APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1984, p. 713–718
0099-2240/84/100713-06$02.00/0

Vol. 48, No. 4

Phyllosphere of Cotton as a Habitat for Diazotrophic Microorganisms

M. G. MURTY

Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore 560012, India

Received 15 February 1984/Accepted 15 June 1984

Positive nitrogenase activities ranging from 0.18 to 0.78 nmol of C_2H_4 cm^{-2} h^{-1} were detected on the leaf surfaces of different varieties of cotton (*Gossypium hirsutum* L. and *G. herbaceum* L.) plants. *Beijerinckia* sp. was observed to be the predominant nitrogen-fixing microorganism in the phyllosphere of these varieties. A higher level of phyllosphere nitrogen-fixing activity was recorded in the variety Varalaxmi despite a low C/N ratio in the leaf leachates. Leaf surfaces of the above variety possessed the largest number of hairy outgrowths (trichomes) which entrapped a majority of microbes. Immersion of plant roots in nutrient medium containing $^{32}P_i$ led to the accumulation of label in the trichome-borne microorganisms, thereby indicating a possible transfer of nutrients from leaf to microbes via trichomes. Extrapolation of acetylene reduction values suggested that 1.6 to 3.2 kg of N ha^{-1} might be contributed by diazotrophs in the phyllosphere of the variety Varalaxmi during the entire growth period.

Colonization of leaf surfaces of plants by a diverse array of microorganisms is now well documented (12, 18). A considerable number of nitrogen-fixing organisms have been reported to occur in the phyllospheres of a wide range of plants (19, 24, 28). These organisms, utilizing carbohydrates excreted by the leaves, fix appreciable amounts of nitrogen which might benefit the plants (19). Investigations in recent years have confirmed the above-mentioned observations in the phyllospheres of mulberry (28), Douglas fir (9), maize and Guatemala grass (3), and rice (8). High fixation rates have been reported also on the phyllosphere of *Spartina alterniflora* (7) and some other C_3 and C_4 graminaceous plants (13).

The information available on phyllosphere nitrogen fixation and its actual contribution to economically important crop plants is scanty and fragmentary, particularly with respect to the mode of nutrient exchange between the plant and the microorganism. In this report, a comparison of acetylene reduction (AR) activities on the leaf surfaces of different varieties of cotton (*Gossypium hirsutum* L. and *G. herbaceum* L.) plants and the contribution made by nitrogen fixers in one of the varieties, namely, Varalaxmi, are made.

MATERIALS AND METHODS

Plant material. Leaves of different varieties of cotton (Table 1) were obtained from pot-grown plants maintained in the open yard (temperature, 30 ± 2°C) of a nearby nursery. Each pot contained 6 kg of a 2:1:1 mixture of soil-sand-farmyard manure for two plants. The pots were watered regularly to 60 to 80% water-holding capacity. Unless otherwise stated, all plants were in the preflowering to flowering stage at the time of experimentation. The samples were collected in the late afternoon to allow maximum accumulation of photosynthate and were quickly brought to the laboratory in sterile petri dishes placed in ice.

Enumeration of nitrogen-fixing bacteria. The nitrogen-fixing bacteria from the leaf surfaces were estimated either by impressing the leaves for 5 min on Burk's modified nitrogen-free solid agar (27) as reported earlier (4) or by plating the serially diluted leaf washings (28) on the above-mentioned medium. The microflora trapped on the membrane filters (Millipore Corp.) were determined by serial dilution and plating of the membrane washings as stated above. Values were expressed on a dry-weight basis (oven dried at 80°C for 24 h) or on a leaf area basis (measured by planimeter).

AR measurements. Rates of nitrogen fixation on surfaces of detached leaves were estimated in 50-ml Erlenmeyer flasks sealed with rubber bungs fitted with serum stoppers as described earlier (13), using the AR technique (16).

