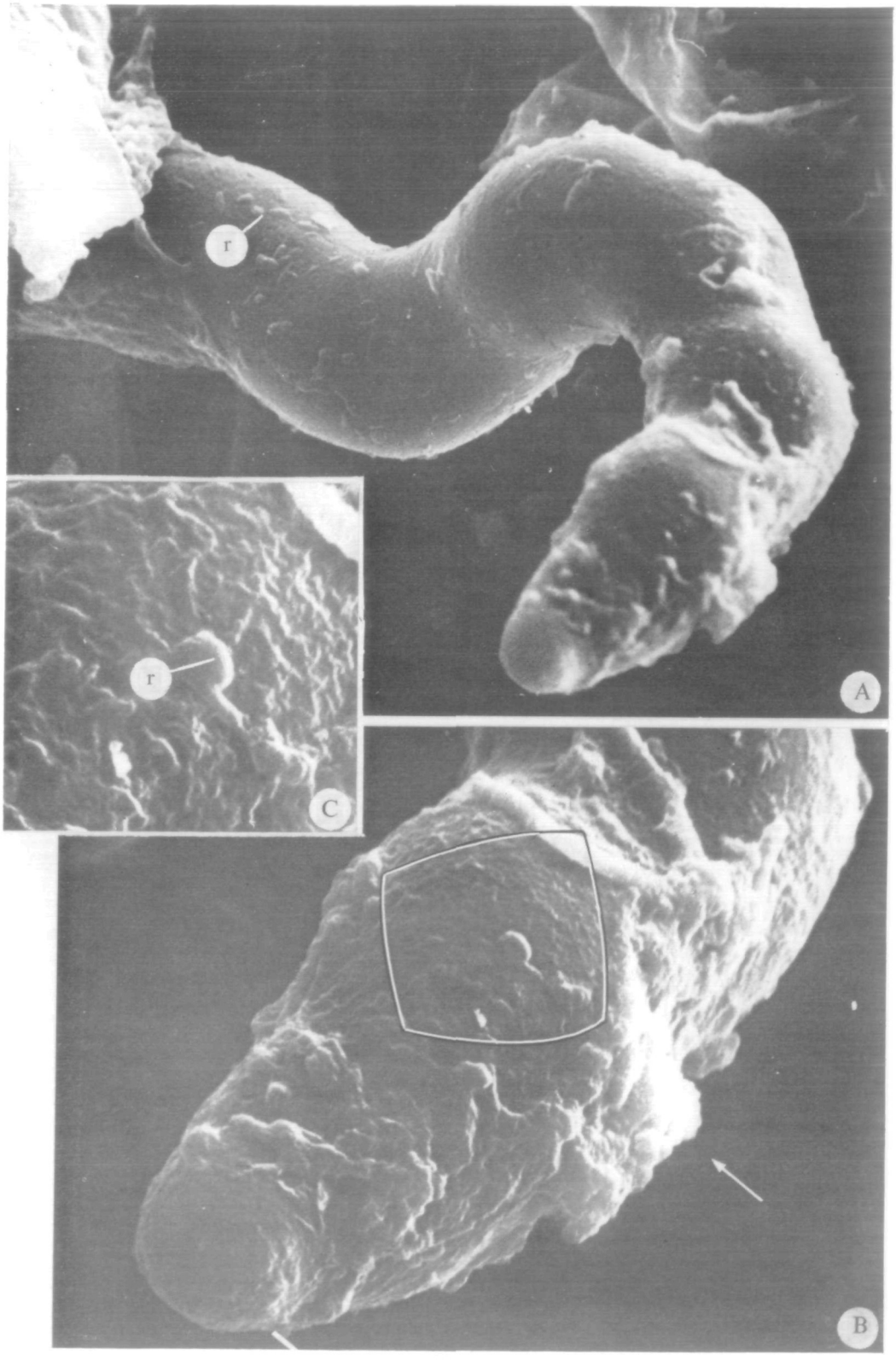
PLATE 5A. Root tip of *T. subterraneum* with the surface covered by an amorphous layer except for a small microcolony of bacteria (b) and one gap (arrow). There has been some collapse in this unpolymerized GMA preparation. ×880. PLATES 5B, C. *T. subterraneum* epidermal surface, unpolymerized GMA preparations. The epidermis is coated with bacteria and small granules. The granules are more prolific on the root hair (h) at the edge of 5B. The longitudinal junction between epidermal cells is prominent. 5B × 550; 5C × 4600. PLATE 5D. Branched root hair which is noticeably constricted behind the branching and has a smoother surface in this zone. ×1800. PLATE 5E. Root hairs with attached bacteria. In places an amorphous film joins the hairs, some small granules (g) are also present on the surface. ×4200.



