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Scanning Electron Microscope Studies of *Agrobacterium tumefaciens* Attachment to *Zea mays*, *Gladiolus* sp., and *Triticum aestivum*

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Scanning electron microscope studies demonstrated that cells of *Agrobacterium tumefaciens* strains attach to cells on the cut surfaces of corn and wheat seedlings and to gladiolus disks. Bacterial cells attached to these monocots in the same manner as they attached to the dicots tested. Of the strains tested, A66 and T37 covered more of the cut surfaces of these monocots in a nonrandom fashion than did cells of other isolates. These bacteria attached to cells of intact monocotyledonous plants and had the greatest affinity for tissues located within the vascular bundles. They attached in large numbers to cells in these areas in all three monocots tested.

When introduced into the wounds of susceptible dicotyledons and gymnosperms, virulent strains of the soil phytopathogen, *Agrobacterium tumefaciens*, cause crown gall tumors (12, 19). Until recently, it had been assumed that monocotyledons were not susceptible to *A. tumefaciens* infections, since large tumors normally do not develop following inoculation with these bacteria (5). However, those plants lacking the oncogenic response have not been analyzed routinely for opine synthase activities. Since the genes which encode the expression of these activities are located in the T-DNA, failure to test for expression may result in the underestimation of the host range of *A. tumefaciens*. This is particularly true if in a given infected species, e.g., maize (7), opine synthesis is an event distinctive from and not contingent upon tumor formation.

After *A. tumefaciens* inoculations, expressions of enzymatic activities encoded by T-DNA genes have been demonstrated in representatives of the families Amaryllidaceae, Liliaceae, and Gramineae (7, 8, 10), and tumors have since been reported on *Asparagus officinalis*, *Narcissus* sp., *Gladiolus* sp., and *Dioscorea bulbifera*, a commercially important monocotyledonous yam (8, 9, 10, 21). The transfer, integration (shown by Southern blot analysis), and expression of T-DNA in *D. bulbifera* (21) provide definitive proof that monocots are susceptible to transformation mediated by *A. tumefaciens*.

The first step in *A. tumefaciens*-mediated plant cell transformation is the actual attachment of the bacterial cell to plant cell walls (12). Monocot cell walls were thought to have fewer bacterial attachment sites than dicot cells (15). This would account for the lack of tumor formation in some monocots. It has been reported that more of these bacteria attach to carrot than to corn or oat protoplasts from suspension cultures (16) and that they do not attach to embryonic leaf fragments of maize (15). However, attachment studies have used cells prepared by many different techniques (6, 13, 14–17, 20) which may have altered their physiological characteristics considerably. It is known that *A. tumefaciens* cells rapidly agglutinate and attach to mechanically isolated protoplasts of *Asparagus officinalis* (6). Given these data, the fact that tumors form on some monocots, and the demonstration of opine synthase activities in other monocots (7–9, 10, 21), we inoculated the cut surfaces of intact gladiolus, corn, and wheat plants with *A. tumefaciens* to examine attachment. We examined these surfaces by scanning electron microscopy to determine whether attachment is a factor restricting transformation in this group of plants. We do not believe that it is, since these bacteria definitely do attach to the monocots tested.

Bacterial strains A66 and T37 were grown in yeast extract broth (7) to a titer of 10^9 cells per ml as determined by viable colony counts. We thank G. Heberlein for *A. tumefaciens* A66 and B. B. Lippincott and J. A. Lippincott for T37. Gladiolus corms, carrots, potatoes, and red wheat (*Triticum aestivum*) seeds obtained from The Andersons of Maumee, Ohio, and corn (*Zea mays* var. yellow Iochief) seeds, purchased from Botzum Seeds of Dayton, Ohio, were surface sterilized, and the seeds were germinated according to procedures described previously (2, 7).

The electron microscopy work was carried out at the Electron Microscope Facility at Bowling Green State University by A. E. Graves and A. C. F. Graves.

Seedlings (4 days postgermination) were removed from the seed and cut through the upper and the lower mesocotyl to form truncate cylinders of tissue having oval ends, both of which could be viewed from above. Gladiolus corm, carrot, and potato disks were excised according to previously described procedures (2, 6). The plant cells were inoculated with 10^8 bacterial cells. The plant tissues were allowed to incubate with the bacteria overnight in MS (18) salts medium; they were then washed vigorously twice by vortexing at speed 7 for 30 s in phosphate-buffered 0.9% saline solution (9 g of NaCl, 2.79 g of Na_2HPO_4, 0.43 g of KH_2PO_4 in 1 liter of distilled, deionized water [pH 7.2]). A set of disks or seedlings of each of the plants was washed three more times at the highest speed on the vortex to remove any bacteria that were not firmly attached.

