

## ROLE OF THE NITROGEN-FIXING BACTERIAL MICROFLORA IN THE EPIPHYTISM OF TILLANDSIA (BROMELIACEAE)<sup>1</sup>

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Nitrogen-fixing activity in the phyllosphere of 12 species of *Tillandsia* from different Mexican habitats was evaluated by the acetylene reduction assay, and nitrogen-fixing microorganisms were isolated and characterized. The leaves from eight of the 12 *Tillandsia* species examined exhibited nitrogenase activity in enrichment cultures. Among the microorganisms implicated—*Agrobacterium*, *Bacillus*, *Erwinia*, *Pseudomonas*, *Rahnella*, *Vibrio*, and *Xanthomonas*—only *Bacillus megatherium* reduced acetylene in pure culture. Our findings suggest that nitrogen fixation in the phyllosphere of the sampled epiphytes occurs under suitable conditions and that most of the bacteria involved are primarily soil and water inhabitants. The results also suggest a relationship between the composition of the nitrogen-fixing microbial communities grown on the leaf and the different development of the leaf area in *Tillandsia* due to the aerial components (wings) of the trichomes.

The epiphyte strategy for higher plants was a subject of inquiry from the point of view of the source of nutrient and nutrient absorption mechanisms. In particular, the epiphytism of *Tillandsia* (Bromeliaceae) is especially interesting because these neotropical plants have completely lost contact with the ground, living in some cases on inert supports such as high-tension wires.

This behavior is possible because the absorbing foliar trichome (a structure that characterizes, at different stages of its development, the Bromeliaceae) reaches in *Tillandsia* the highest degree of evolution both morphologically and functionally. Thanks to these trichomes that thickly cover the foliar surface, the *Tillandsia* do without the absorbing function of the roots which, if occurring, act only as an adhesion system to the host (Benzing, 1980; Brighigna, Cecchi Fiordi, and Palandri, 1990). In some species called 'atmospheric', the roots are typically absent or poorly developed.

Once the *Tillandsia* absorption apparatus has been identified (Schimper, 1888; Mez, 1904) and its functioning mode, both at structural (Mez, 1904) and ultrastructural level (Dolzmann, 1964, 1965; Brighigna, 1974, 1976; Benzing et al., 1976; Brighigna, Cecchi Fiordi, and Palandri, 1978; Brighigna et al., 1988), has been established, it becomes important to know how these epiphytes can satisfy their nutritional requirements, particularly for nitrogen.

The importance of the phyllospheric microflora in nutrition of tropical vegetation is well known (Ruinen, 1961, 1974). Preliminary investigations to verify the presence of nitrogen-fixing bacteria on *Tillandsia* were carried out on specimens acclimatized in greenhouses of the Botanical Garden in Florence (Favilli et al., 1975). The results of those investigations stimulated further research on nitrogen-fixing associations as soon as we obtained samples of 12 Mexican species of *Tillandsia* that had been just col-

lected in their growing areas and were thus ecologically more interesting.

In the present work we evaluate further the composition of the nitrogen-fixing bacterial microflora and determine if a relationship exists between that composition and: a) the morphological characteristics of a particular species of *Tillandsia*, which can facilitate its establishment and the subsequent development on the leaves; and b) the type of habitat occupied by the *Tillandsia* species. The role of the nitrogen-fixing microflora in the nitrogen budget of the *Tillandsia* is also discussed.

### MATERIALS AND METHODS

**Sampling**—Twelve species of *Tillandsia* were collected in various Mexican localities characterized by different climatic conditions. The plants were compared with well-determined samples at Herbario Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City. After greenhouse storage for a few days, the plants were sent, by air carrier, to the Department of Botany in Florence, where they arrived 20 days after collection. Leaves were removed from each *Tillandsia* and then cut longitudinally with sterile forceps and scissors. Pieces of leaves were placed in sterile glass containers and brought immediately to the lab for microbiological analysis.

