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Applications of Computer-Assisted Image Analysis for Microscopic Studies of the *Rhizobium*-Legume Symbiosis

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Abstract

Applications of computer-assisted image analysis are described for various developmental stages of the root-nodule symbiosis between *Rhizobium* and legumes. These range from the simplest of morphometric measurements of subcellular components and irregular cell shapes in light, scanning electron, and transmission electron micrographs, to more difficult and complex measurements involving video microscopy of dynamic motion activities (real-time recordings of bacterial motility, flagellar rotation, cytoplasmic streaming and length of root hairs, and time-lapse recordings of root hair growth and maturation). A "horizontal growth station" is described which enables documentation of root hair growth by time-lapsed video microscopy while legume seedlings are growing geotropically on an agar surface under microbiologically controlled conditions. This image analysis technology enables the investigator to perform quantitative microscopy with relative ease and should find wide application in microscopic studies of the *Rhizobium*-legume and other symbiosis.

Keywords: *Rhizobium*, legume, image analysis, digitizing morphometry, video microscopy

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1. Introduction

Microscopy is a major research tool in symbiontology. The digital computer is having a major impact on microscopy since this partnership of computer and microscope greatly facilitates quantitative evaluation of images, thus removing the criticism of this science as being primarily descriptive. The intent of this invited paper is to illustrate various applications of computer-aided image analysis to an audience of skilled microscopists, using the *Rhizobium*-legume symbiosis as the model system. Essential background information is, of necessity, included. Additional, useful information on general and electronic aspects of video microscopy, image analysis, and computer-enhanced video microscopy is available and recommended for those who intend to use this technique (Caldwell, 1985; Inoué, 1986; DeYoung, 1988; Jarvis, 1988; Commare, 1989; Inoué, 1989).

Image analysis

Image analysis is a new technology which utilizes the digital computer to derive numerical information regarding selected image features (Inoué, 1986). Although image analysis technology cannot add anything that is not already present, its ability to extract the maximum amount of data from the image, as well as to quickly store, retrieve, and electronically transmit that data, makes it an invaluable research tool for the microscopist (DeYoung, 1988).

The basic image analysis system consists of a personal computer, proprietary image analysis software, and a digitizing tablet with mouse. The simplest routine for image analysis is the point counting of recognized structures within an image. This technique would customarily be performed by hand; the benefit of the computer is to provide greater speed and accuracy in acquiring the data, with provision for direct storage and retrieval of the data for statistical and graphical treatment (Inoué, 1986). For morphometric image analysis, the investigator calibrates the magnification of enlarged micrographs, stores this information in computer memory, acquires and stores morphometric data such as contour lengths, areas, shapes, size distribution, and finally analyzes the data.

In recent years, video microscopy has become a popular alternative or supplement to photomicrography to record microscopic images. This form of documentation uses a high resolution TV camera attached to the microscope to generate a video image. This image is displayed directly on a monitor for immediate viewing and also can be simultaneously and permanently stored on videocassette tape using a videotape recorder.

To perform image analysis from video images, optical video digitization is required (Caldwell, 1985; Inoué, 1986; DeYoung, 1988). This involves converting the analog video signal from the TV camera into a computer-readable one using an electronic digitizer "video card" installed within the computer. The digitized image consists of a matrix of pixels. Each pixel in the electronic image represents a spot of light on the computer monitor having one of 256 possible shades of grey. In comparison, the human eye can resolve only about 40 shades of grey. The more pixels in a given area, the greater the resolution. A high resolution image requires at least 512 pixels $\times$512 horizontal lines. Since each pixel requires 8 bits of memory, a digitized image contains more than 0.25 megabytes of data.

Videotape recording is a very efficient way of storing thousands of images as individual frames on a videotape cassette, but loss of image quality does occur in playback. Video microscopy recordings are most commonly made in "real time, " i.e., the video display is accomplished at the same rate as video recording. As an option, certain videotape recorders can be equipped to record slow events in "time-lapse" where intervals of lapsed time exist between each recorded video frame. For image analysis, the videotape recorder must be equipped with a digital "freeze-frame" control built in (most recorders have this) to hold the video image of an individual frame for a brief period while optical digitization is performed.

The proprietary image analysis software can be a major cost of the system, and should be chosen after careful consideration of the types of analyses conducted, compatibility with computer hardware, after-sale service and support, self-instruction tutorials, and provision for updated software at regular intervals (DeYoung, 1988). The latter tip for buyers is very important, since this young technology is still in a phase of rapidly developing improvements. Useful comparisons of various image analysis systems by different manufacturers are available (Inoué, 1986; Anonymous 1988a and 1988b).