Tissues were prepared for viewing by scanning electron microscopy using standard fixation techniques for biological materials. The procedure was as follows. Tissues were fixed in 0.1 M phosphate buffer containing 3.5% gluteraldehyde for 1.5 to 2 h on ice, rinsed two times in 0.1 M sodium

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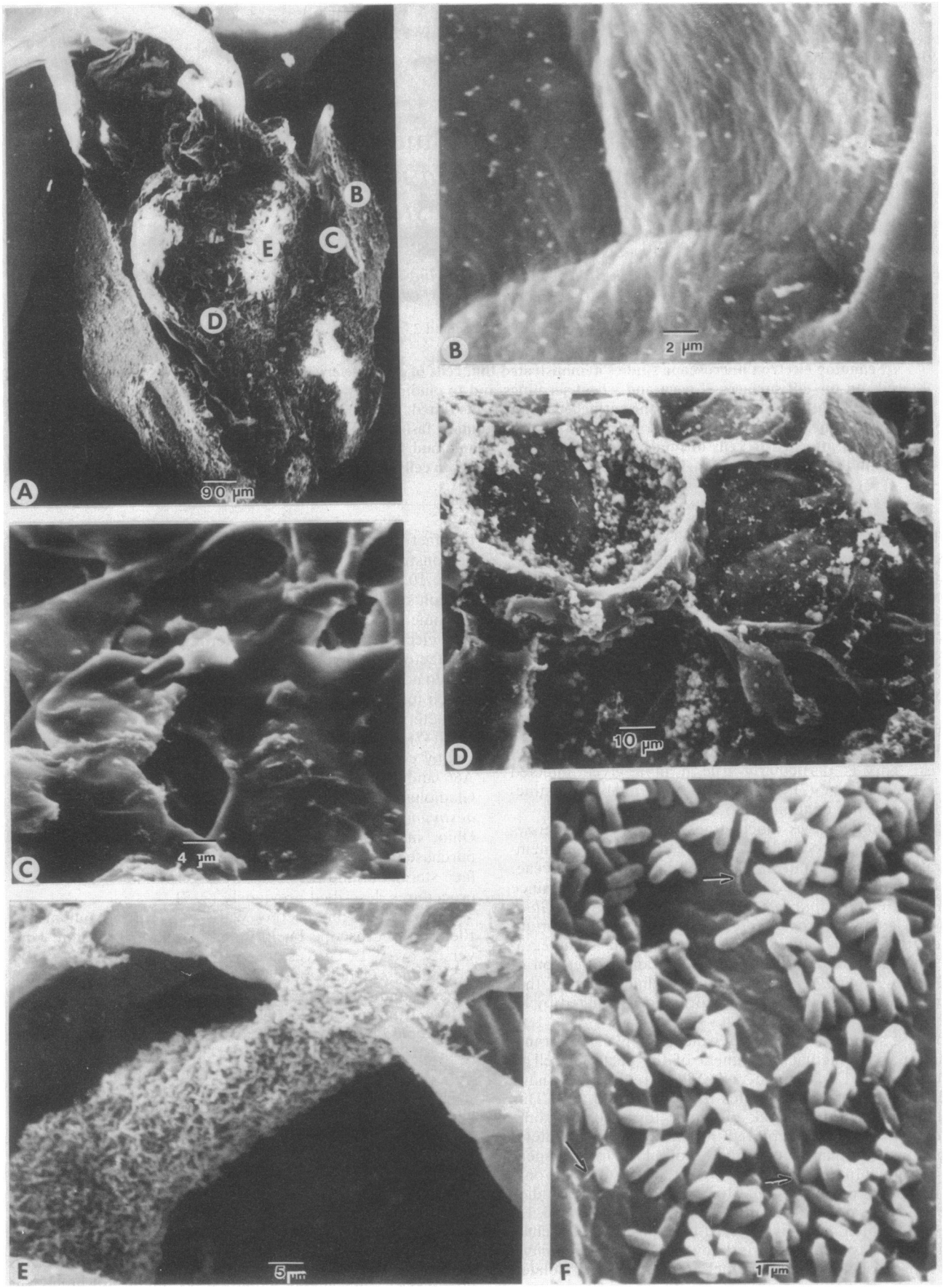


FIG. 1. Scanning electron photomicrographs of attachment of *A. tumefaciens* A66 to intact cells of 4-day-old *Zea mays* seedlings. (A) Corn mesocotyl. Each letter defines a specific region of the mesocotyl, which is shown in detail in the ensuing photomicrographs. (B) Epidermis. (C) Tissue layer directly beneath the epidermis. (D) Storage tissue. (E) Masses of bacteria covering vascular bundles. Bacteria attached to other bundles located in the lower mesocotyl and hypocotyl in masses such as those shown. (F) Detail enlargement of mesocotyl from which most of the bacteria had been removed, showing bacterial attachment to cell walls. Arrows indicate strands that attach bacteria to the cell walls.

