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INTERACTION OF *AGROBACTERIUM VITIS* WITH GRAPEVINE ROOTSTOCKS

BY

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ABSTRACT

Interaction of *Agrobacterium vitis* with grapevine rootstocks. - *Agrobacterium vitis* is a major grape pathogen causing gall formation on grape trunks at or above graft unions and grape-specific necrosis on roots. Recently, *A. vitis* strains from Galicia, in the North-West of Spain, have been described. The crown gall disease in Galicia has not been adequately studied although the region has become a successful viticultural region. Crown gall susceptibility of grape appears to be determined by genetic make-up of the plant and pathogen although it is not known which steps in cycle of disease induce the resistance. Among the strains from Galician vineyards tested in assays with *V. vinifera* cultivars, no difference in attachment was found.

Key-words: Electron microscopy, *Agrobacterium vitis*, grapevine rootstock, attachment, crown gall, Galicia, Spain.

INTRODUCTION

Crown-gall tumor induction by *Agrobacterium* sp. requires a specific association between the bacteria and plant wound sites (LIPPINCOT & LIPPINCOTT, 1969). Although it is known that certain *A. tumefaciens* biovar 1 and *A. rhizogenes* strains can cause grapevine crown-gall, *A. vitis* is considered to be the main causal agent (BURR & KATZ, 1983; KERR & PANAGOPOULOS, 1977). *A. vitis* group is distinguished from other *Agrobacterium* species by differences in DNA homology and metabolic characteristics (OPHEL & KERR, 1990). Among the potential *A. vitis* host range factors, two have been studied in considerable detail: the polygalacturonase associated with grape tissue necrosis (BURR *et al.*, 1987a,b; MCGUIRE *et al.*, 1991; RODRIGUEZ-PALENZUELA *et al.*, 1991) and the tartrate degradation that provides a selective advantage for grape colonization (RUFFNER, 1982; SALOMONE *et al.*, 1998).

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Infection of plants by *Agrobacterium* is a multistage process (ZUPAN & ZAMBRINSKY, 1995, 1997). It represents the only known example of DNA transfer from the bacteria to plants in nature. Not all steps of the infection process for *A. vitis* have been identified, although many are known to be very similar to those of *A. tumefaciens*. The first step is chemotactic attraction of the bacteria towards wounded plant cells and attachment (BRISSET *et al.*, 1991; BROEK & VANDERLEYDEN, 1995). In grapes, freeze injuries often provide the wounded cells. Certain phenolics and other compounds released by wounded cells induce the vir genes of *Agrobacterium*. Attachment of bacteria to plant cells is really a two-step process (MATTHYSSE, 1983; PU & GOODMAN, 1993). First step *Agrobacterium* adheres to the plant cell surface as a single cell, subsequently in response to plant factors, the bacteria elaborate cellulose fibrils that entrap other bacteria resulting in the formation of bacterial aggregates (MATTHYSSE *et al.*, 1981). These fibrils also allow the bacteria to bind very tightly to the plant cell surface. The transfer of plasmid T-DNA and incorporation in plant chromosome is the next step followed by the activation of plant-gall inducing genes (oncogenes) on the T-DNA and rapid multiplication of plant-cells. *A. vitis* can also enter in the plant through root scions and systemically infest the whole plant (LEHOCZKY, 1968; BURR & KATZ, 1984; STELLMACH, 1990; STOVER *et al.*, 1997), in some cases even when no crown gall symptoms are evident (BURR & KATZ, 1983; TARBAH & GOODMAN, 1987).