The Rhizobium-legume symbiosis

Rhizobium is a genus of bacteria capable of infecting, nodulating, and entering into a nitrogen-fixing symbiosis with leguminous plants. Microscopy has played a central role in documenting the events of the infection process in this symbiosis. The first event recognized is the attachment of the bacteria to the root epidermis, especially root hairs. Later, excreted metabolites from the bacteria cause deformations in root hairs, such as corkscrews, branches, and tightly curled structures resembling shepherd crooks at their growing tip. The rhizobia within the overlap of the shepherd crook penetrate the

root hair wall and occupy the lumen of an intracellular, refractile tube called the infection thread. The infection thread grows to the base of the root hair cell where it penetrates and enters adjacent host cells in the outer cortex of the root. At the same time, inner cortex cells are stimulated to grow and divide, eventually forming an organized mass of infected plant tissue which protrudes from the root surface as a visible nodule. Rhizobia are released from the infection thread within some of the nodule cells and enlarge into bacteroids. These pleomorphic bacteroids are provided with organic acids from plant photosynthate to produce metabolic energy for fixation of atmospheric nitrogen into ammonia, which is then excreted by the bacterial symbiont and assimilated into amino acids by the plant. This plant-microbe symbiosis is a major benefit to agriculture since it provides nitrogen fertilizer for production of leguminous crops.

2. Materials and Methods

A schematic diagram of the image analysis and video microscopy systems used in this study is presented in Fig. 1.

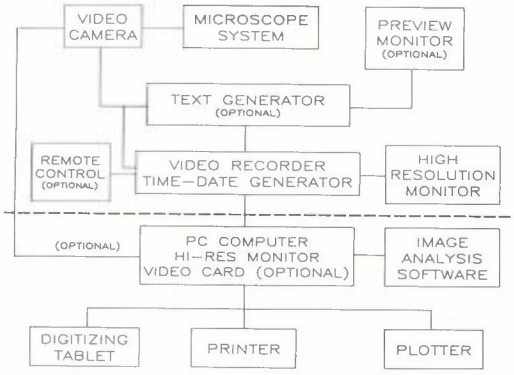


Figure 1. Schematic diagram of video microscopy (above dotted lines) and image analysis (below dotted line) system used in this study.

The digital computer and peripheral hardware consist of an IBM-compatible personal computer with enhanced resolution graphics monitor, 640K random access memory, 20 MB hard disk, internal TARGA optical digitizer video card interface with video overlay module for direct screen measurements of video images (Truevision Inc., Indianapolis, IN) and mouse

modified for operation with Bioquant software, dot matrix printer, and 6-pen plotter.

The image analysis software is the Bioquant System IV (R&M Biometrics, Nashville, TN) containing menu-driven programs for auto-tutorial training, digitizing morphometry, statistical analysis, graphic display, automatic video counting, and 3-dimensional reconstruction and rotation functions.

The video microscopy system consists of a Zeiss Photomicroscope I, a Wild M5A Stereomicroscope, a Panasonic WV-1850 high resolution (800 horizontal line) black & white video camera with ER NEWVICON tube, a C-mount adapter to connect the TV camera to the microscope phototubes, a FOR.A VTW-100 video text generator to introduce text on the video tape, a 9 inch diagonal RCA TC-1910A black & white monitor to preview the text before video recording, a Panasonic AG6050 time-lapse video recorder with 6 time-lapse modes (6:1 to 240:1 time compression) and real time mode, an internal time-date generator to superimpose date and time (month-day-year and hour-minute-second) over recorded images, a Panasonic AG-A6000 remote controller to operate recording functions up to 3 meters away from the videotape recorder, and a 14 inch diagonal Audiotronics 14VM971 monochrome video image monitor with 800 horizontal line resolution.

For electron microscopy, specimens were examined with a Philips CM10 transmission electron microscope and a JEOL 35CF scanning electron microscope.

The bacterial symbionts were *Rhizobium trifolii* strains BAL, 0403, and ANU843, *R. phaseoli* strain CIAT 899, and *R. leguminosarum* strain 127C53. The legume symbionts were Louisiana Nolin white clover (*Trifolium repens*), a black bean line 21–58 (*Phaseolus vulgaris*), and Alaska peas (*Pisum sativum*). The bacteria were cultured on BIII agar and the plants were either grown axenically or with the bacterial symbionts under microbiologically controlled conditions. In most cases, white clover was grown in slide cultures using Fahraeus nitrogen-free medium without agar, whereas beans and peas were grown in flask cultures containing the same medium solidified with 1% agar. Details of the media composition, and methods used to culture the symbionts and prepare specimens for light and electron microscopy are described elsewhere (Dazzo, 1982).

3. Results and Discussion

A variety of microscopic examinations of the *Rhizobium*-legume symbiosis were performed to illustrate the applications of image analysis, first with

selected micrographs to illustrate frequently used measurement modes, and later applying video microscopy to solve special problems inherent in analyzing objects in motion.

