

# Root Hair Cell Enhancement in Tissue Cultures from Soybean Roots: A Useful Model System

IN VITRO RHIZOBIUM SYMBIOSIS<sup>1, 2</sup>

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## ABSTRACT

A technique for obtaining large numbers of root hair cells in cell cultures from soybeans is described. The cells were grown on agar containing the Prairie Regional Laboratory B5 (PRL-B5) medium for periods longer than 60 days. Mixed populations of cultured root hair cells and cortical cells were used to study the *in vitro* association between soybean cells and *Rhizobium japonicum*. The advantages of these types of root cell cultures in studies of symbiosis are discussed.

Although cultures from soybeans and peas have been described in the literature (1, 4), the differentiation of such cultures to give cells which yield root hairs has not been documented. We wish to describe techniques yielding relatively large numbers of root hairs in cultures from soybean roots. The cultures enriched with root hair cells were obtained during our studies on the association between *Rhizobia* and soybean cells. In the present communication, we extend our previous observations on the *in vitro* association between *R. japonicum* and cultured soybean cells (13, 16) and indicate some advantages of using cultures enriched in cells with root hairs, for studies of *in vitro* symbiosis. Plant cultures enriched in cells with root hairs may also prove of use in other *in vitro* studies of root cell physiology.

## MATERIALS AND METHODS

Primary cultures of *Glycine max.* (L.) Merr. Harosoy and Acme were used. These cultures were obtained as follows. Seeds were germinated in the dark aseptically for 3 days at 26 C and pieces of the hypocotyls 3 to 4 mm long were removed. The hypocotyl tips were placed on the surface of 1% agar with PRL-B5 medium containing 1  $\mu\text{M}$  2,40D (5). After 15 days, epidermal and cortical tissue was stripped off with a pair of fine forceps, and the stele was discarded. The epidermal and cortical tissue masses were cut into fine pieces with iris scissors. The cells were then passed through a 200- $\mu\text{m}$  nylon mesh screen and placed on 1% agar containing B5 medium with 1  $\mu\text{M}$  2,4-D and 1  $\mu\text{M}$  kinetin. After 2 weeks, the cells were resuspended in PRL-B5, centrifuged, and replaced on fresh agar medium with B5, but devoid of added hormones. This manipulation was apparently enough to bring about the desired type of differentiation, namely, enrichment of root hair cells.

These cultures could be left on agar for up to 2 months. Alternatively, cells were placed in suspension in the same medium in Erlenmeyer flasks and shaken at 100 rpm in a model G2L environmental incubator gyrotory shaker (New Brunswick Scientific). All cultures were incubated at 26 C in the dark.

The *Rhizobia* studied in association with cultured soy cells were obtained from USDA Laboratories at Beltsville, Md. or the Nitragin Co. (Milwaukee, Wis). The *R. japonicum* (Kirchner) Buchanan strains used were USDA R138 and 61A76. These were usually maintained on agar slants (17) and then grown in suspension cultures as needed, using the medium of Valera and Alexander (17). We also used B5 medium with 1 mM KNO<sub>3</sub> and 2 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to maintain the *Rhizobia*. *Rhizobia* in pure culture grew poorly in this low nitrate version of the B5 medium. (Unpublished observations in our own and other laboratories show that pure cultures of both of these strains of *Rhizobia* can also reduce acetylene at substantially low rates in different suspension media.)

When the *Rhizobia* were maintained in association with soybean cells in the low nitrate version of the B5 medium, they multiplied rapidly and healthy symbiotic cultures were obtained in suspension. These cells were termed symbiotic because the cultures could reduce acetylene to ethylene after washing exhaustively with 300 to 400 ml of B5 medium without nitrate but with 2 mM ammonium sulfate (pH 6). We have now substituted this with ammonium succinate. The washing was performed by placing the dispersed plant cells on 60- $\mu\text{m}$  nylon mesh screens so that not only extraneous bacteria but even small plant cells were removed. Soybean cell clumps infected with *R. japonicum* did not exceed 20 mg after drying and weighing. The weights were obtained after carrying out acetylene reduction experiments on the washed cells. The cells were washed with B5 medium salts and with extra KCl for osmolarity. It was necessary to add extra substrates for carrying out acetylene reduction on washed cells.

The washed plant cells had many bacteria within the plant cell cytoplasm, but few, if any, attached to the plant cell walls as indicated by examinations with a phase contrast microscope. These observations were from cells kept in suspension with *R. japonicum* from 4 to 5 weeks. The attachment of *Rhizobia* to the outside of plant cell walls was observed only during the first 48 hr of mixing the cultures. After this period, plant cells were invaded, often in a destructive manner. However, as indicated below, symbiosis can be achieved in large numbers of cells in culture.