The in situ experiments were conducted with pot-grown plants by enclosing the shoots in 1-liter polyethylene bags (Fig. 1). The bags were checked for leaks by pressing the aerated bags under water. Freshly generated C_2H_2 was then injected through the air-tight bag to make a 10% (vol/vol) acetylene atmosphere inside the bag. The incubations were carried out for 2 h under field conditions. Neither evacuation nor flushing of the bags with argon was done. The bags were kneaded gently so that the gases inside could be mixed well. Needle punctures made during injection were sealed immediately with a quick-drying sealant. At the end of the incubation period, the bagged pots were carefully returned to the laboratory and the C_2H_4 produced was measured by gas chromatography. Acetylene blanks and plants minus acetylene were always included for determination of background ethylene. Ethylene was measured in a Perkin-Elmer model F11 gas chromatograph fitted with a hydrogen flame ionization detector and a Porapak N column (125 by 0.15 cm). Nitrogen gas (1 kg cm^{-2}) served as carrier gas. The oven temperature was adjusted to 70°C. The values were normalized for dry weight as well as for leaf area.

In situ collection of leaf leachates and C/N estimation. Plastic troughs of 20-cm diameter were carefully fixed to the bases of plants through narrow slits, and the leachates were collected into the troughs by applying a fine spray of distilled water (200 ml each time) to the leaves with an atomizer sprayer. Leachates were collected and pooled from two test plants at three points in the diurnal cycle, i.e., at 9, 12, and 15 h. The volume of leachates in each case was separately reduced to 5 ml by lyophilization and membrane filtered (0.45-μm pore size; millipore) to eliminate any microorganisms.

The carbohydrates in the leachates were determined colorimetrically by the anthrone method (29), and ninhydrin-

TABLE 1. Leaf characteristics of different varieties of cotton plants and the *Beijerinckia* sp. population on the surfaces

Variety	Leaf characteristics	*Beijerinckia* sp. population ($\times 10^2$) g of dry leaves^{-1} [a]
G. hirsutum (L.) var. Varalaxmi (high-yielding hybrid of Laxmi X SB-289E)	Medium-sized, trilobed, and hairy due to trichomes	7.0
G. hirsutum (L.) var. Laxmi (female parent of var. Varalaxmi)	Large, trilobed, and hairy due to trichomes	6.9
G. hirsutum (L.) (hybrid variety of unknown origin)	Small, entire or occasionally lobed; trichomes sparsely distributed	6.6
G. herbaceum (L.) var. Africana	Small, highly dissected without trichomes	4.9

[a] Values are representative of duplicate samples taken at two different intervals during the growth of the plants and approximate 10^3 cm^{-2} on a leaf area basis.

positive compounds were determined according to Alberti and Bartley (1). The C and N values were derived (3) by using glucose and glutamic acid, respectively, as reference standards. After the experiment was over, the leaves were separated and dried, and the values were expressed based on the dry weight of leaves.

Scanning electron microscopy. Scanning electron microscopy was carried out as previously described (11) with some modifications. Leaf samples of 3-mm^2 size were fixed in 2.5% glutaraldehyde (Polaron Equipment Ltd.) in 100 mM potassium phosphate buffer (pH 7.2) at 5°C for 2 days. The samples were rinsed in the same buffer and dehydrated by passing through graded series of ethanol of 50%, 75%, and absolute ethanol at room temperature. The final dehydration process was carried out by immersing the samples in 90% acetone for 20 min followed by distilled acetone, with three changes for 3 h each time. They were then fixed on glass slides (1 cm^2), coated by vaporizing a layer of carbon (about 100 nm) in an evaporator, and finally sputter coated (Polaron Sputter Coater, E5000) with gold-palladium (about 200 nm). The specimens were viewed with a Cambridge Stereoscan 150 electron microscope operating at 15 kV.