The first example is a transmission electron micrograph of portions of a white clover root hair infected with *R. trifolii* BAL (Fig. 2A). Shown are the

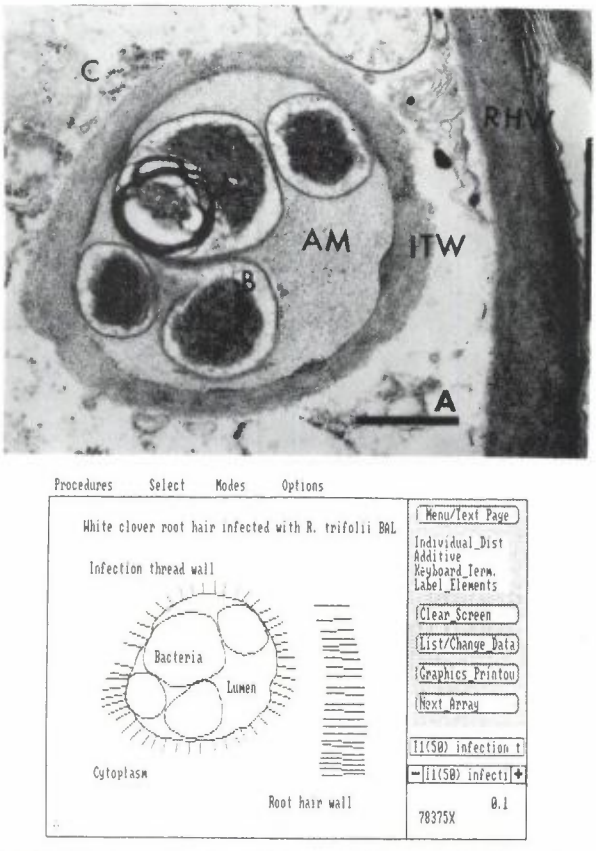


Figure 2. (A) Cross-sectional transmission electron micrograph of a white clover root hair infected with *R. trifolii* BAL. RHW root hair wall; ITW infection thread wall; B bacteria; AM amorphous matrix within lumen of infection thread; C root hair cytoplasm. Bar is 0.5 μm.
(B) Digitized tracing of selected areas of Fig. 2A and Bioquant System IV menu selections as they appear on the computer monitor during image analysis.

root hair wall and the infection thread in cross-section, with root hair cytoplasm lying between the two wall structures and bacterial cells embedded in the amorphous matrix of the infection thread lumen. (Surrounding the infection thread wall is a continuous membrane not resolved in this micrograph). Figure 2B shows the Bioquant System IV digitizing morphometry

menu selections as they appear on the computer monitor, and within the measurement screen (large square on the left) are video lines approximating the path traced on the tablet with the mouse. In this case, we entered the magnification of the 8 × 10 inch micrograph into the computer (shown in the lower right-hand corner), chose the appropriate measurement mode and traced the mouse over the print on the tablet surface. This took about 15 min of user time. Replicate measurements were stored as an array of data values and then analyzed statistically, requiring only seconds of computer time. Individual distances measured as straight lines between two points registered with the cursor button of the mouse showed that the average wall thickness of the root hair and infection thread were 0.41 μm (n=23; s.d.=0.04) and 0.16 μm (n=49; s.d.=0.04), respectively. The area and perimeter of the infection thread lumen were measured simultaneously by tracing the inner face of the infection thread wall. These values were 1.78 μm^2 and 4.91 μm, respectively. The combined area of the bacteria in cross-section was 1.09 μm^2, thus filling 61% of the area of the infection thread lumen.

The next image analysis was performed on a phase contrast photomicrograph of a white clover root hair infected with *R. trifolii* 0403 (Fig. 3). This

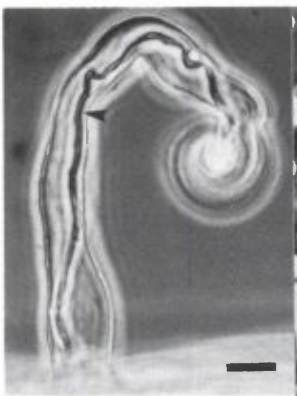


Figure 3. Phase contrast photomicrograph of a white clover root hair infected with *R. trifolii* 0403. Arrow points to the infection thread. Bar is 10 μm.

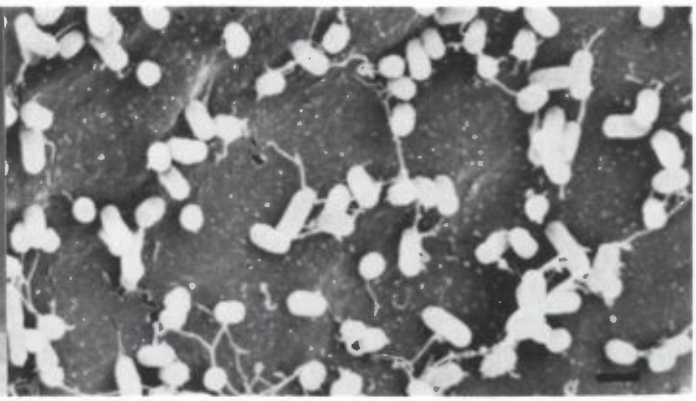


Figure 4. Scanning electron micrograph of an area of the white clover root epidermis colonized by *R. trifolii* ANU843 cells. Bar is 1 μm.

specimen was chosen to illustrate the ease of measuring irregular lengths, as would exist along the curved line of the refractile infection thread within the root hair. The in-focus length of this structure within the root hair was measured in 15 sec to be 155 μm.