Different *Vitis* sp. often respond differently to infection by *A. vitis* (STOVER *et al.*, 1997; SÜLE *et al.*, 1994). Avirulence of the bacterium or resistance of the plant may be due to a failure of some steps described earlier, in addition to field conditions offering different attachment ability responses. In this study, we have tried to determine whether attachment is a factor restricting the ability of *A. vitis* to infect *Vitis* genotypes.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

We have used 194-628v and 266-271v *A. vitis* strains, both isolated from Galician vineyards (LASTRA, 1998), and NCPBB3554 from Spanish collection of type strains (IVIA, Valence, Spain). They were stored in distilled water containing 10% Skim Milk and 5% glycerol at -80°C. Bacteria were grown on yeast-extract-peptone-agar (PA) medium (0.5% bactopeptone, 0.3% yeast extract, 11.49 ml glycerol 87% and 2% agar) containing 1 mM cycloheximide (Actidione, Fluka Biochemica, Switzerland).

Plant material

Three representative rootstocks from Galician vineyards: Albariño, Godello and Mencía, and grape cuttings from Viticulture and Enology Station of Leiro (Ourense, Galicia) where *A. vitis* is not detected were used. They were cleaned in a water bath at 50°C in order to eliminate the endophytic flora and then sprayed with fungicide. The cutting were stored at 4°C for 10 days to allow the root formation and then planted in pots containing a mixture of 75% soil and 25% vermiculite. The plants were maintained

in nurseries with controlled conditions of temperature (25°C) and humidity. When the new shoots were 10-15 cm long, the plants were wounded in roots, shoots and stem with a sterile lance and inoculated with 20 ml of a bacterial suspension containing 10^8-10^9 cfu/ml into each wound.

Immunoblots and dot-immunobinding

Bacterial suspensions used for inoculations were tested with monoclonal antibody Ab F21-1D3G7C8 against *Agrobacterium vitis* (BISHOP *et al.*, 1989) kindly provided by Thomas Burr (N.Y. State Agricultural Experiment Station, Cornell University, Geneva, USA). Suspensions were spotted on a nitrocellulose membrane, fixed with 10% acetic acid and 25% ethanol, rinsed with distilled water and blocked with PBST buffer (10 mM $NaPO_4$, 4.5% NaCl, 0.5% Tween and 5% Bovine Serum Albumin). The membrane was then incubated for 1 h at room temperature in *A. vitis* specific monoclonal antibody diluted to 1 µg/µl in PBST. After 3 washes in PBST buffer without BSA the membrane was incubated for 1 h with peroxidase labeled anti-mouse IGg (Amersham Pharmacia Biotech). Following 4 washes, peroxidase activity was assessed with 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Fluka Chemie, Buchs, Switzerland) in 100 mM Tris-HCl, pH 7.5 containing 0.03% H_2O_2.

Electron microscopy

Cells from *A. vitis* suspension were deposited with a toothpick on a drop of water. Formvar (0.5%)-coated 75-mesh grids were placed on top of the drop for 15 to 20 seconds to allow the adhesion of bacterial cells. Grids were then stained for 20 to 30 s with a freshly prepared 1% solution of potassium phosphotungstate (pH 7.0) and washed twice for 10 s in a drop of water. The grid was air dried and examined on a Zeiss EM10 electron microscope.

Scanning electron microscopy of bacteria on Vitis sp.

Segments from different parts of grape plant were surface-sterilized with 50% clorox for 10 min, rinsed with sterile distilled water, cut into 0.5 cm segments and incubated in 4 ml solution of 10^8-10^9 cfu/ml. The suspension was prepared in phosphate buffer 10 mM (KH_2PO_4 and $Na_2HPO_4.2H_2O$) pH 6 to avoid osmotic alteration. Samples were removed at 15 min, 30 min, 2 and 6 hours, rinsed in phosphate buffer and prepared for electron microscopy. Segments were fixed in Sörensen buffer containing paraformaldehyde 2% and glutaraldehyde 1.5% for 1 hour at 20°C and then 3 hours at 4°C, post-fixed with 1% OsO_4 in the same buffer for 1 hour at room temperature and then dehydrated in ethanol. Specimens were critical-point dried, sputter-coated with gold and then viewed on a Zeiss 940A SEM. Controls were performed by inoculating the grape segments and tomato as a host plant with distilled water and *Escherichia coli*.