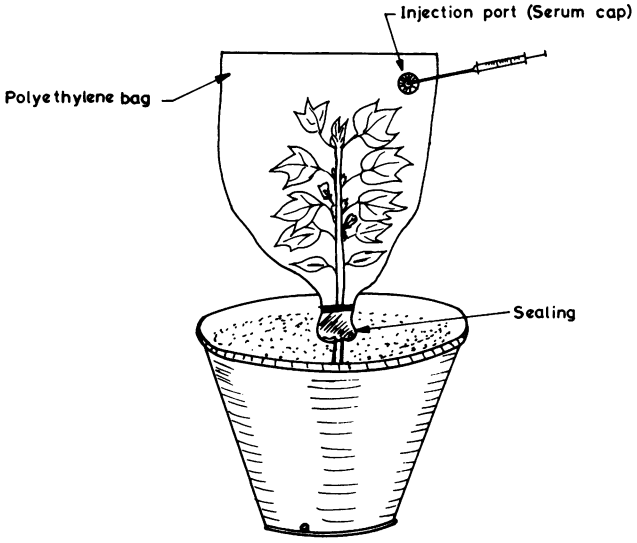

FIG. 1. Diagram showing experimental setup for in situ measurement of AR activity on cotton (var. Varalaxmi) leaf surfaces.

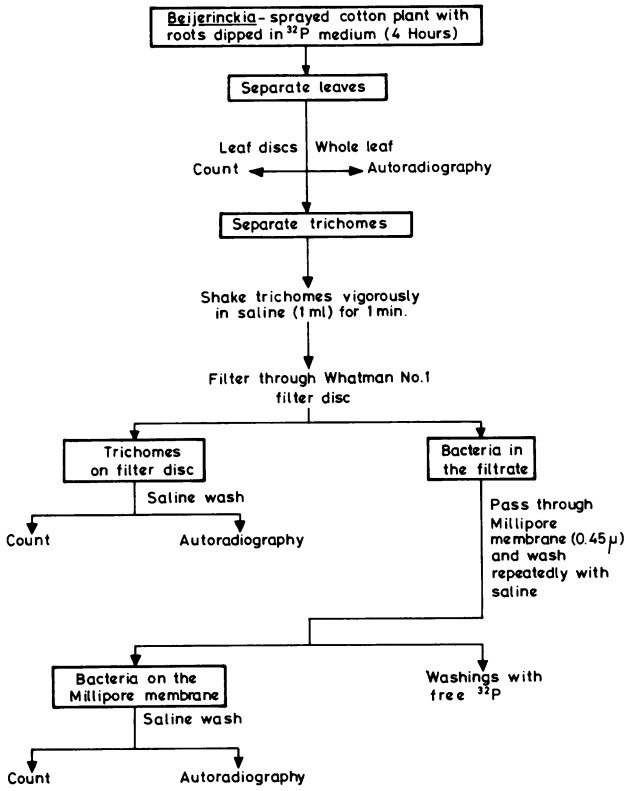

FIG. 2. Schematic flow diagram showing the method of studying $^{32}P_i$ transfer from cotton (var. Varalaxmi) leaf trichomes to the attached microbes.

TABLE 2. Nitrogen fixation (AR) on the leaf surfaces of different varieties of cotton plants

Variety	Acetylene reduced[a]	
	nmol of C_2H_4 g^{-1} h^{-1}	nmol of C_2H_4 cm^{-2} h^{-1}
G. hirsutum var. Varalaxmi	156.25 ± 22.28 110.71 ± 14.30[b]	0.75 ± 0.11 0.78 ± 0.10[b]
G. hirsutum var. Laxmi	83.93 ± 7.58	0.47 ± 0.04
G. hirsutum (hybrid variety of unknown origin)	45.54 ± 6.24	0.25 ± 0.03
G. herbaceum var. Africana	35.71 ± 6.47	0.18 ± 0.03

[a] Values are means of eight replicate samples ± standard deviations.
[b] Values for in situ experimental plants (means of four replicate samples ± standard deviations). When values were expressed on a dry-weight basis, the whole shoot was taken into consideration; for area, leaves alone were considered.