**Scanning electron microscopy**—Small pieces of the leaf blade of each *Tillandsia* were fixed in a phosphate-buffered 3% glutaraldehyde solution (pH 7.4), dehydrated in an ethanol series, and subjected to critical-point drying utilizing a CPD 020 Balzers Union Instrument. The samples were coated with gold and observed in a scanning electron microscope, Philips 515.

**Transmission electron microscopy**—A small quantity of *Bacillus megatherium* biomass isolated from *Tillandsia bartramii*'s phyllosphere was fixed in phosphate-buffered 2.5% glutaraldehyde + 4% paraformaldehyde, postfixed in 2% OsO<sub>4</sub>, dehydrated, then embedded in Spurr's epoxy resin. Ultrathin sections obtained using Reichert ultra-

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microtome U3 were stained with uranyl acetate (Gibbons and Grimstone, 1960) then observed under a Philips electron microscope 300 operating at 80 kV.

**Media**—The combined carbon medium (Rennie, 1981) was modified replacing sucrose by glucose and prepared in two solutions. Solution 1:  $K_2HPO_4$ , 0.8 g;  $KH_2PO_4$ , 0.2 g; NaCl, 0.1 g;  $Na_2FeEDTA$ , 28.0 mg;  $Na_2MoO_4 \cdot 2H_2O$ , 25.0 mg; yeast extract, 100.0 mg; mannitol, 5.0 g; glucose, 5.0 g; sodium lactate, 0.5 ml (60% v/v); distilled water, 900 ml. Solution 2:  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $CaCl_2$ , 0.06 g; distilled water, 100 ml. The solutions were autoclaved separately, cooled and mixed; filter sterilized biotin ( $5 \mu g l^{-1}$ ) and para-aminobenzoic acid ( $10 \mu g l^{-1}$ ) were added and the final pH was adjusted to 7.0.

**Nitrogen-fixing activity**—Nitrogen-fixing activity was evaluated by the acetylene reduction method (Burris, 1974). Small pieces of leaves (1.5–2.0 cm in length) were placed in 26-ml glass vials containing 1 ml of sterile distilled water and sealed with serum caps. The gas phase consisted of air containing 12.5% of acetylene by volume. The vials were incubated in light at  $32 C \pm 2$ . The ethylene formation was measured after 24 hours of incubation by gas chromatographic analysis (flame ionization gas chromatograph, Fractovap, Carlo Erba, Italy). Nitrogen-fixing activity was evaluated as  $nmol C_2H_4 h^{-1} g^{-1}$  (dry weight) of leaf.

Nitrogen-fixing activity was also estimated by placing pieces of leaves in vials containing 5 ml of enrichment medium (Rennie, 1981).

After incubation for 24 hours at  $32 C \pm 2$ , acetylene was introduced into the vials (12.5%) and ethylene formation was estimated as described above.

**Isolation and characterization of nitrogen-fixing bacteria**—Nitrogen-fixing bacteria were isolated from enrichment cultures prepared by placing small pieces of leaves in vials containing 5 ml of semisolid N-free culture medium (Rennie, 1981). After incubation for 24 hours at  $32 C \pm 2$  the vials were tested by the acetylene reduction assay (ARA). The vials showing bacterial growth and nitrogenase activity of more than  $10 nmol C_2H_4 h^{-1} ml^{-1}$  were considered to contain nitrogen-fixing bacteria. Bacteria were isolated from enrichment cultures by successive plating on N-free solid Rennie medium (1981).

The strains isolated were identified using the criteria recommended by Krieg and Holt (1984) in *Bergey's Manual of Systematic Bacteriology* as well as by using the API (Appareil et Procédes d'Identification—API System S.A.A., France) tests 2OE, 2ONE (Rennie, 1980), and 5OCHB (Analytab, 1979; McCarley and Rennie, 1980; Logan and Berkeley, 1981, 1984).