We also used our cultures for studying attachment of *Rhizobia* to different types of soybean root cells. Inspection of the live cultures for this purpose was facilitated by using Zeiss dark field optics.

Inspections of stained sections were also made. Fixation and staining of sectioned material often added uncertainties for examination of external bacterial attachment as well as internal

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cytoplasmic profiles of the plant cell vesicles. In our opinion, live preparations were better suited for such examinations. Examinations with interference contrast optics (Zeiss) allowed rapid evaluation of the cultures for possible use in acetylene reduction experiments.

## RESULTS

Root cell and root hair cell proliferation was obtained when cells were placed in medium which was lacking 2,4-D and kinetin. These cells were then maintained in culture for long periods of time when returned to medium containing  $1 \mu\text{M}$  each of kinetin and 2,4-D. The combination of higher 2,4-D or kinetin interfered with expression of root hair cell morphology in the root cell culture. Figure 1a is a phase contrast photograph of cells started as indicated under "Materials and Methods" and then suspended in B5 medium without the 2,4-D. The suspension culture was 2 days old, and the "hair" appendage is quite short (arrow) in the young root hair cell. The major criterion for identification of root hair cells is that the main cell body is kidney-shaped, with a single, narrow appendage or "hair," on the concave side. The cell in Figure 1a also shows clumps of *Rhizobia* attached to one side of the main cell body.

Root hair cells could be obtained more easily from cultures derived from *Glycine max* variety Acme than from cultures derived from variety Harosoy. Figure 1, b through f, was made with dark field illumination. In Figure 1b, a cluster of 3-week-old cells is indicated with a few cells showing the early morphology of root hair cells. Figure 1b also illustrates that suspension cultures with root hair cells can be used for studying the initiation of bacterial attachment. This dark field photograph shows a plant culture which has been infected with *R. japonicum* R138 (USDA 3I-1b-138), 3 hr previously. The walls of the plant cells and the attached bacteria on the exterior of these cells are clearly outlined as shown in the magnified inset. It should be noted that bacteria neither attach exclusively to root hair cells, nor attach to all root hair cells. This phenomenon has also been noted *in vivo* (3).

Root hair curling was not apparent for soybean cell cultures within this time. Some curling was noted after longer periods of association as indicated below. When plant cells were cultured together with *R. japonicum* for longer periods of time, longer appendages were observed (Fig. 1c). Such long root hair appendages have not been noted in cultures without added *Rhizobia* and containing the modified PRL-B5 medium. It should be noted that these preparations were from suspension cultures. The preparations were also low in nitrate since the plant cells were resuspended in the low nitrate B5 medium. This was done to encourage infection of the plant cells by *Rhizobia* and to give derepression of rhizobial nitrogenase. The root hair cells formed large rows (Fig. 1c), and in the majority of our preparations, such rows contained appendages all oriented in one particular direction. An enlargement made from the same negative as Figure 1c shows root hair appendages more clearly (Fig. 1d). This figure shows presumed infection thread material in the

appendage of the root hair cells, as seen with dark field optics. Round cortical cells to the back of the root hair cells were also seen (Fig. 1, c-f) and these cells contained *Rhizobia*. A morphological criterion is difficult for these cells, other than the observation that these were next to distinctly epithelial cells, they were round and were invaded. On the other hand, many root hair cells (Fig. 1d) appeared not to contain infection threads or *Rhizobia*. The few root hair cells which have infection threads in Figure 1, c and d are indicated by arrows. Cells in the cultures exposed to the bacteria for periods longer than 3 days had few *Rhizobia* attached to the exterior surfaces (Fig. 1, c-f).