Colonization of bacteria on the root epidermis can be best documented by scanning electron microscopy. Figure 4 is a micrograph illustrating the patchy colonization of *R. trifolii* ANU843 on a portion of the white clover root epidermis after 4 days incubation. Despite sufficient incubation of the symbionts, a confluent layering of bacteria did not develop. Two measurement modes of image analysis were applied to this micrograph. The touch counting mode conveniently uses the mouse cursor button to enumerate repeated objects in an image, and in 2-4 min counted 110 bacterial cells in the print (calculated density of 68 bacteria per 100 μm^2 area of epidermal surface). The consecutive distance mode was used to measure the extent of crowding represented by the straight line distance between neighboring bacterial cells within a patch. The center of the cell pole attached to the root surface was used as a reference point for each cell. The analysis required 5–7 min and showed that the average distance between bacteria within a patch of colonization was 0.97 μm (n=95; s.d.=0.39 μm), which is approximately one cell length.

Only a portion of the plant cells within root nodule tissue is infected with the rhizobial symbiont, and this portion will vary according to plant genotype, bacterial strain, and adverse environmental conditions. Figure 5 represents a light micrograph of a toluidine-stained section through a bean root nodule infected with *R. phaseoli* CIAT 899. An image analysis requiring 20–25 min showed that 57% of the area is occupied by infected cells, 33% is occupied by uninfected cells, and 10% is occupied by intercellular spaces.

The Bioquant System IV software can calculate several useful measurements of shape options from the tracing of perimeters. For instance, a measurement of cell distortion can be easily derived from cells whose perimeter completely lies within the micrograph. This measurement is called the "shape factor" representing the amount by which a structure varies from a circle independent of its size. The maximum value is 1.00 for a perfect circle and the minimum value is 0.00 for a line. The shape factor values for infected and uninfected cells in Fig. 5 were 0.89 (n=18; s.d.=0.04) and 0.77 (n=16; s.d.=0.08), respectively. However, this analysis only applies to the section examined. A more comprehensive evaluation of cell shapes could be accomplished by serial sectioning, followed by tracing perimeters in micrographs and using image analysis for 3-dimensional reconstruction and rotation of this structure about any axis.

Branching of root hairs in axenically grown white clover seedlings occurs at a frequency of approximately 1%. When cultured with the *R. trifolii* symbiont, this frequency can increase significantly. This morphological response is interpreted as a bacterial-induced relocation of the growing tip of the root

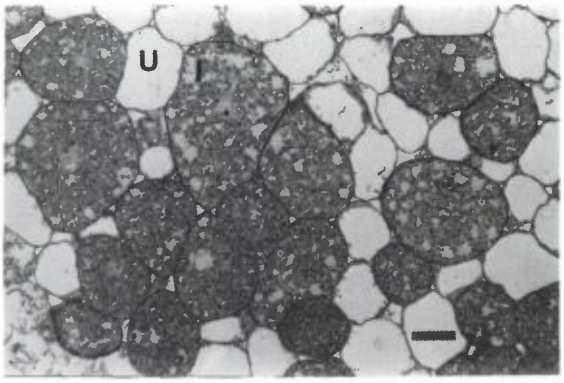


Figure 5. Brightfield micrograph of a toluidine-stained section through a bean nodule infected with *R. phaseoli* CIAT 899. I infected nodule cell; U uninfected nodule cell. Bar is 100 μm.

hair. Figure 6 is a light micrograph showing several branched root hairs on a white clover root grown with *R. trifolii* 0403. The angle of the branch appears to be repeated. The vertex and angle mode was used to measure the variability in this angle. For this procedure, three points were entered for each branched hair with the mouse cursor button so that two points represented the position of each tip and the vertex intersect represented the point where the branch converges with the originally straight hair. Image analysis requiring 4–5 min indicated that the angle of the branch for 7 root hairs was 66.7 degrees, with a standard deviation of 11.2 degrees.

The morphological transformation of the bacterial symbiont from vegetative rods into pleomorphic bacteroids within host nodule cells is a dramatic process coupled to succinate metabolism (Urban and Dazzo, 1982; Gardiol et al., 1987). Figure 7 is a scanning electron micrograph of the cut surface through a pea nodule showing vegetative rods and considerably enlarged, "Y"-shaped bacteroids of *R. leguminosarum* 128C53. A two-step procedure requiring 1 hr was used to calculate the cell volume of the rod and bacteroid forms of the microsymbiont. First, the total length and diameter were measured from each cell using the length mode. Then the calculation procedure of the Bioquant System IV was programmed to convert these data into cylindrical volumes. The results indicated that the average volumes of the vegetative rod and bacteroid cell were 0.12 μm^3 (n=6; s.d.=0.04 μm^3) and 0.86 μm^3 (n=8; s.d.=0.21 μm^3), respectively. Assuming that cell distortions were not introduced by specimen preparation, the average cell volume of the pea bacteroid is 7.17 times more than the vegetative cell of *R. leguminosarum* in this root nodule.