RESULTS AND DISCUSSION

The *in vitro* assessment of sensitivity of the three green-house-grown rootstocks, Mencía, Godello and Albariño revealed that Albariño is more sensitive to tumor development following artificial inoculation with *A. vitis* (Fig. 1a,b). A basal crown gall of 6-7 cm^2 diameter was detected in Albariño rootstock (Fig. 1b) and the plant vigor

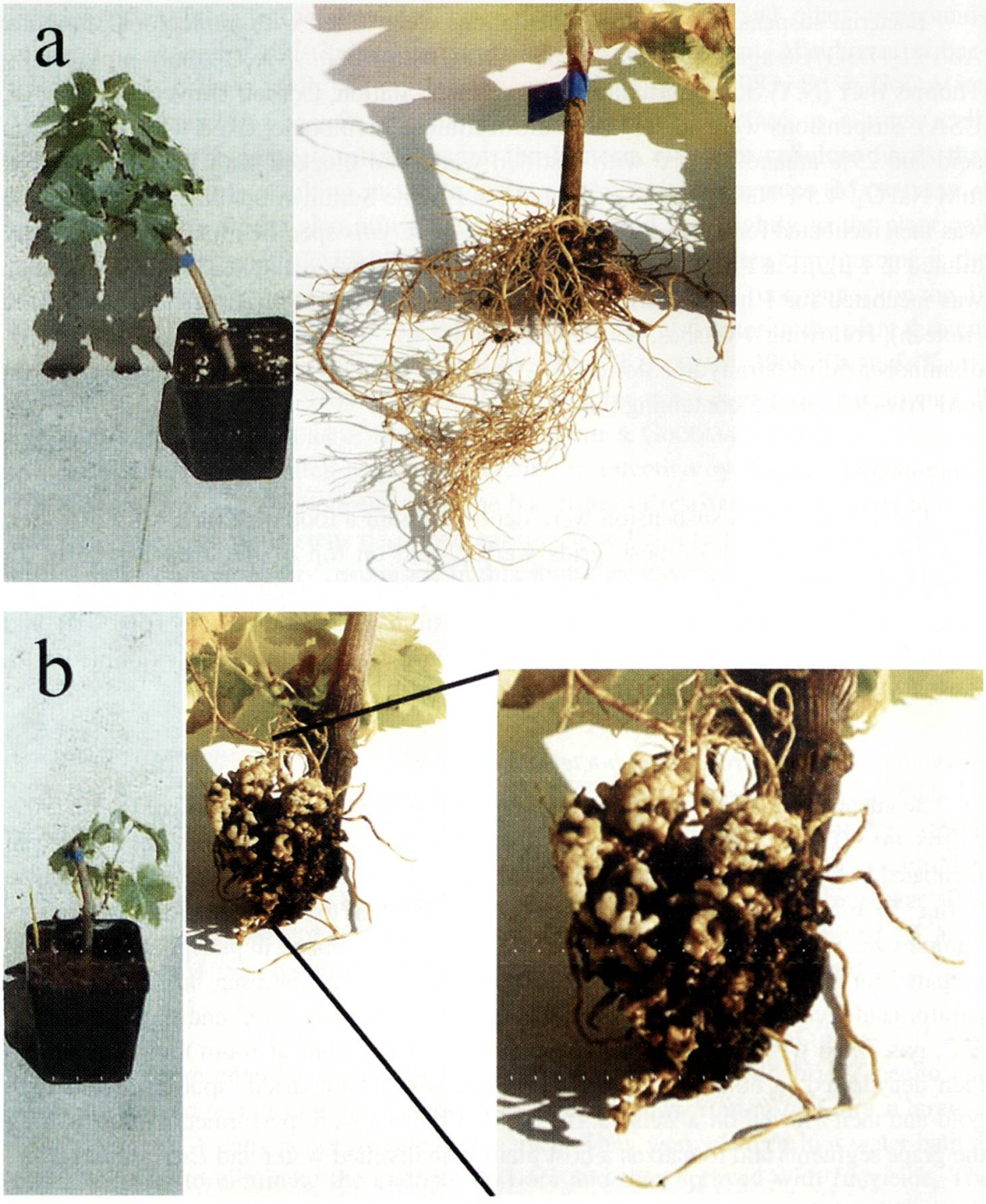

FIG. 1.
Effect of inoculations of *A. vitis* in Albariño rootstoock. (a) Control; (b) Crown gall in basal trunk.

was considerably reduced (Fig.1a,b). When dilacerate of these tumor was plated in the ROY & SASSER medium (1983), colonies with *A. vitis* morphology were found. In infected plant the root system was found to be poorly developed (Fig. 1) maybe due to an early induction of root decay. It has been reported that *A. vitis* incites decay of grape roots (BURR *et al.*, 1987a; MCGUIRE *et al.*, 1991) which is related to specific activation of polygalacturonase production. This specific induction of polygalacturonase has not been described in other Agrobacteria. No tumors were detected in the other rootstocks.

Although adequate microscopy techniques are not available to perform a quantitative determination of the differences in attachment ability, nevertheless it is possible to evaluate the morphology and characteristics of cell to cell contact. We can also approximately estimate changes in interaction between the bacteria and cell surface. *A. vitis* cells did attach to exposed cut surface of the three rootstocks tested. The distribution of bacterial cells and binding efficiency showed no apparent difference. This non host-specific binding has been reported for *A. tumefaciens* since binding to suspension of cultured cells of non susceptible crown-gall monocot plants such as asparagus, wheat, corn and bamboo have been reported (GRAVES *et al.