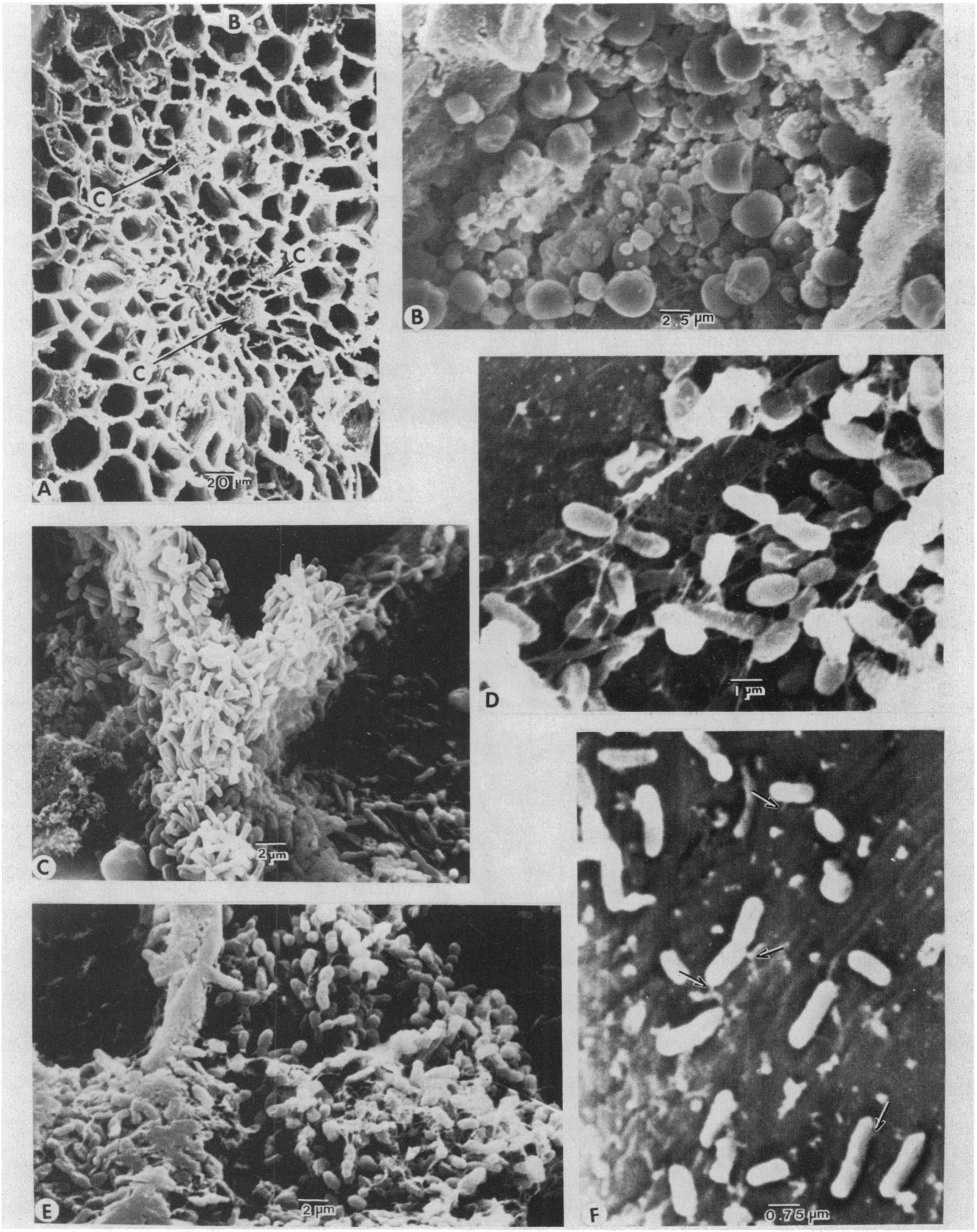