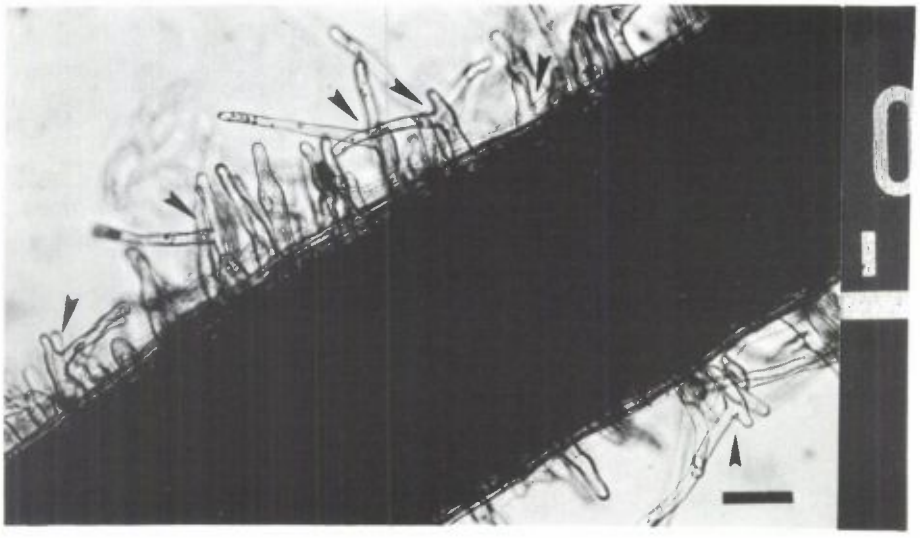


Figure 6. Light micrograph of a white clover root inoculated with *R. trifolii* 0403. Arrow points to branched root hairs. Bar equals 50 μm.

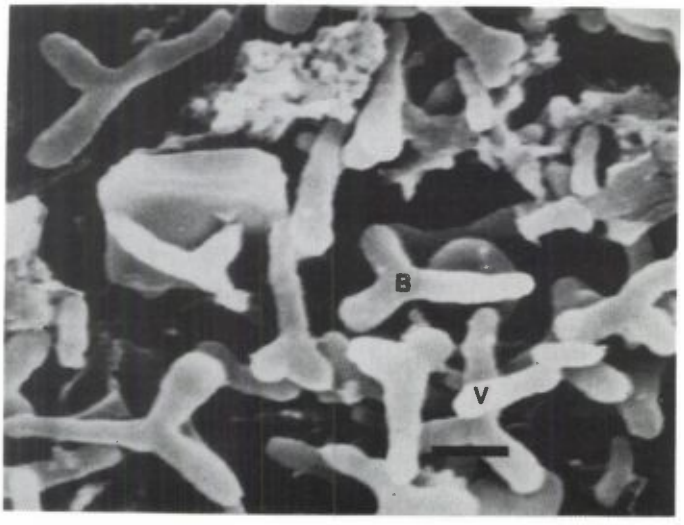


Figure 7. Scanning electron micrograph of the cut surface within a pea root nodule infected with *R. leguminosarum* 128C53. V vegetative rod-shaped bacteria; B enlarged "Y" shaped bacteroids. Bar is 1 μm. (From Dazzo, 1980, and reproduced with permission of Academic Press).

Traditionally, root hair density (number per constant root length) and length are measured directly with an ocular micrometer and compound microscope (Carlson, 1969), or examining enlarged photomicrographs with a microscope (Itoh and Barber, 1983; Foehse and Jungk, 1983). The root hair counts of a root segment having constant length would then be multiplied by a factor of two because although present around the root, the root hairs were visible in only one plane of the photomicrograph. The total root hair surface may be calculated from the length, diameter, and number of root hairs, and corresponding calculations made for the roots. Finally, these may be combined to arrive at an approximation of the total surface contact between the root system and the soil (Carlson, 1969).

Video microscopy combined with image analysis can significantly improve the accuracy of these measurements and alleviate much of the tediousness in acquiring the primary data for these calculations. In Fig. 8A, we have photographed a freeze-frame video image of an axenically grown white clover

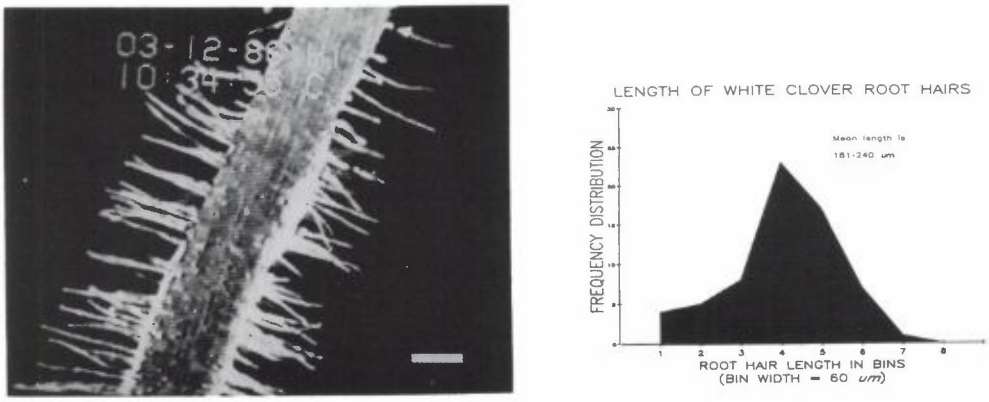


Figure 8. (A) Video image of root hairs on a white clover root magnified by darkfield stereomicroscopy. Numbers at the top are data from the time-date generator superimposed on the video image. Bar equals 200 μm.
(B) Frequency distribution of root hair lengths on the root in Fig. 8A.