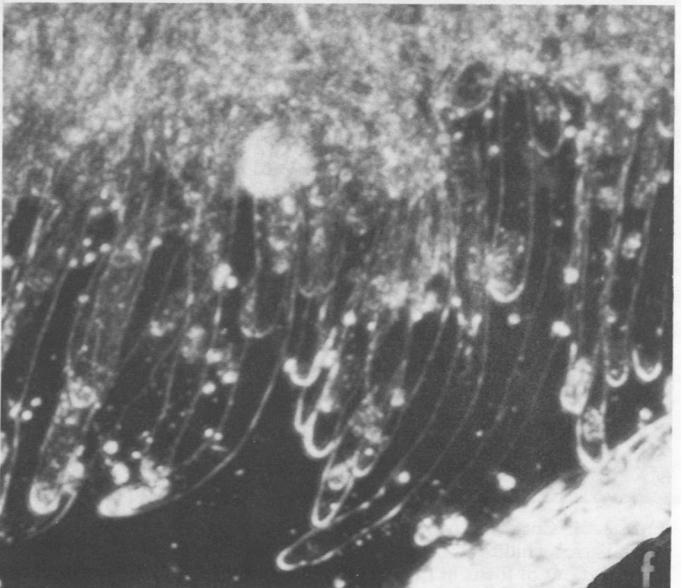
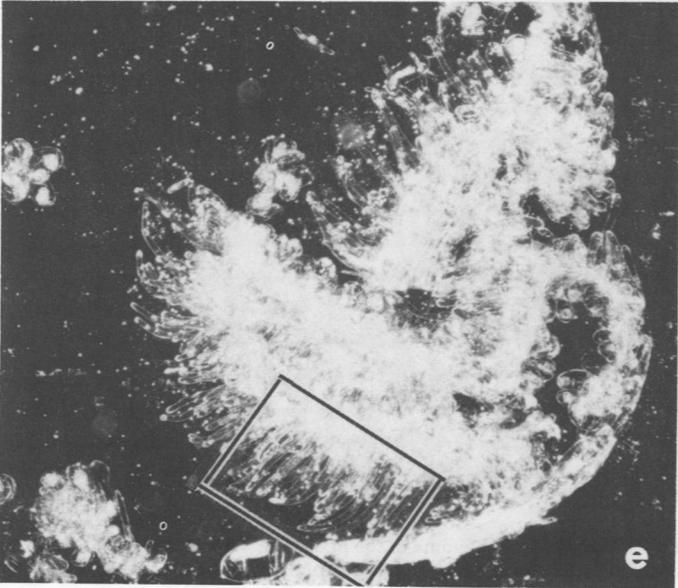
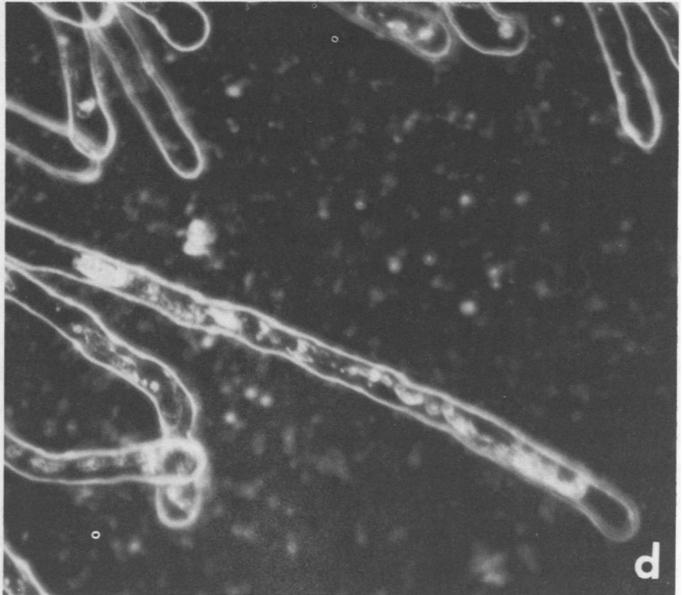
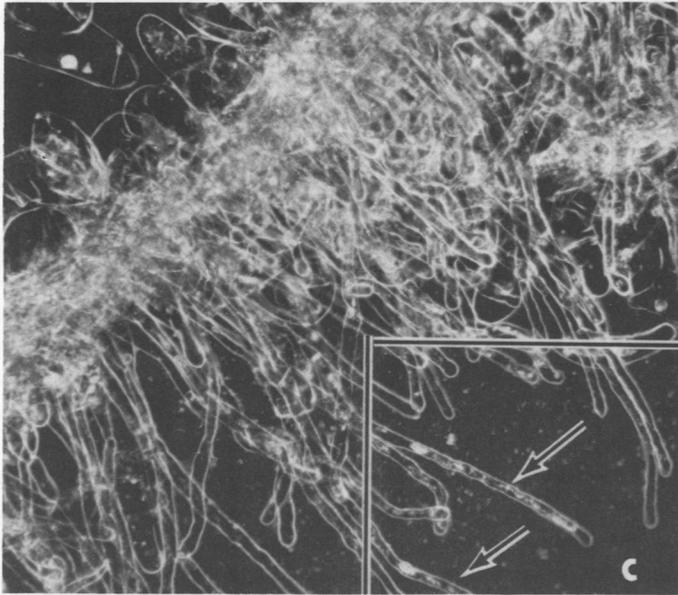
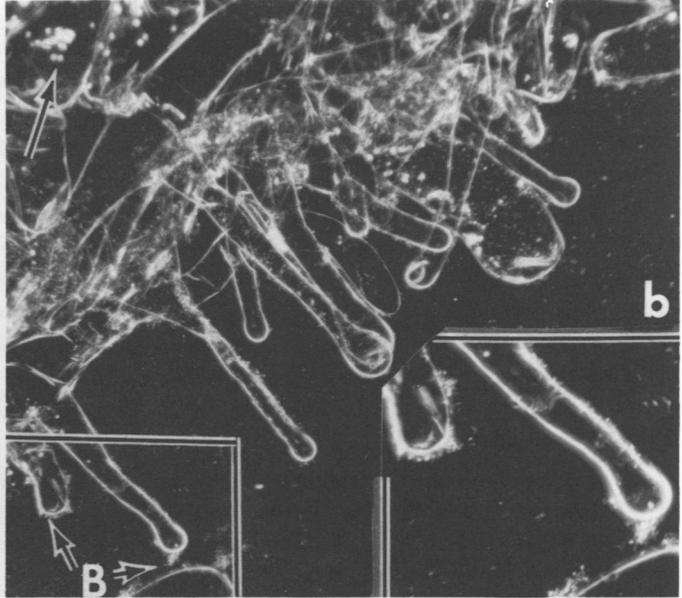
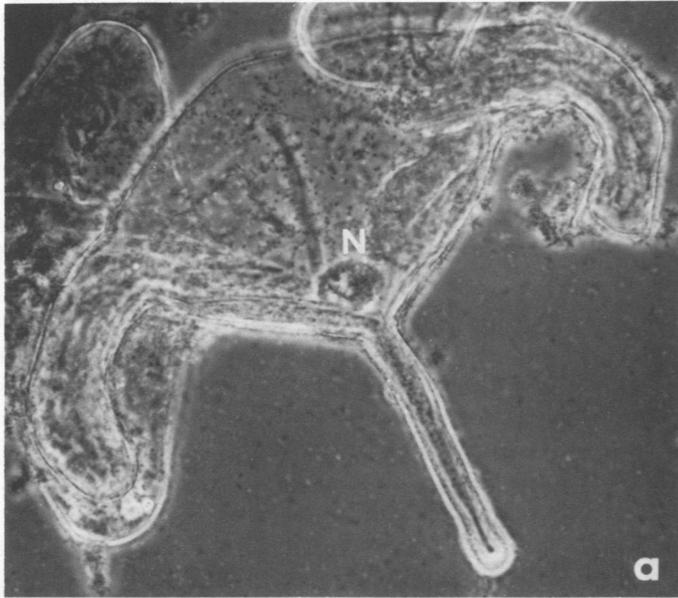
Figure 1, c and d illustrates the following. (a) Attraction of *Rhizobia* toward the plant cells, and the subsequent attachment of *Rhizobia* to the plant cell surfaces are distinct phenomena. (b) The actual infection of the plant cells in culture with initial destructive invasion, and the differentiation of some of the plant cells for symbiosis are separate events. Since each event is chronologically separated, the earlier phenomena (attraction and attachment of bacteria) can be studied independently of the latter (infection and differentiation). This chronology holds for both callus as well as suspension cultured soybean cells.