$^{32}P_i$ partition among plant parts, leaf trichomes (hairy outgrowths), and microbes. Uptake of $^{32}P_i$ from leaf trichomes by the microbes was measured with intact plants. As the native microflora on the leaf surfaces was observed to be insufficient to measure the transfer of $^{32}P_i$ from the leaf trichomes, the leaf microflora of 2-week-old pot-grown plants was enriched by inoculating (14) with *Beijerinckia* sp. (approximately 10^8 cells ml of saline $^{-1}$ [0.85%, wt/vol]). The plants were then dug out and the roots were washed with cold (sterile) distilled water. The root systems were dipped in 100 ml of mineral solution (1 mM each KNO_3, $MgSO_4$, and $CaCl_2$) containing about 100 μCi of $^{32}PO_4^{3-}$ (Bhabha Atomic Research Centre). The mineral solution was used here to enhance the $^{32}P_i$ uptake by the plants. Incubations were carried out for 4 h at room temperature under continuous illumination. The roots were shielded from light. After incubation, the trichomes were completely scraped from the leaf surfaces under a magnifying glass using a fine blade, and the microbes attached were dislodged (Fig. 2).

Radioactivity was measured in a Beckman LS-100 liquid scintillation counter. When whole plant was used, the material was homogenized and used for radioactivity determination. The counts for trichomes and microbes were taken by directly dropping the filter disks or Millipore membranes containing the samples into the scintillation fluid after drying them. The values were expressed based on dry weight of the original tissue from which they were separated.

Autoradiography was carried out by exposing an X-ray film (Indu, India) to leaves, trichomes (fixed by using an adhesive tape on a piece of paper), or Millipore membranes with microbes for 2 h. The film was later developed in developer solution for 6 min, transferred to fixative for 30 min, washed for about 2 h, and dried overnight.

RESULTS

***Beijerinckia* sp. population and leaf characteristics of different varieties of cotton.** *Beijerinckia* sp. was the only nitrogen-fixing aerobic bacterium that could be isolated from the leaf surfaces of all varieties checked (Table 1). Among the varieties used, only leaves of the variety Africana were completely devoid of trichomes. However, the *Beijerinckia* sp. population was comparable on the leaves of all varieties irrespective of the nature and shape of the leaf surface.

Nitrogenase activity on the leaf surfaces of different varieties of cotton. Although the population of *Beijerinckia* sp. was apparently uniform on all varieties (Table 1), the levels of AR activity differed with variety (Table 2). In general, the varieties of *G. hirsutum* which had trichomes on the leaf surfaces recorded somewhat higher levels of AR activity when compared with the variety Africana, which was devoid of trichomes.

An experiment for measuring nitrogenase activity of the intact leaves was conducted with the variety Varalaxmi, which showed the highest AR activity. The experimental setup for measuring in situ AR activity of the leaves is shown in Fig. 1. No significant difference in AR levels between detached and intact leaves was observed in the variety Varalaxmi when compared on a leaf area basis (Table 2). However, the values for intact leaves based on dry weight of the plant were somewhat lower than those of detached samples. This could be due to the inclusion of stem biomass while normalizing the values.

C/N estimation in the leaf leachates of cotton, variety Varalaxmi. The amounts of total carbohydrates and ninhydrin-positive compounds in the leaf leachates increased with time points of collection (Table 3). However, the C/N ratio remained almost constant irrespective of the time.

Surface morphology of cotton (variety Varalaxmi) leaf. Scanning electron microscopy of the upper surface of cotton leaves revealed a sparse microbial population (data not shown), whereas the lower surface possessed a large number of long filamentous trichomes (Fig. 3a) with many microbes attached to them (Fig. 3b and c). The trichomes were clustered when the leaf was young (Fig. 3a), but as the leaf matured the trichomes were separated and uniformly distributed on the surface (Fig. 3b).