*, 1988). The basis of the increased sensitivity of Albariño is not known but we observed that the first step of infection is not determining. Avirulence of the bacterium or resistance of the plant can be caused by either the lack of T-DNA transfer or a failure of some subsequent step leading to symptom formation.

Microscopic studies showed varying degrees of attachment, with bacteria attaching individually early and in cluster after longer incubation (Figs 2,3) The basis of clustering of bacterial cells to the plant cell surface is believed to be due to cellulose fibrils produced by *Agrobacterium* (MATTHYSSE, 1983). These fibrils initially anchor the bacteria to the plant surface (Fig. 2b) and then entrap additional bacteria, resulting in the formation or large bacterial clusters (Fig. 2d, Fig. 3b) held close to the plant cell wall and plasma membrane by these fibrils. In control inoculations of *A. vitis* in tomato plants we could observe the same attachment characteristics. The attachment was not observed when we inoculated with *Escherichia coli*. Electron and scanning microscopic analyses confirmed the morphology of *A. vitis* as expected (Fig. 4).

We conclude from these data that wound-exposed portions of host vine cell walls constitute the natural attachment site. The apparent attachment of *A. vitis* to cell walls which occurs in all combinations of host/pathogen suggests that attachment *per se* is not sufficient for tumor initiation. Further research is needed to understand pathogen biology of *A. vitis* and answer the question of how do nontumorogenic strains differ from tumorogenic strains. This information will not only be of academic interest but may provide useful information for developing novel disease control strategies.