The last two illustrations of Figure 1 are from the *same* slide mount. The low power picture (Fig. 1e) shows the appearance of Harosoy cell clusters invaded by *R. japonicum* R138. These clusters were maintained on agar for 2 months prior to photography. Figure 1e indicates that many soybean cells can indeed be invaded by *R. japonicum*. Numerous root hair cells in the middle of the picture are devoid of bacteria. The bright bodies within the round cortical cells are structures formed by the invading *Rhizobia* in the plant cell cytoplasm. The dark field optics show these clearly in Figure 1e, and 1f (also see Fig. 2). Figure 1, e and f illustrate the fact that *Rhizobia* reside for long periods in cultured soybean root hair cells. An invaded, round cortical cell is seen in the bottom half of Figure 1e and in the center of Figure 1f, and is the brightest cell in the picture. The *R. japonicum* invade and then move within the cells immediately behind the root hair cells. The culture illustrated in Figure 1, e and f was that used to demonstrate acetylene reduction *in vitro* (Fig. 4).

A further examination of the cultures was made with interference contrast optics. Figure 2a is a light microscope picture taken at high magnification with interference contrast optics of a symbiotically differentiated cell. The cell walls can be seen clearly, and there are no *Rhizobia* attached on the outside of the cell wall. The cell membrane (M) is barely visible due to the shadow effect of the lighting. The peribacterial structures around the bacteria (BV) are seen in the parts of the cell which were in complete focus. Figure 2a illustrates the utility of interference contrast optics for monitoring tissue culture cells infected with *Rhizobia*. Figure 2b is a picture showing the senescence of this type of symbiotic differentiation. The bacteria emerging from the plant cell are morphologically distinct from the free *Rhizobia* in the background. This distinct morphology may be due to the peribacterial structures indicated as BV in Figure 2a, as well as inclusions accumulating within symbiotic bacteria.