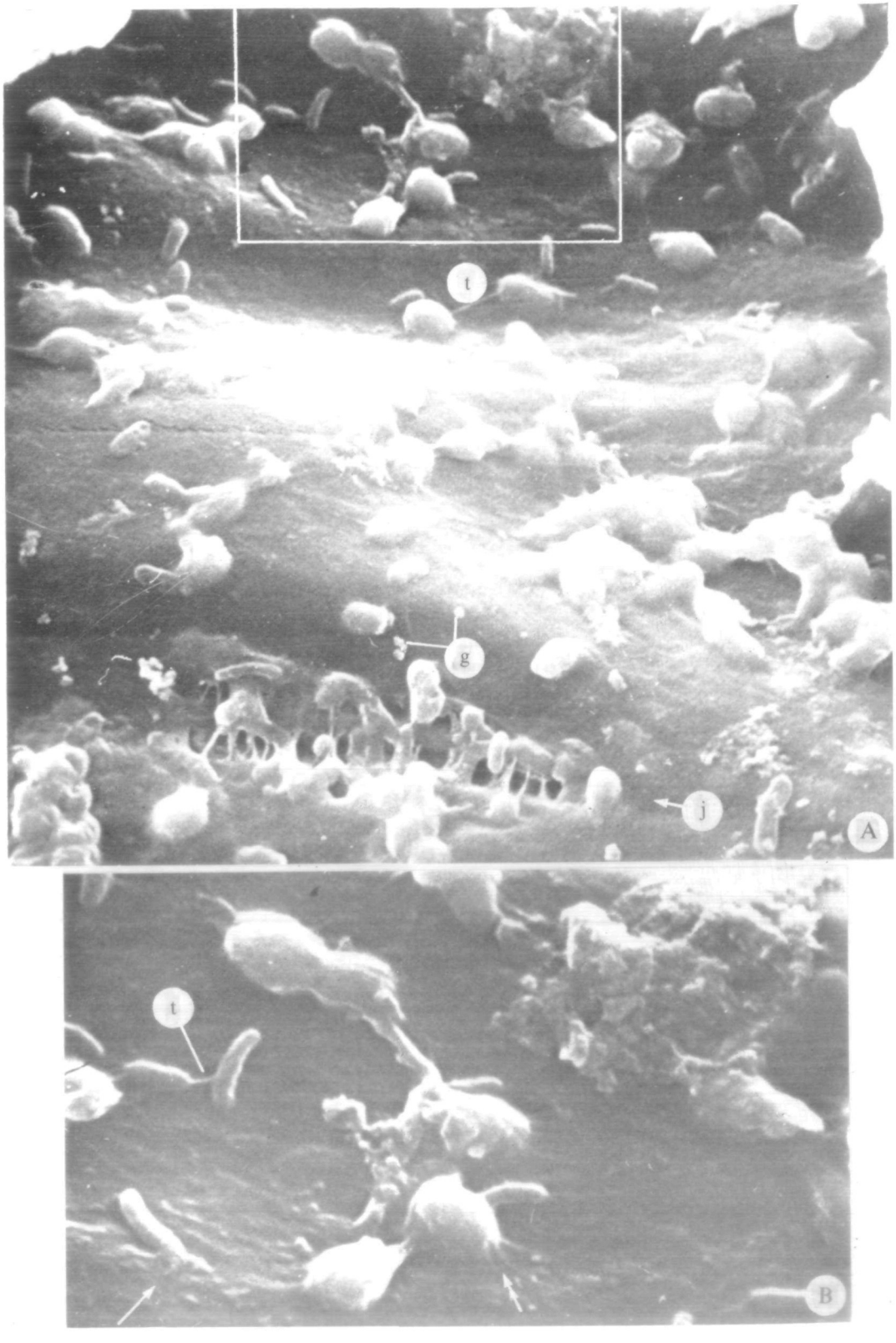
DART—PLATE 1



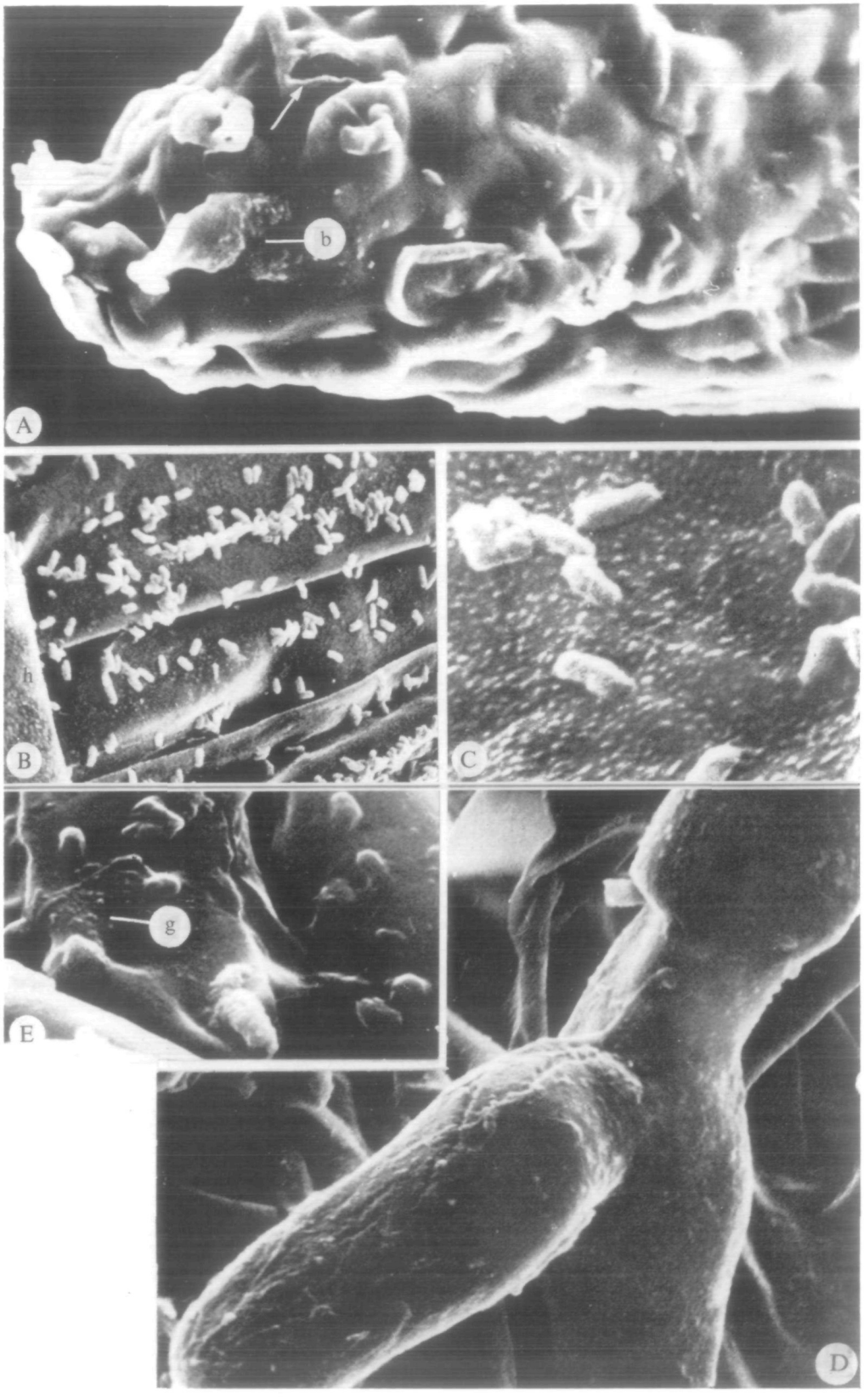
DART—PLATE 2



DART—PLATE 3



DART—PLATE 4



DART—PLATE 5