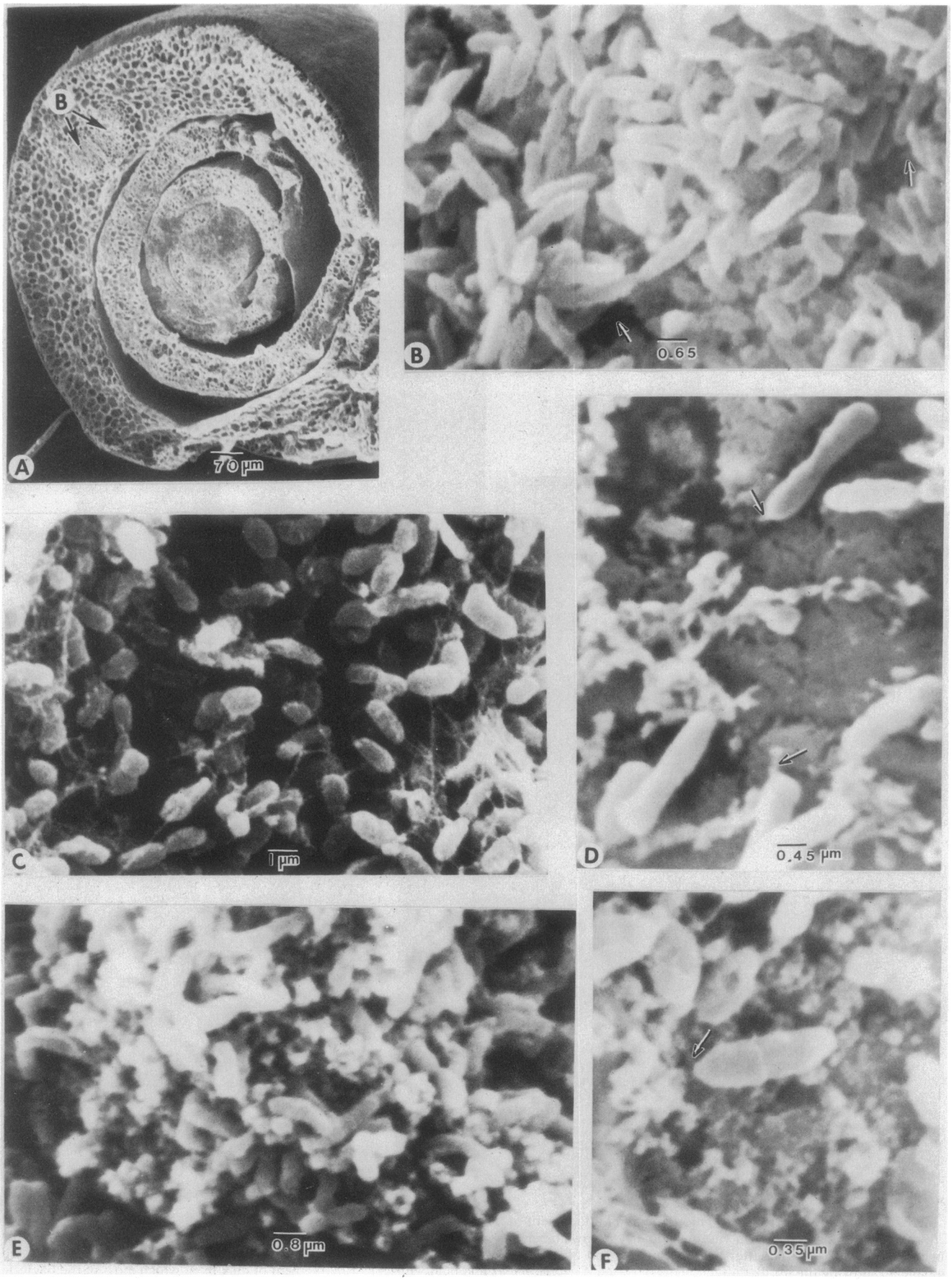