$^{32}P_i$ transfer experiments. Nearly 1% of the total label used was transferred from leaf to microbes via trichomes (Table 4). This was further confirmed by autoradiography (Fig. 4a, b, and c). Filtration of trichomes immediately after scraping revealed <1% of the counts in the filtrate (data not shown), whereas as much as 10% of the total counts was recovered in the bacteria (Table 4). This might rule out the possibility that some label leaked out into the medium through broken ends of trichomes and was taken up by the bacteria. These experiments thus indicated a possible permeation of nutrients from leaf to microbes across the walls of trichomes. Further, a dilution plating carried out with the Millipore membranes used for trapping microbes from leaves exposed to the label verified the presence of microorganisms. The microbial population level, irrespective of whether the leaves were exposed to label, remained almost constant. The microbial population was $1.4 \times 10^4 \pm 0.3$ and $1.7 \times 10^4 \pm 0.4$ g of dry leaves^{-1}, respectively, in cotton plants (variety Varalaxmi) exposed and not exposed to $^{32}P_i$ (counts normalized to weight of original leaf tissue from which they were dislodged, with values being means of three replicate samples ± standard deviations) (see Fig. 2).

TABLE 3. Estimation of carbohydrates, ninhydrin-positive compounds, and C/N ratios in leaf leachates of cotton (var. Varalaxmi) plants

Time of leachate collection (h)[a]	μg/g of dry leaves[b]				
	Total carbohydrates	Ninhydrin-positive compounds	Total C	Total N	C/N
Morning (9)	17	27	17.8	2.6	6.9
Noon (12)	44	70	46.2	6.7	6.9
Evening (15)	62	95	63.6	9.0	7.0

[a] Leachates were collected in situ from the same test plants at different time points on a diurnal cycle and checked for C/N ratios individually. See text for details.

[b] Values are means of duplicate samples.

DISCUSSION

My findings clearly establish that nitrogen fixation, as determined by the AR technique, takes place in the phyllospheres of different varieties of cotton plants. *Beijerinckia* sp. was observed to be the predominant nitrogen-fixing organism on the leaf surfaces of these varieties. Values of about 10^6 nitrogen-fixing bacteria cm^{-2} on leaf surfaces of citrus (24) and mulberry (28) have been reported. However, these are much higher than the values obtained for *Beijerinckia* sp. (about 10^3 cells cm^{-2}) on cotton leaves. It has also been reported that nitrogen fixation could be supported only if the C/N ratio of the leaf leachates was >10 (3, 23). Leachates from leaf sheaths of Guatemala grass had a very high C/N value of about 600, whereas leachates of leaf blades had a value of <10 (3). The latter was considered unfavorable for supporting fixation.