In order to ensure minimal interference from external *Rhizo-*

FIG. 1. Morphology of soybean root cells grown under a variety of culture conditions. a: Appearance of young root cells differentiating in suspension culture; a root hair cell with appendage (A) and curved sides is shown in the center. The nucleus (N) is at the base of the root hair appendage. *Rhizobia* are attached on the root hair surface. Phase contrast,  $\times 800$ . b: Attachment of *R. japonicum* R138 4 hr after addition to soybean root cells which were in suspension culture for about 3.5 weeks; arrow at top left indicates aggregation of plant organelles. Arrows B, at bottom left, indicate attached bacteria. This portion is enlarged at the right. All of 15 distinguishable root hairs are oriented to one side (originally  $\times 280$ ). c: Culture of soybean root cells, 40 days after addition of *Rhizobia*; although *Rhizobia* are distinguished in the culture medium, very few show external attachment to root hairs. Opaqueness in the main cell mass is due to intracellular *Rhizobia*. Of the 80 root hairs that are distinguished, a small degree of curling is noticeable in only 10 to 12 and bacterial "threads" are visible in perhaps five.  $\times 120$ . d: Enlargement from the negative used in Figure 1c; note that few *Rhizobia* are attached to plant cells although many *Rhizobia* are present in the background. e: Extensively invaded mass of soybean cells after 60 days in culture with *Rhizobia*; note that only cortical cells behind the root hair cells show opaqueness due to symbiotic bacteria.  $\times 45$ . f: A portion of the cell mass indicated by the lines shown in Figure 1e was enlarged. Only four of 50 root hairs in the lumen appear to be curled.  $\times 170$ . Acetylene reduction from this culture is indicated in Figure 3.