root magnified by stereomicroscopy under darkfield illumination to visualize the laterally projecting root hairs. The root is focused along its optical median plane to minimize error of measuring root hairs appearing "short" because they lie diagonal to the plane of focus, and information from the time-date generator is superimposed at the top of the image. For image analysis, the live or prerecorded video image in a freeze-frame is "grabbed" with the Bioquant "Hold Image" feature and converted to a digitized image by the computer Targa interface for measurements with the mouse cursor overlay.

The length of each root hair is measured in the individual distance mode simply by registering two points on the digitized image using the mouse cursor button: one point at the base and the other at the tip of the root hair. The computer then measures the straight line distance between the two points. (The length mode would be used if the hair were deformed instead of straight). The average hair length was 213 μm (n=65; s.d.=81 μm) and the density of root hairs in-focus was 23 root hairs per mm of root length. Once entered into the computer (5–10 min), these data are immediately amenable to a frequency distribution of root hair lengths as shown in Fig. 8B. We are applying this approach to measure the effect of various excreted metabolites from pure cultures of *Rhizobium* on growth and development of root hairs on its symbiotic host, white clover (Dazzo et al., 1987; Dazzo et al., 1988; Hollingsworth et al., 1989). The major advantages of video microscopy for this type of measurement are that the photographic intermediate is eliminated and the videorecording can be analyzed at a later convenient time. These factors become very important when many samples must be examined.

Another major application of video microscopy is the image analysis of moving objects. These techniques were used to examine 4 examples of behavioral motion of *Rhizobium trifolii* and white clover: angular velocity of lateral flagellar rotations, velocity of swimming bacteria, cytoplasmic streaming and growth of root hairs.

Unlike most flagellated bacteria which have left-handed filaments and swim by counter-clockwise rotation, *Rhizobium* has peritrichous, complex flagella that form right-handed helical bundles that rotate only in the clockwise sense (Gotz and Schmitt, 1987). To measure the angular velocity at which the bacteria rotate their flagella in the clover root environment, we examined *R. trifolii* ANU843 cells tethered by single lateral flagella to the underside coverslip surface of 1-day old seedling slide cultures. The rotating bacteria were videorecorded in real time at 60 frames/sec and played back in slow motion at 0.2 sec increments. A sequence of freeze-frame video images of a cell in clockwise rotation is shown in Fig. 9A–C. Analysis of 6 tethered cells (requiring 1 hr) indicated that they rotate their flagella at an average rate of 5.5 rotations/sec (s.d.=0.6 rotations/sec).

Track reconstruction is used to measure movement parameters (e.g., velocity, X-Y coordinate direction, etc.) of living cells which have been videorecorded for image analysis. This is a three-step process (Gualtieri et al., 1988). First, the cells are focused and videorecorded in either real time or time-lapse, depending on their speed of motion. Then, the video images of focused cells are segmented into consecutive lapsed times by frame advance to reconstruct tracks spatially and chronologically. The simplest way to do

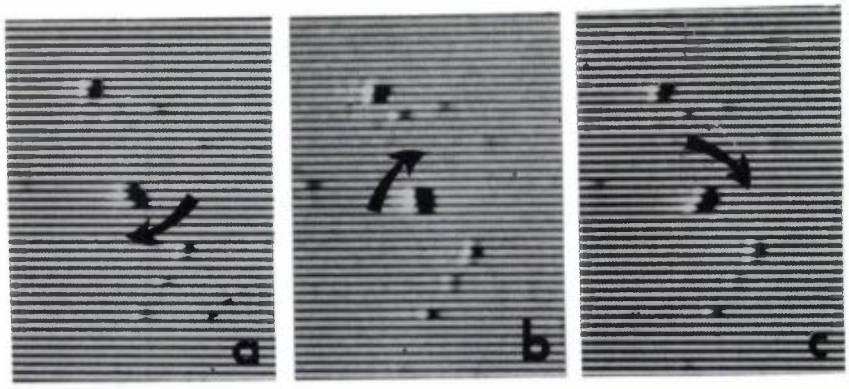


Figure 9. Series of video images of *R. trifolii* ANU843 rotating clockwise while tethered to a cover slip in slide culture with white clover roots.

this is by marking the tracks of the recorded object at consecutive frames on an acetate overlay placed directly on the monitor. Because this method introduces slight error due to parallax, some computer-assisted image processing systems have been developed which can perform the track reconstruction automatically. Finally, measurements of track segmentation are obtained by image analysis using the cursor overlay or the digitizing tablet. For the Bioquant System IV, the "DELTA function" is used to calculate the change in position from the previous element to the current element using X, Y coordinate arrays registered at the center of gravity of the encircled moving object.