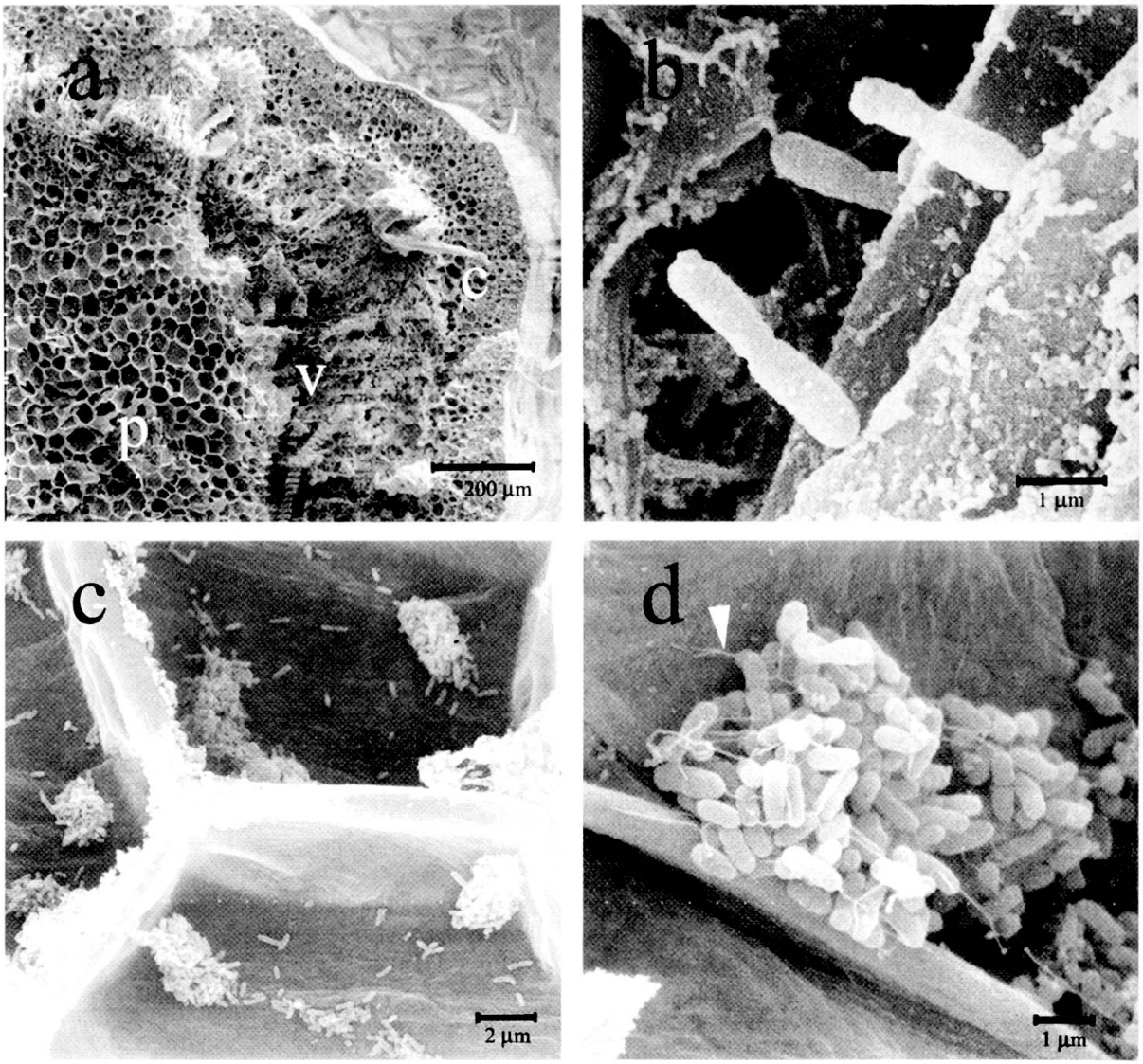

FIG. 2.

Scanning electron photomicrographs of attachment of *A. vitis* cells to stem of different vine rootstocks. a) Cross-section of *Vitis* stem (c: cortex; v: vascular bundle; p: pith); b) Individual attachment of bacteria to plant cell after 15 min of incubation (Mencía x 266-271v); c) Clusters formed 30 min after inoculation (Albariño x 194-628v); d) Detailed enlargement of clusters showing cellulose strands maintaining the bacterial cluster and attachment to plant cell, two hours after inoculation (Mencía x 194-628v). Arrows indicate bacterial attachment strands extending to cells.