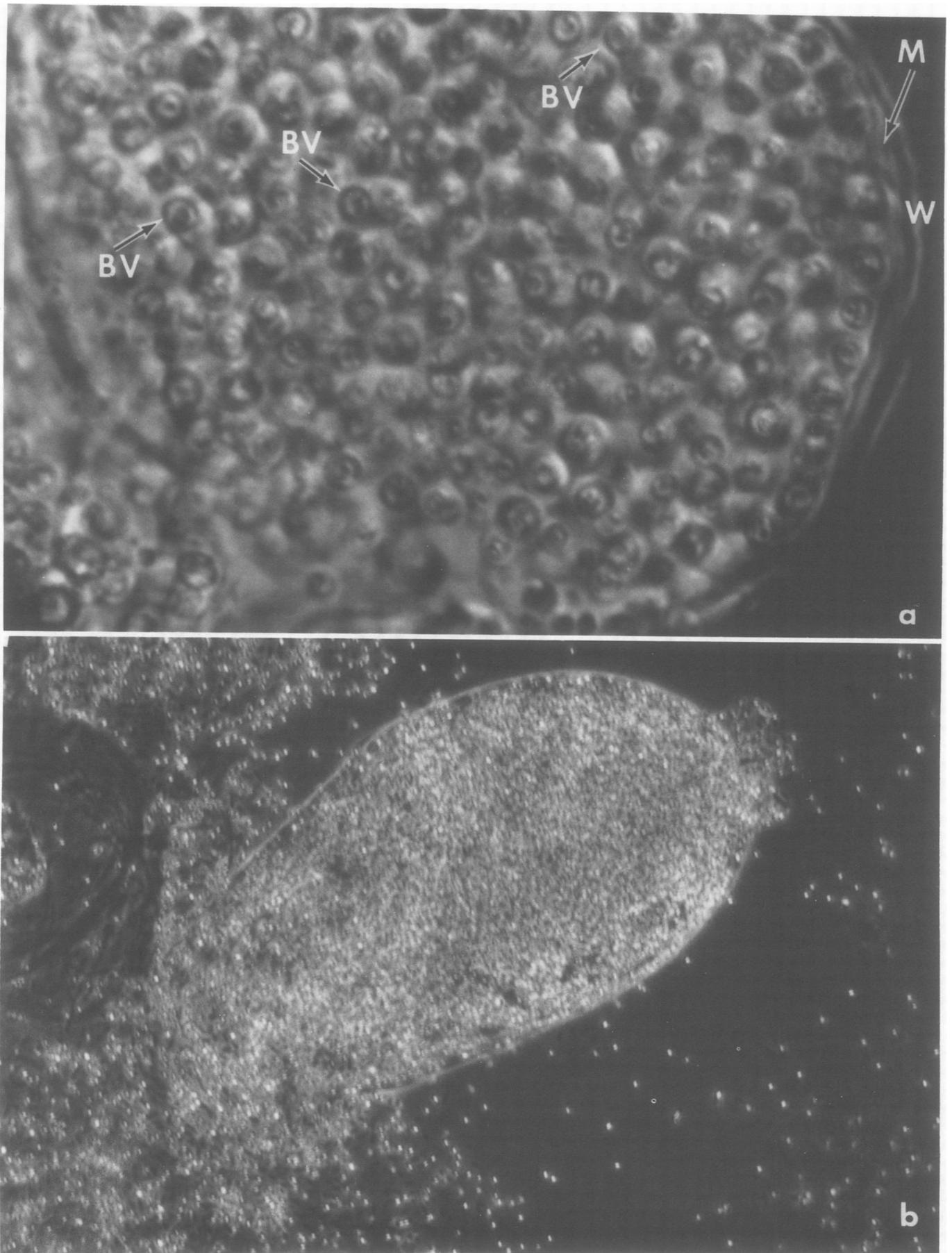


FIG. 2. a: Cortical soybean cell photographed with interference contrast optics to show peribacterial structures formed around intracellular bacteria; arrows indicate three peribacterial structure profiles (BV) which are in focus; M: plasmalemma of the plant cell; W: plant cell wall. Another plant cell is out of focus in the background.  $\times 1750$ . b: Senescent cortical cell after infection by *Rhizobia*; morphology of bacteria emerging from the plant cell is distinct from the morphology of free bacteria in the background. The free *Rhizobia* do not contain inclusions, and their entire profiles are dark. This phase contrast picture also shows another plant cell devoid of bacteria and with a prominent nucleus at the left (original at  $\times 600$ ). Both enlargements were made from Kodachrome transparencies.

bia in those cultures which had been invaded by the bacteria, the invaded plant cells were placed on nylon filters (60- $\mu$ m mesh) and copiously washed as detailed under "Materials and Methods." When the plant cell surfaces were free of *Rhizobia* as indicated by inspection with the light microscope at high power, the cells were sampled for acetylene reduction.

Figure 3 demonstrates acetylene reduction by the plant cell culture shown in Figure 1, e and f which had been invaded during a 2-month cultivation period with *Rhizobia*. The culture was placed in suspension and then subdivided into a number of vials which contained various carbon substrates. Each carbon substrate is indicated at optimal concentration. (Data obtained for other concentrations for each substrate have not been presented.) Each bottle contained 12 to 15 mg of plant cells, dry weight. Control vials containing fresh preparation of *Rhizobia* or plant cells alone did not show net reduction of acetylene in this period. Another control consisted of measuring acetylene reduction by *Rhizobia* collected from the washed cultures. The washings were centrifuged and the pellets resuspended in succinate medium. The acetylene reduction from the combined rhizobial pellet was a 100-fold lower than the control culture (C) of Figure 3. This combined pellet was resuspended in 2 ml of B5 with no nitrate medium, and assayed in similar vials to those used for the experiment of Figure 3. In contrast to those situations in which free *Rhizobia* have been induced to give nitrogenase activity in pure culture (4, 7, 9, 10), the infected plant cells started reducing acetylene as early as the vials were assayed. The acetylene reduction activity indicated in Figure 3 for pyruvate succinate,  $\alpha$ -ketoglutarate, and glutamate was linear from the start of the experiment to a period ending at 70 hr. There was no further increase in acetylene reduction with incubation up to 200 hr. The additions of various substrates were made because the cell suspensions with *Rhizobia* were washed copiously and then removed for assay. Thus, substrates were likely to be depleted. Pyruvate, succinate, and  $\alpha$ -ketoglutarate were better substrates than glutamate or malate.

The control cells (C) did not contain any substrate. They did, however, show linear acetylene reduction for 48 hr, and presumably the energy for this was supplied by endogenous substrate(s).