A focal plane in which swimming cells of *Rhizobium* remain in focus for relatively long "runs" in the external environment of its host symbiont lies just beneath the tethered cells on the coverslip in slide cultures. Track reconstructions of 1/12 sec segments depicting *R. trifolii* ANU843 cells swimming in the white clover root environment are shown in Fig. 10. Image analysis of these curvilinear tracks (requiring 1–1.5 hr) indicate that the bacteria swim in the slide culture at an average velocity of 52.3 μm/sec (s.d.=0.9 μm/sec).

Cytoplasmic streaming in root hairs is readily apparent from the flowing motion of refractile organelles within these cells examined by phase contrast microscopy. Video microscopy of this behavioral motion for image analysis is best achieved with the root hairs located at the optical median plane of the root where these elongated cells lie parallel to the focal plane. Before track reconstruction, it is best to preview the video sequence to choose organelles that remain refractile and are not obstructed in motion. To examine the effect of the bacterial symbiont on cytoplasmic streaming of white clover

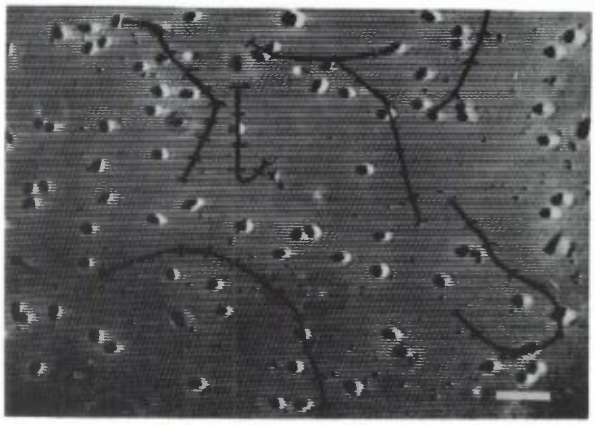


Figure 10. Track reconstructions of video images of *R. trifolii* ANU843 swimming in the white clover root environment of slide cultures. Bar is 10 μm.

root hairs, we performed video microscopy on 1-day old slide cultures of axenically grown seedlings and seedlings grown with *R. trifolii* ANU843. Real time recordings were used to make track reconstructions in 2 sec segments. Movement of refractile organelles within a root hair relative to a fixed line is depicted in freeze-frame video images shown in Fig. 11. Image analysis requiring 1–1.5 hr showed that velocities of cytoplasmic streaming in root hairs grown with and without rhizobia were 6.0 μm/sec (n=35; s.d.=2.8 μm/sec) and 4.4 μm/sec (n=14; s.d.=3.1 μm/sec), respectively. This 35% increase in velocity of cytoplasmic streaming suggests an early metabolic response of host root hairs to the bacterial symbiont.

Documentation of root hair grown under microbiologically controlled conditions was the most challenging and difficult problem for this study. This was solved by time-lapse video microscopy of root hairs on intact white clover seedlings lying flat on sterile plastic plates containing a 2 mm layer of Fahraeus medium solidified with optically clear, 0.7% purified agarose. The seedling plate was mounted vertically by metal clips so the plate cover faced the microscope stage of our specially designed "horizontal growth station" (The Microscope Company, Medina, Ohio) (Dazzo et al., 1988) (Figures 12A and 12B). This permitted geotropic growth of the undisturbed root with minimal condensation on the plate. Darkfield illumination with a voltage--regulated fiber optic ring source was used to minimize illumination and heating of the specimen. Video recordings were made in timelapse at a 240:1 time compression. The station was designed so that the horizontally mounted stereomicroscope and attached TV camera rest on surfaces which slide easily over the formica surface for focusing adjustments. The root hairs were