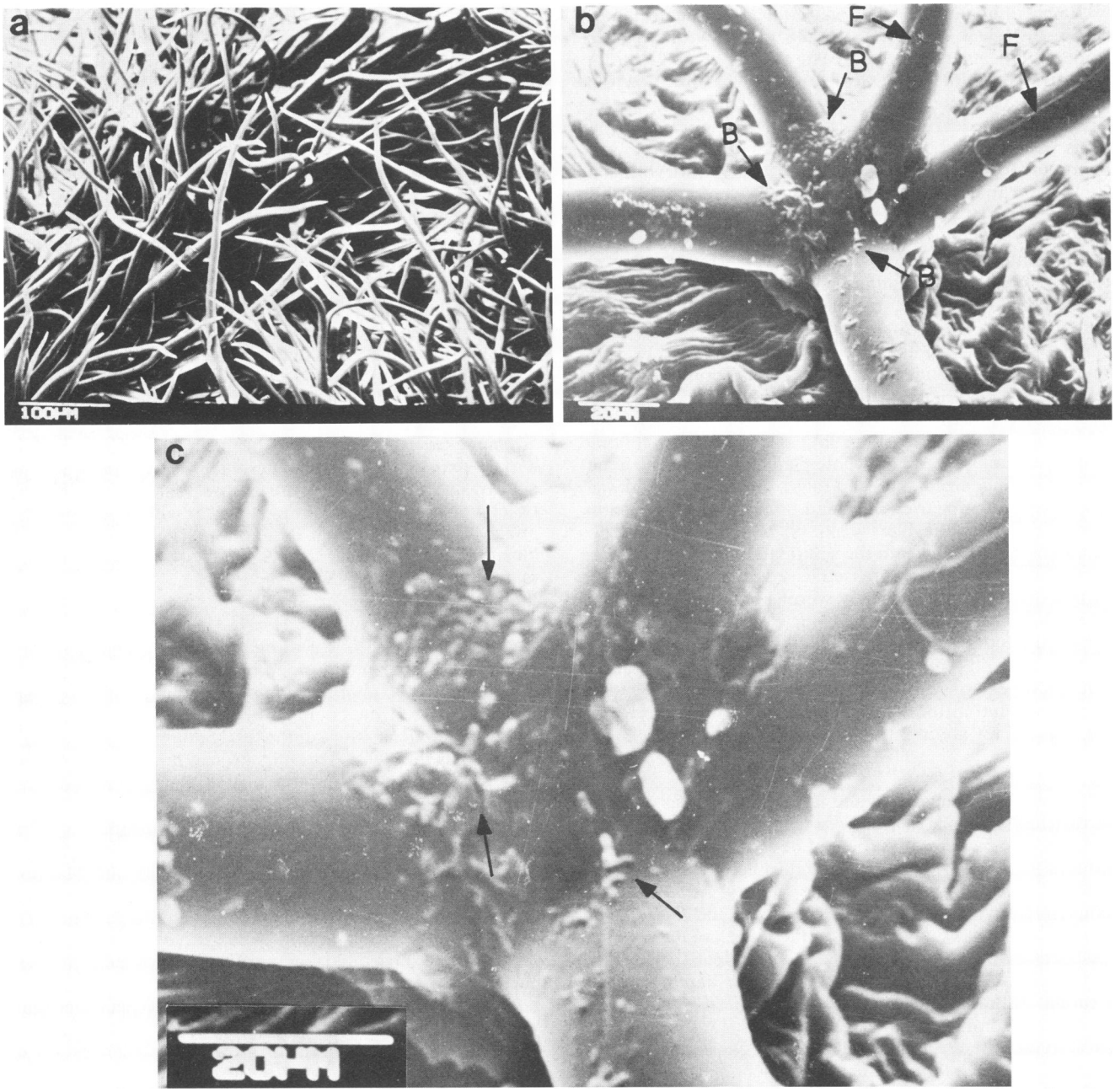

FIG. 3. Scanning electron micrographs of lower surfaces of cotton (var. Varalaxmi) leaf. (a) Young leaf with trichomes crowded all over the surface. (b) Trichome of a matured leaf with microorganisms attached on the surface. Trichomes are well separated here. B, Bacteria; F, fungal hyphae. (c) Higher magnification of the specimen in (b) showing bacteria (arrows).

Variety Varalaxmi, which showed high AR activity, nevertheless had a low C/N value (around 7) in the leaf leachates. However, the carbon estimated here is from total sugars and hence does not reflect the total carbon per se from other nonsugar substrates that may also be available (23) on the leaf surfaces. This may increase the C/N value by a few units. Furthermore, in a given environment, the nature of the compounds which serve as energy sources and the efficiency of nitrogen-fixing organisms to utilize them may play equally important roles in the fixation process apart from the C/N ratio.