The nitrogen-fixing activity of the isolated strains was estimated in vials containing 5 ml of modified Rennie medium (1981) from which yeast extract was omitted and incubated at  $32 C \pm 2$  under anaerobic or microaerophilic conditions.

## RESULTS

All species of *Tillandsia* tested, their ecological types, collection sites, and habitats are presented in Table 1. In

TABLE 1. *Species examined and collection data*

Species	Type <sup>a</sup>	Locality	Habitat
<i>T. albida</i>	atm	Meztlitlan—Mexico	Arid
<i>T. andrieuxii</i>	int	Amecameca—Mexico	Semihumid
<i>T. bartramii</i>	int	Oaxaca—Mexico	Semihumid
<i>T. circinnatoides</i>	atm	Coscatlan—Mexico	Arid
<i>T. deppeana</i>	tk	Lacuna Atesca—Mexico	Humid
<i>T. gymnototria</i>	tk	Acultzingo—Mexico	Humid
<i>T. imperialis</i>	tk	Puerto del Aire—Mexico	Humid
<i>T. juncea</i>	tk	Acultzingo—Mexico	Humid
<i>T. macdougallii</i>	int	Rio Frio—Mexico	Semihumid
<i>T. recurvata</i>	atm	Meztlitlan—Mexico	Arid
<i>T. schiedeanana</i>	int	Tequila—Mexico	Humid
<i>T. usneoides</i>	atm	Amecameca—Mexico	Semihumid

<sup>a</sup> atm = atmospheric; int = intermediate; tk = tank.

'tank' *Tillandsia* living in humid environments the wide and inflated bases of the leaves overlap at the edges forming niches (Pittendrigh, 1948) in which water is impounded. Within the tank, organic debris of animal and plant origin accumulates. The trichomes of these species are thick but have a modest development of the aerial components (wings) which do not overlap, leaving some of the leaf area uncovered.

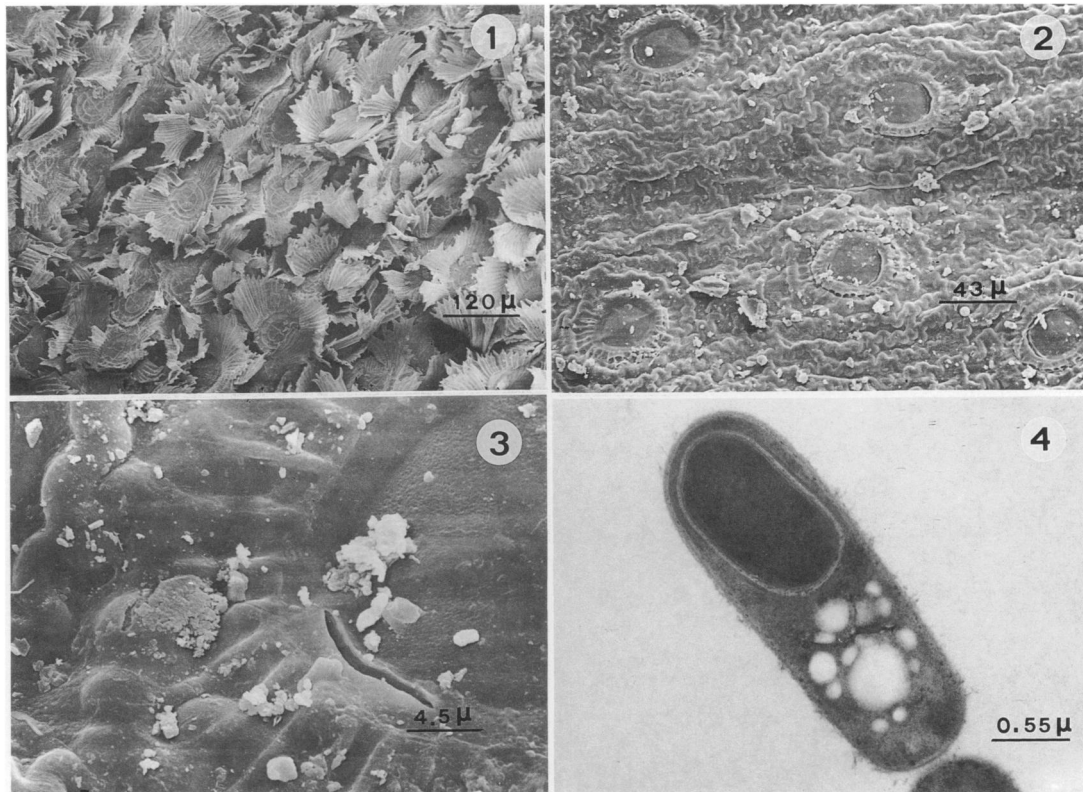
In the 'intermediate' *Tillandsia* occurring in mesic and semihumid environments, tanks are reduced or absent. The leaves are troughlike and are densely covered with trichomes, whose highly developed wings overlap to form a continuous layer that acts as protection for microorganisms and significantly increases the leaf area. Leaf epidermis of *T. macdougallii* (Fig. 1) is an example of both the atmospheric and the intermediate species, for the frequency of the trichomes and the high development of their wings. The 'atmospheric' *Tillandsia* living in xeric environments are tankless and their leaves are covered by trichomes having well-developed overlapping wings. The *T. imperialis*, tank, has an epidermal structure typical of species from humid habitats. The trichomes have poorly developed wings that only partially cover the leaf surface without substantially increasing the leaf area (Figs. 2, 3).