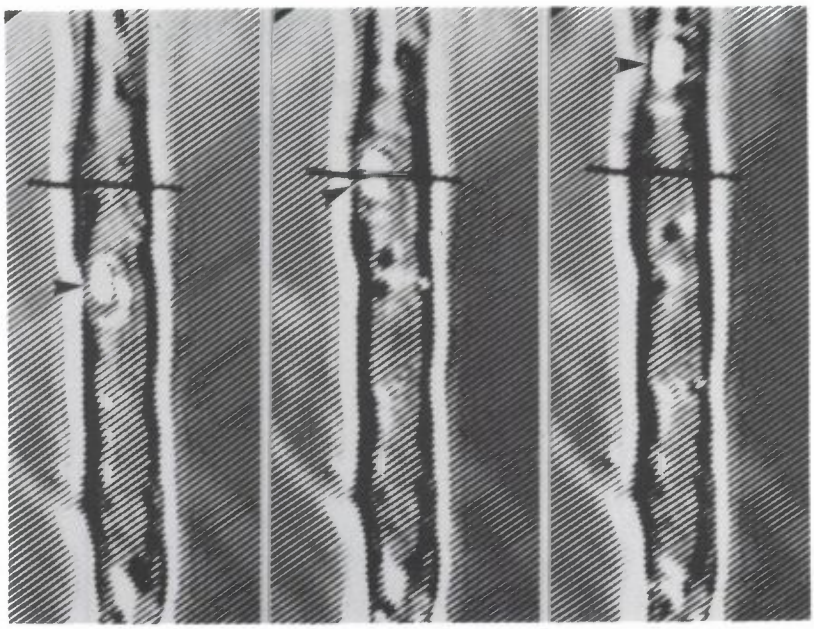
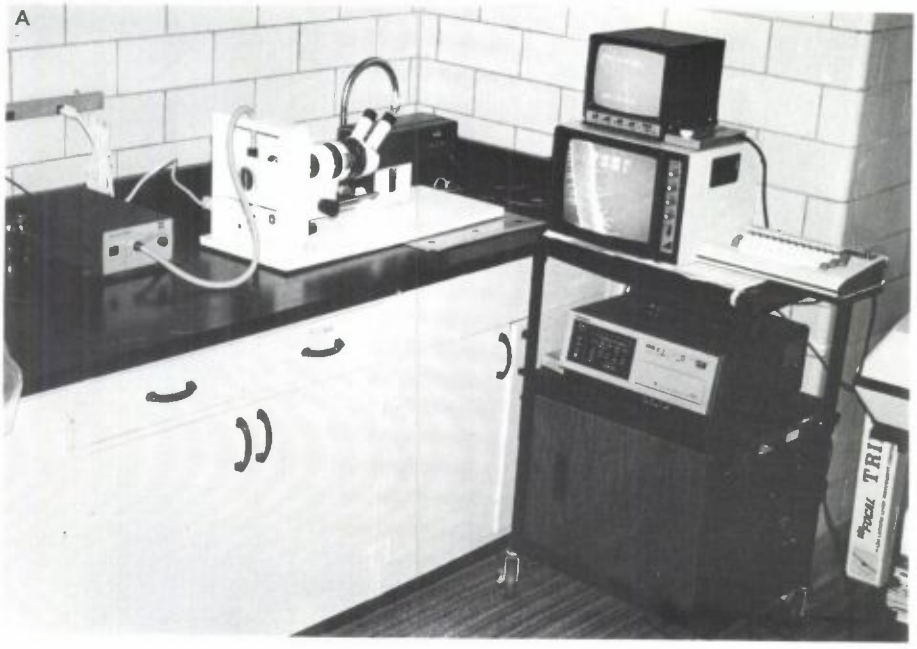


Figure 11. Series of video images of a white clover root hair depicting movement of a refractile organelle (arrow). A black line traversing the hair is drawn as a fixed position reference to the moving organelle.

measured by image analysis in the length mode from recorded, still-frame, magnified images at 1-hr segments recognized by the time-date generator display. Data acquisition required approximately 1 hr. The growth kinetics of three root hairs grown axenically are depicted in Fig. 13. The results indicate that they grew at approximately the same rate (42 μm/hr) for 6–8 hr before reaching their mature length. This approach should provide a very sensitive measure of the impact of the bacterial symbiont and its excreted metabolites on growth and development of host root hairs.

In summary, image analysis applied to achieve quantitative microscopy should find wide use in studies of microbial symbioses. The applications increase with developments in hardware and software, but in the end, it is the creativity of the investigator which primarily limits the use of this powerful technique.



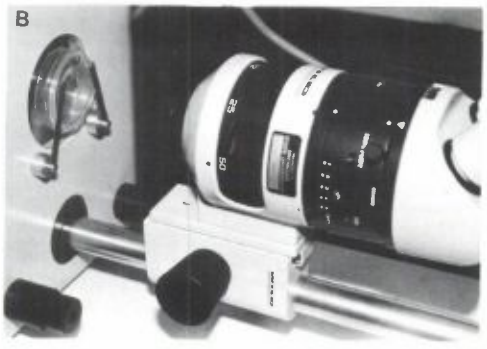


Figure 12. (A)Video microscopy equipment used to record root hair growth in time lapse under microbiologically controlled conditions. Shown from left to right are the fiber optic illuminator, horizontal growth station with stereomicroscope and TV camera, B/W text preview monitor on top of the high resolution image monitor with time-date generator displayed at the top of the screen, keyboard for text generator, and time-lapse video recorder.
(B)Close-up of the microscope stage on the horizontal growth station with the vertically mounted seedling plate supporting the clover plant. The fiber optic ring behind the stage projects darkfield illumination on the seedling root during time-lapse recordings. The plate is inverted to prevent condensation on the plastic surface.

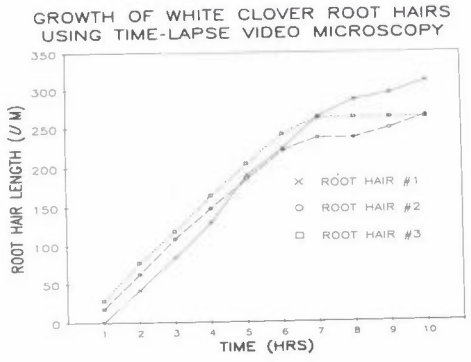


Figure 13. Growth of white clover root hairs using time-lapse video microscopy.

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