The values for AR activity expressed on a leaf area basis seem to represent a more direct proportional relationship with the surface population density (25). However, no correlation could be observed between the *Beijerinckia* sp. populations and AR activity in this investigation. Similar observations have been made with various graminaceous plant leaves (13, 16). This was attributed to variations in the wettability of leaf surfaces and the efficiency of the organism under such conditions. Nevertheless, the efficiency of *Beijerinckia* sp. isolated from variety Varalaxmi was observed to be somewhat lower (1.2 mg of N g of sugar utilized^{-1}) when compared with the standard *Azotobacter* species (about 10 mg of N g of sugar utilized $^{-1}$).

Overestimations of nitrogen fixation in grasses due to the usage of excised roots have been reported (2, 17). The values obtained with the intact plant system for the variety Varalaxmi were, however, found to be well within the range reported for the detached leaves.

AR activity was greatest in the variety Varalaxmi, which had a large number of trichomes, whereas variety Africana had no trichomes and recorded the lowest AR. Trichomes have been implicated in the mechanical and chemical defense reactions of some plants against their pests (6, 26). However, the trichomes observed on cotton leaf surfaces in this study do not seem to be glandular in nature; i.e., they do not seem to play any defensive role. The data, on the other hand, suggest that they may enhance the trapping efficiency of plant surfaces in the concentration of air-borne microorganisms in general and possibly aid in the transfer of nutrients to the attached microbes. It would be of interest to determine whether such trichomes on some varieties of plants offer a better-protected milieu to the nitrogen fixers than do their counterparts which lack these structures.

Epiphytes, mainly cyanobacteria of a freshwater macrophyte, *Myriophyllum spicatum*, have been reported to add 7.5 to 12.5 μg of N mg of plant dry weight^{-1} year^{-1} (5). The nitrogen contribution made by the phyllosphere nitrogen-fixing microorganisms of various plants from temperate latitudes was estimated to be 0.1 to 10% of the total plant requirement for nitrogen (25).

Assuming (i) a steady phyllosphere AR activity during half of the growth period (i.e., 120 days), (ii) an average leaf area of 50 cm^2 and 300 leaves per plant in a hectare containing about 8,000 plants, and (iii) a molar C_2H_4/fixed-N_2 conversion ratio of 3, extrapolation of the reported figures for cotton variety Varalaxmi ranging from 0.5 to 1.0 nmol of C_2H_4 cm^{-2} h^{-1} gives 1.6 to 3.2 kg of N_2 fixed ha^{-1} growth period^{-1}. Such extrapolations, however, should be accepted with caution since assumption (i) has to be checked. Moreover, the C_2H_4/N_2 conversion ratio may be affected by different factors (10). Hence, a true estimate of overall nitrogen contribution to cotton by phyllosphere nitrogen-fixing microorganisms in this study is difficult due to the lack of data for $^{15}N_2$ incorporation.

It has been recently reported that a lectin-like factor with a molecular mass of about 19.5 kilodaltons is involved in the selective retention of *Beijerinckia* sp. on cotton leaf surfaces (14, 15). Also, a fungus belonging to the genus *Alternaria* has been consistently observed on the leaf surfaces of the variety Varalaxmi. An extracellular polysaccharide secreted by this fungus stimulates AR activity of *Beijerinckia* sp. under cultural conditions (M. G. Murty, Ph.D. thesis, Indian Institute of Science, Bangalore, India, 1982). The polysaccharide is suspected of protecting the bacterial nitrogenase from excess oxygen. Further work on these questions should help to provide a better understanding of leaf-microbe interactions, ultimately leading to the improvement of phyllosphere nitrogen fixation in general and its possible exploitation in agricultural practices in particular.

TABLE 4. Determination of $^{32}P_i$ transfer from cotton (var. Varalaxmi) leaf trichomes to the attached microbes[a]

Sample	Radioactivity (cpm g of dry tissue^{-1})
Whole plant	2.2×10^6
Leaves	4.0×10^4
Trichomes	4.4×10^{3b}
Microbes	4.3×10^{2b}

[a] See Fig. 2 and text for details.
[b] Counts are normalized to the weight of original leaf tissue from which trichomes or microbes were separated.