With respect to the nitrogen fixation on the leaves of the *Tillandsia* species examined, nitrogenase activity has been detected only when leaf pieces were incubated in enrichment, N-free medium (Table 2). The microbial species isolated from each *Tillandsia* species are given in Table 3.

## DISCUSSION

For all epiphytes, especially the tropical ones belonging to the *Tillandsia*, the literature has indicated rainwater as a certain and varied source of various nutrients (Tukey, 1970) because it acts as a vehicle for inorganic salts and organic molecules, including nitrogenous compounds, leached from the host plant. Moreover, Benzing et al. (1976) showed that small organic nitrogen molecules could be absorbed by trichomes of *Bromeliaceae*.

In the arid zones most of the upper layer of the soil that reaches the *Tillandsia* by means of wind is retained on the epiphytes by the trichome wings, as commonly observed by epiphyte collectors. The evidence that this



Figs. 1–4. 1. SEM image of the adaxial leaf page of *T. macdougallii*. Note the microhabitat created by the absorbing trichomes. They are close set and have highly developed wings.  $\times 84$ . 2. SEM image of the adaxial leaf page of *T. imperialis*. Because of the scanty frequency of the absorbing trichomes and the limited development of their wings the leaf surface remains uncovered.  $\times 230$ . 3. Detail of an absorbing trichome of *T. imperialis*, SEM. Debris of various kinds of microorganisms persist even after the preparation of the leaf sample.  $\times 2,200$ . 4. *Bacillus megatherium* cell isolated from the phyllosphere of *T. bartramii*. The spore and spheric stores (polyhydroxybutyrate) are evident. TEM photograph.  $\times 18,000$ .

debris represents a source of mineral nutrition to the *Tillandsia* (but also of most of the phyllospheric microflora) is demonstrated by the occurrence of *Bacillus* and *Pseudomonas*, typical soil inhabitants.

The nitrogen-fixing activity of the phyllospheric microflora can contribute substantially to the nitrogen nutrition of the plants, especially in oligotrophic environments (Ruinen, 1965; Vasantharajan and Bhat, 1968; Bhat, Limaye, and Vasantharajan, 1970; Edmisten, 1970; Jones, 1970; Murty, 1983, 1984).