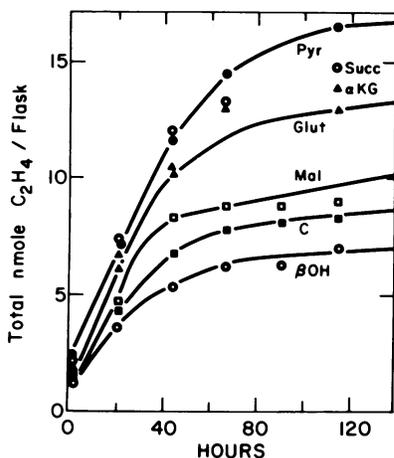


FIG. 3. Acetylene reduction by a symbiotic culture of soybean cells (var. Harosoy) and *Rhizobia* (*R. japonicum* R138). The preparations were from the culture illustrated in Figure 1, e and f. The preparations were washed and analyzed as described in the text. The gas phase consisted of 1.8% acetylene, 2.5% O<sub>2</sub>, and the remainder argon. Samples containing 12 to 15 mg of cells, dry weight, were used in 20-ml vials. Each vial also contained 0.1 ml of substrate at optimal concentrations as follows: pyruvate (Pyr), 10 mM; succinate (Succ), 25 mM;  $\alpha$ -ketoglutarate ( $\alpha$ KG), 25 mM; glutamate (Glut), 10 mM; malate (Mal), 25 mM;  $\beta$ -hydroxybutyrate ( $\beta$ OH), 50 mM; C: infected cells without added substrate.

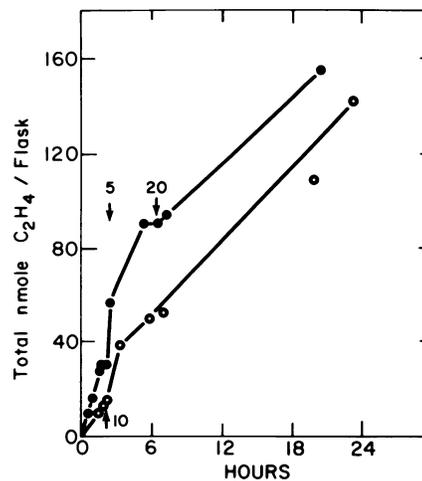


FIG. 4. Effect of changing O<sub>2</sub> concentration on acetylene reduction by washed, symbiotic cell cultures. Acme soybean cells were used with *R. japonicum* 61A76. Cells (15 mg dry weight) were added to duplicate flasks in one treatment (○—○). Vials in the second treatment (●—●) also received 15 mg of cells dry weight. Vials in both treatments were initiated with 2% O<sub>2</sub>, 1.8% acetylene, and the remainder argon. At 2.5 hr, pure O<sub>2</sub> was injected to a calculated concentration of 5% for one set of vials (●) and 10% for another set of vials (○). At 6.5 hr, the O<sub>2</sub> for the former vials was increased to 20%. Data points are the mean from duplicate vials. It should be noted that one set of vials (●) gave superior reduction rate with 2% O<sub>2</sub> (see text). These preparations were not as uniform as those used for the experiment of Figure 3.

The rate of acetylene reduction with  $\beta$ -hydroxybutyrate was below that of controls without substrate(s). Synergistic *R. japonicum* metabolize this substrate to give acetylene reduction (13). The poor acetylene reduction obtained with  $\beta$ -hydroxybutyrate cannot be easily explained at the present time. After infection, plant cells may not be permeable to this substrate.

The viability of the plant cells invaded by *Rhizobia* is illustrated further by the experiment shown in Figure 4. In this experiment, acetylene reduction was monitored after addition of different quantities of O<sub>2</sub> in the measuring vials. The combination of Acme plant cells and *R. japonicum* 61A76 was used. The experiment was conducted with plant cells which had been infected 50 days previously and kept on agar until the experiment was carried out. The agar culture was suspended in liquid medium and washed copiously with B5 medium to reduce the *Rhizobia* which had not invaded the cells. Duplicate vials were set up for each of two types of treatments containing various concentrations of O<sub>2</sub>. Fifteen mg of infected root cells were added to the vials. Both treatments were started with 2% O<sub>2</sub>, 1.8% acetylene, and the remainder argon. After 2.5 hr, a calculated 5% O<sub>2</sub> concentration was presented to one set of vials whereas 10% O<sub>2</sub> was added to another set. Acetylene reduction was followed over a period of 1 day (Fig. 4). The O<sub>2</sub> content of the vials containing 5% O<sub>2</sub> was increased to 20% after a period of 6 hr. Both sets of vials exhibited similar rates of acetylene reduction after 8 hr. (Two vials per set were used and results averaged.) One set of vials (●) gave superior acetylene reduction with 2% O<sub>2</sub> than the other (○). This may be due to better association between plant cells and bacteria in cell clusters sampled in the former case. The experiment showed that larger quantities of O<sub>2</sub> were needed when symbiotic association was made in culture by *Rhizobia* reducing the acetylene in association with plant cells. It was necessary to increase the O<sub>2</sub> concentration gradually and 10% O<sub>2</sub> was the final optimal concentration when these infected soybean cultures were not shaken during the assay. When free *Rhizobia* in pure culture reduced acetylene, very low quantities of O<sub>2</sub> were needed as illustrated with the cowpea *Rhizobium* 32H1 by Evans and Keister (4). In

this case, the cultures were shaken (4). In tests with synergetic culture, intermediate levels of O<sub>2</sub> were needed by *R. japonicum* strains 61A76 or R138 (14). When cultures of plant cells infected with *Rhizobia* are shaken at 90 rpm, acetylene reduction is not inhibited as in the case of free *Rhizobia* (4).