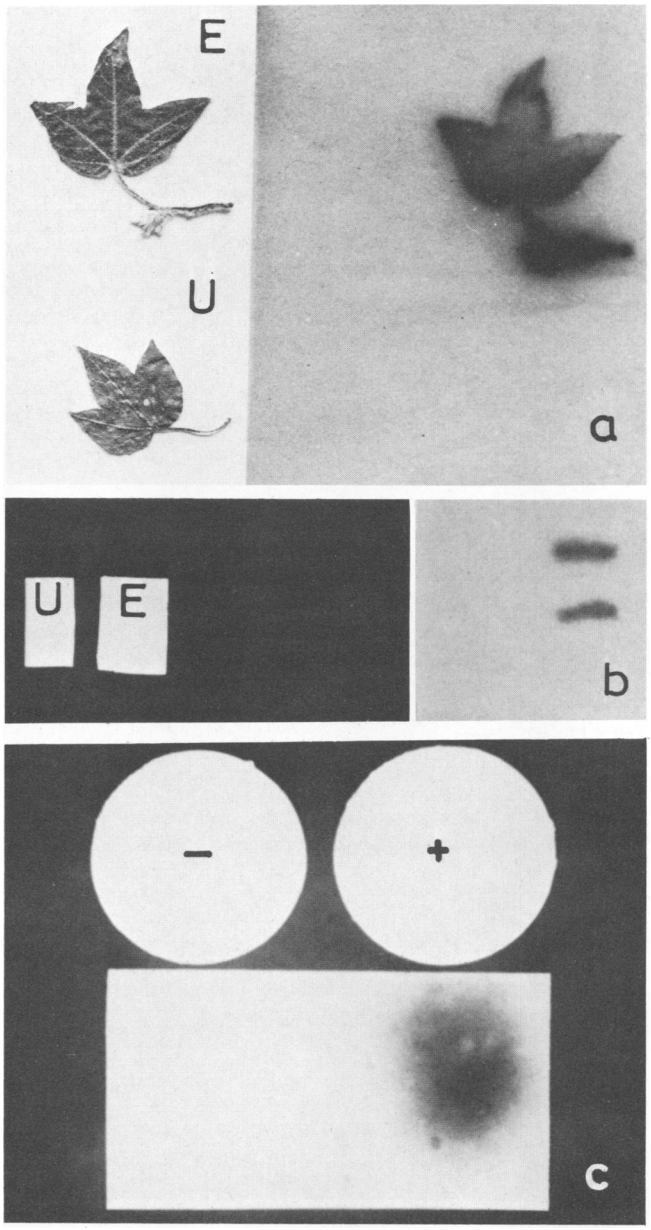

FIG. 4. Autoradiograms showing the transfer of $^{32}P_i$ label from cotton (var. Varalaxmi) leaf via trichomes to microbes. (a) Leaves from plants exposed (E) or unexposed (U) to the label. (b) The leaf trichome fraction processed as in Fig. 2, from exposed (E) and unexposed (U) plants. Here, two strips (approximately 10 by 2 mm) of Whatman filter paper retaining trichomes from each treatment were fixed to white paper sheets and autoradiographed. (c) Millipore membrane showing the presence of label in the entrapped microbes from leaves of plants artificially inoculated and exposed to the label (+). The control (−) is from the leaves of plants either sterilized and exposed or inoculated but not exposed to the label.

ACKNOWLEDGMENTS

I am very grateful to the late V. N. Vasantharajan for his constant encouragement. Constructive comments rendered by P. K. Yadava during the preparation of this manuscript are greatly appreciated.

I also thank the Indian Council of Agricultural Research, New Delhi, for a Senior Research Fellowship.

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