FIG. 2. Scanning electron photomicrographs of attachment of *A. tumefaciens* T37 cells to cells of intact gladiolus corm disk tissues. (A) Cross-section of gladiolus corm vascular bundle. Arrows labeled C indicate bacterial masses. (B) Storage tissue outside the vascular bundle (marked in panel A by the letter B). (C) Enlargement of the area labeled C in panel A. (D and E) Detailed enlargements of areas indicated in panel C showing the fibrillar attachments between *A. tumefaciens* and plant cells. (F) Enlargement of disk from which most of the bacteria had been dislodged, showing bacterial attachments to plant cell walls. Arrows indicate bacterial attachment strands extending to cells.


A
B
70 µm
B
0.65
C
1 µm
D
0.45 µm
E
0.8 µm
F
0.35 µm

phosphate buffer (pH 7.2) for 15 min each, and postfixed in a mixture of equal parts of 2% osmium tetroxide and 0.2 M sodium phosphate buffer (pH 7.2) for 1.5 to 2 h (or overnight) on ice. The tissues were then rinsed again in 0.1 M sodium phosphate buffer for 15 min and, finally, dehydrated in successive ethanol solutions of 25, 40, 60, 80, and 95%. The final dehydration was done in 100% ethanol for 15 min, in 100% ethanol for 1 h, and in 100% ethanol overnight on ice. Specimens were critical-point dried, sputter-coated with gold-palladium, and then viewed on a Hitachi HHS.

While *A. tumefaciens* cells did attach to exposed corn, gladiolus, wheat, and carrot cells, their distribution was not uniform over the cut surfaces (compare Fig. 1B, C, and D and Fig. 2B with Fig. 1E and F and Fig. 2C, D, E, and F). The bacteria attached specifically to cells of tissues in and around the vascular bundles in corn (Fig. 1E and F), gladiolus (Fig. 2C, D, and E), and wheat (Fig. 3B). In carrot cells, the bacteria attached to the vascular tissue. The bundles were difficult to distinguish in the potato disks. The bacteria attached by fibrillar connections not only to each other but to the cut cell surfaces as well (Fig. 2D and E and Fig. 3B, C, and E). Strands were seen running from the bacteria to the plant cell walls (Fig. 1F, Fig. 2F, and Fig. 3B, D, and F). In both plant groups, these strands looked identical, and there were one or several strands attaching each bacterial cell (Fig. 2F and 3D). However, there were areas in all of these plants where the bacteria did not attach.

Before these data can be placed in proper perspective, it is important to note that factors such as age, cell type, cell cycle stage, and other physiological conditions play an important role in events leading to attachment of *A. tumefaciens* to plant cell surfaces (4, 11). It has been reported that the attachment of these bacteria to the cell wall fragments derived from *Helianthus* cotyledons seems to be age dependent; fewer bacteria attach to older cell fragments than to younger ones (3). With respect to corn, the data are varied and often contradictory. For example, these bacteria do not attach to leaf protoplasts or leaf fragments derived from 7- to 21-day-old plants (15). However, they did attach to exposed tissues from 4-day-old seedlings. And while fewer of them attach to corn protoplasts from cell cultures than to carrot protoplasts (16), they do attach abundantly to the vascular bundles in the seedlings. It is interesting that in *Zea mays*, the vascular bundles of the very young internodes are the tissues which regenerate when wounded (1); they are also the tissues to which the bacteria adhere.

Parenchyma cells in some monocots lose the ability to differentiate at a very early stage in their development (22). Given these data, it is possible that the cells of some monocots (such as members of the family Gramineae) differentiate rapidly and lose the ability to respond readily to *A. tumefaciens* infections. Thus, at any particular time, it is possible that only a few cells in a wound are in the competent stage and that, if all are transformed, the cells might not be of sufficient number to effect tumor formation. Should the cells to which the bacteria are capable of adhering pass through the competent stage and become senescent, their binding ability may be lost. In this case, their transformation efficiency would be reduced significantly, particularly if T-DNA transfer to individual cells is contingent on the attachment of a population of bacteria to cell masses of a critical size.