INTERACTION D'*AGROBACTERIUM VITIS* AVEC DIFFÉRENTS CÉPAGES DE LA VIGNE

Agrobacterium vitis, un de plus importants agents pathogènes de la vigne, provoque la formation de tumeurs (galles) et des nécroses spécifiques au niveau des racines. Récemment, des souches de *A. vitis* provenant de Galicie, au Nord-Ouest de l'Espagne, ont été décrites. Bien que cette région possède une tradition viticole et pro-

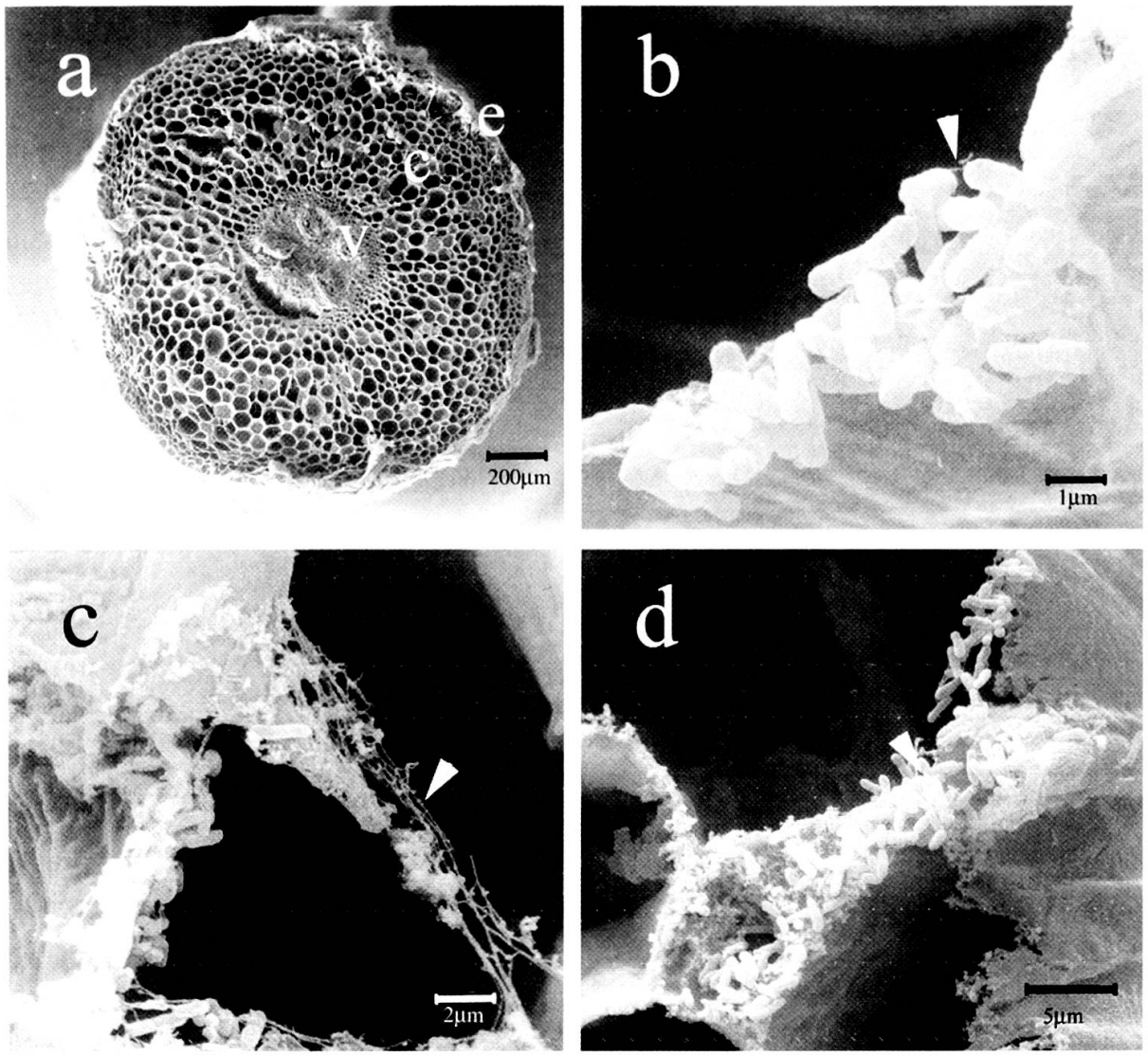

FIG. 3.

Scanning electron photomicrographs of attachment of *A. vitis* cells to roots of different grapevines rootstocks. a) Cross-section of *Vitis* roots (e: epidermis; c: cortex; v : vascular bundle); b) Clusters formed after 30 min of bacterial inoculation (Albariño x 266-271v); c) Fibrilar attachments between *A. vitis* and plant cells after 