### DISCUSSION

We have shown that root hair cells can be grown and maintained in tissue culture. The runs of these cells, in *cis*-orientation (hair appendages oriented to one side only), as illustrated in Figure 1, c and d, can be picked out from culture dishes by fine forceps with the aid of a low power dissecting microscope and used for further study as needed. At this time, we do not know how this orientation occurred in our cultures. However, we have pictorial records of numerous times that such orientation has been noticed.

In studies of plant cell culture-rhizobial associations, actual infection and exhibition of bacterial threads in root hair cells occurred infrequently. There was evidence for curling of the hair cells in such cultures, but the number of curled hair cells was less than 2%. This may indicate that there is no directionality in suspension culture with respect to some unknown chemical gradients as might be formed *in vivo*. However, there might be other types of preformed chemical gradients which, even in uninfected root cells, give *cis*-oriented hair appendages. The *in vitro* infection of *Trifolium pratense* cultures grown on agar, it is contended, is associated with root hair curling by *Rhizobium trifolii* (12).

The attachment of *Rhizobia* to the various cell types in culture, however, can be studied with some success (15). An assay method has been developed, but not published, for estimating early attachment and binding of *Rhizobia* to soybean cells in culture using radioactive isotopes for labeling nucleic acids with tritiated nucleotides. Rapid turnover problems and interspecific exchange can be avoided by this tactic.

Apparently, the *in vitro* system of studying symbiotic nitrogen fixation has been difficult to repeat (2, 10) since it was first reported by Holsten *et al.* (6). The main difficulty has been to obtain acetylene reduction from suspension cultures in which symbiotic rhizobial invasion could be easily demonstrated. The culturing of soybean cells on agar and acetylene reduction by *Rhizobia* added to the undifferentiated callus have been reported by a number of workers (1, 2, 10, 12). No studies in the cell biology of these associations have been published. In this present context, we have emphasized the cell biology of tissue culture system in selected studies of infection and *in vitro* symbiosis. No leghemoglobin formation (color) has been observed in our cultures.

We have been able to study two modes of *in vitro* symbiotic infection of legume cells by *Rhizobia* (on agar and in suspension culture) and to confirm the fact that in both cases, acetylene is reduced. We have also reported that *Rhizobia* in synergetic cultures reduced acetylene (13, 15).

The differentiated cultures illustrated in Figure 1 are easier to infect for symbiosis than cultures which remain as callus tissue and do not differentiate. We obtained cultures in which root hair cells were obvious (also see ref. 12). We have also obtained cells analogous to cortical cells. The physiological criteria for viable cells were as follow. (a) Washed preparations reduced acetylene without added substrate (Fig. 3). (b) Acetylene reduction re-

quired higher O<sub>2</sub> than that for synergetic (13) or free-living rhizobial cultures (4, 7-9). (c) Different quantities of lectin can be sampled with different culture types from our laboratory (see below). (d) We have also shown (unpublished) incorporation of <sup>3</sup>H-thymidine and <sup>3</sup>H-arginine in two different batches of uninfected plant cultures.

Other types of cells were also obtained. These were very large cells and did not interact with *Rhizobia*. Their origin is not known. The ploidy of various cell types is also not known.

Differentiation of root cells was obtained by the initiation of the cultures in normal concentrations of 2,4-D and kinetin. The cultures were then placed in media devoid of hormones, and were finally retransferred to normal hormone concentration. Acme cell cultures showed more root hair cell differentiation than Harosoy cultures. We have also noted that when cultures were infected with *Rhizobia*, larger numbers of root hair cell clusters were obtained. The nature of the factors which are useful in enrichment of root hair cells in association with *Rhizobia* is unknown. However, it should be emphasized that these differentiated cell types can be obtained without addition of the bacteria. We hoped that preparations such as ours will be useful in a variety of plant physiology experiments, *e.g.* root cultures from soybean variety Acme which were enriched in root hair cells elaborated soybean agglutinin at concentrations between 12.8 to 9.8 mg lectin/g fresh weight (11). The same study has indicated callus cultures derived from Acme Var. soybeans elaborated 6.8 to 7.7 mg soybean agglutinin/g. These values compare well with the soybean agglutinin content of cotyledons at 9.1 mg/g.

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