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LITERATURE CITED

1. **Aloni, R., and T. Plotkin.** 1985. Wound-induced and naturally occurring regenerative differentiation of xylem in *Zea mays* L. Planta (Berl) **163**:126–132.
2. **Anand, V. K., and G. T. Heberlein.** 1977. Crown gall tumorigenesis in potato tumor tissue. Am. J. Bot. **64**:153–158.
3. **Bouckaert-Urban, A.-M., G. Brouwers, L. Thoelen, and J. C. Vendrig.** 1982. Influence of a crown-gall tumor initiation enhancer on bacterial attachment to the host plant cell wall. Planta **156**:364–368.
4. **Braun, A. C.** 1975. The cell cycle and tumorigenesis in plants, p. 177–196. *In* J. Reinert and H. Holzer (ed.), Cell cycle and cell differentiation. Results and problems in cell differentiation, vol. 7. Springer-Verlag, New York.
5. **DeCleene, M., and J. DeLey.** 1976. The host range of crown gall. Bot. Rev. **42**:389–466.
6. **Draper, J., I. A. Mackenzie, M. R. Davey, and J. P. Freeman.** 1983. Attachment of *Agrobacterium tumefaciens* to mechanically isolated *Asparagus* cells. Plant Sci. Lett. **29**:227–236.
7. **Graves, A. C. F., and S. L. Goldman.** 1986. The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*: detection of T-DNA specific enzyme activities. Plant Mol. Biol. **7**: 43–50.
8. **Graves, A. C. F., and S. L. Goldman.** 1987. *Agrobacterium tumefaciens*-mediated transformation of the monocot genus *Gladiolus*: detection of expression of T-DNA-encoded genes. J. Bacteriol. **169**:1745–1746.
9. **Hernalsteens, J. P., L. Thia-Toong, J. Schell, and M. van Montagu.** 1984. An *Agrobacterium*-transformed cell culture from the monocot *Asparagus officinalis*. EMBO J. **3**:3039–3042.
10. **Hooykaas van Slogteren, G. M. S., P. J. J. Hooykaas, and R. A. Schilperoort.** 1984. Expression of Ti-plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature (London) **311**:763–764.
11. **Lipetz, J.** 1966. Crown gall tumorigenesis. II. Relations between wound healing and the tumorigenic response. Cancer Res. **26**: 1597–1605.
12. **Lippincott, B. B., and J. A. Lippincott.** 1969. Bacterial attachment to a specific wound site as an essential stage in tumor initiation by *Agrobacterium tumefaciens*. J. Bacteriol. **97**:620–628.
13. **Lippincott, B. B., M. H. Whatley, and J. A. Lippincott.** 1977. Tumor induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. Plant Physiol. **59**:388–390.
14. **Lippincott, J. A., and B. B. Lippincott.** 1976. Nature and

FIG. 3. Scanning electron photomicrographs of attachments of *A. tumefaciens* A66 to intact wheat seedling tissues and strain T37 to carrot disk and potato disk tissues. (A) Cross-section of 4-day-old wheat seedling mesocotyl. (B) Detailed enlargement of bacteria attached to tissue in area labeled B in panel A. Arrows indicate strands running to plant cells. (C) Bacteria attached to carrot disk vascular tissue. Enlargement of carrot disk from which most of the bacteria had been removed, showing detail of bacterial attachment to plant cell. (D) Masses of bacteria attached to surface of potato disk. (E) Detailed enlargement of potato disk, showing bacterial attachment to potato cell. Arrows indicate strands that attach bacteria to the plant cell wall.

specificity of the bacterium-host attachment in *Agrobacterium* infection, p. 438–451. *In* B. Soldhein and J. Raa (ed.), Cell wall biochemistry related to specificity in host-plant pathogen interactions. Universitetsforlaget, Oslo.
15. **Lippincott, J. A., and B. B. Lippincott.** 1978. Cell walls of crown-gall tumors and embryonic plant tissues lack *Agrobacterium* adherence sites. Science **199**:1075–1078.
16. **Matthysse, A. G., and R. H. G. Gurlitz.** 1982. Plant cell range for attachment of *Agrobacterium tumefaciens* to tissue culture cells. Physiol. Plant Pathol. **21**:381–387.
17. **Matthysse, A. G., K. V. Holmes, and R. H. G. Gurlitz.** 1982. Binding of *Agrobacterium tumefaciens* to carrot protoplasts. Physiol. Plant Pathol. **20**:27–33.
18. **Murashige, T., and F. Skoog.** 1962. A revised medium for rapid growth and bio-assays with tobacco cultures. Physiol. Plant. **15**:473–497.
19. **Nester, E. W., M. P. Gordon, R. M. Amasino, and M. F. Yanofsky.** 1984. Crown gall: a molecular physiological analysis. Annu. Rev. Plant Physiol. **35**:378–413.
20. **Ohyama, K., L. E. Pelcher, and A. Schaefer.** 1979. *In vitro* binding of *Agrobacterium tumefaciens* to plant cells from suspension culture. Plant Physiol. **63**:382–387.
21. **Schafer, W., A. Gorz, and G. Kahl.** 1987. T-DNA integration and expression in a monocot crop plant after induction of *Agrobacterium*. Nature (London) **327**:529–532.
22. **Swamy, B. G. L., and D. Sivaramakrishna.** 1972. Wound healing responses in monocotyledons. I. Responses *in vivo*. Phytomorphology **22**:305–314.