2 hours of inoculation (Mencía x 194-628v); d) Masses of bacteria attached to wounded surface of roots plant cell (Godello x 194-628v). Arrows indicate bacterial attachment strands extending to cells.

duise une grande quantité de vin, cette maladie de la vigne n'a pas suffisamment été étudiée. La susceptibilité à cette infection paraît être déterminée génétiquement, mais on ignore à quelle étape du cycle infectieux le pathogène induit la résistance. Le simple attachement de *A. vitis* sur les différentes parties étudiées du cep ne semble pas suffisant pour déclencher la formation de tumeurs.

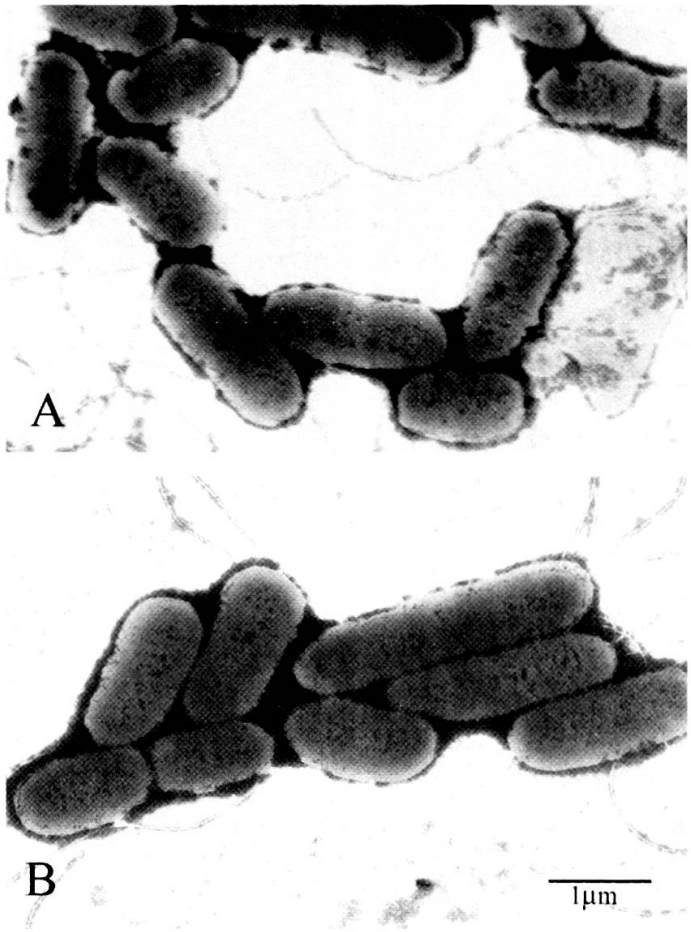

FIG. 4.
Electron micrograph of negatively stained *A. vitis* strains (A) 266-271v and (B)194-628v.

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