TABLE 2. Nitrogen-fixing activity on leaves of the 12 *Tillandsia* species examined incubated in enrichment medium (Rennie, 1981)

<i>Tillandsia</i> species	nmol $C_2H_4$ $h^{-1} g^{-1}$ (d.w.) leaf
<i>T. deppeana</i>	662.24
<i>T. gymnototria</i>	0
<i>T. imperialis</i>	65.61
<i>T. juncea</i>	0
<i>T. andrieuxii</i>	296.66
<i>T. bartramii</i>	4,225.38
<i>T. macdougallii</i>	229.70
<i>T. schiedeana</i>	2,349.44
<i>T. albida</i>	0
<i>T. circinnatoides</i>	8,311.21
<i>T. recurvata</i>	0
<i>T. usneoides</i>	548.37

Even if the phyllosphere is a variable habitat as regards the nutrients and the space available, conditions favorable for nitrogen fixation, such as high carbon/nitrogen ratio, availability of energy sources, optimal temperature, and high humidity, are frequently present (Ruinen, 1970; Murty, 1984). *Tillandsia* are able to modify humidity and temperature of their leaf-area through the functional characteristics of the trichomes, whose raised wings reflect sunlight (Benzing, 1980), disperse heat (Brighigna, in press), and retain little drops of water. Moreover, the establishment and nitrogen-fixing activity of microorganisms are influenced by factors such as microclimate, leaf surface morphology, wetability of the leaf surface, protection from oxygen produced by cohabitating microorganisms, and the nutrition value of the foliar leachate (Ruinen, 1974). Consequently, the role of the tillandsioid trichome in offering a protected environment and an avenue for nutrient exchange between leaf interior and the phyllosphere is particularly noteworthy.

The levels of nitrogen fixation observed confirm the presence of a nitrogen-fixing microflora in eight of the 12 species of *Tillandsia* examined. The lack of nitrogen-fixing activity on four *Tillandsia* species could be due to one or more stress conditions present in the period between the collection of the plants and the analysis for nitrogen-fixing activity. This is in agreement with the findings that under environmental conditions such as long periods of extreme sun or drought, nitrogen-fixing bacteria did not adapt well

to the foliar environment (Ruinen, 1971; Bentley and Carpenter, 1980; Jones, 1982).

The low diversity of bacteria species isolated from *Tillandsia* collected in different environments indicates a lack of specificity among epiphytic microorganisms and species of *Tillandsia*. The presence of *Bacillus megatherium* (Fig. 4) on a tank species (*T. imperialis*), an intermediate species (*T. bartramii*), and an atmospheric species (*T. usneoides*) confirms what Vosnyakonskaya and Khudyakov (1960) presented as regards the capacity of epiphytic microflora to colonize different plants.

Among the microbial species found on the leaves of *Tillandsia*, *Bacillus licheniformis*, *B. subtilis*, *B. megatherium*, and *B. brevis* constitute the most significant group because they are present in 75% of the species examined.

The predominance of *Bacillus* species in the *Tillandsia* phyllosphere may be related to sporulation capacity (an important characteristic for survival in unsuitable and limiting environments), ability (at least for some *Bacillus* species) to synthesize nitrogenase and thus be self-sufficient, when necessary, as regards to nitrogen nutrition, and the high capacity to degrade many complex biopolymers (assuming such an important role in the biological cycles of carbon and nitrogen). Such characteristics exhibited by the genus *Bacillus* make it one of the most suitable for the extreme environment in which *Tillandsia* lives. However, we note that lacking among the species of *Bacillus* living on *Tillandsia* are several recognized in literature as phyllospheric nitrogen-fixing, e.g., *Bacillus polymyxa*, *B. circulans*, and *B. azotofixans* (Ruinen, 1961; Kononkov, Umarov, and Mirchink, 1980; Sedlin, van Elsas, and Penido, 1984; Balloni et al., 1987).

It is interesting that *Bacillus megatherium*, which is considered of slight importance as a diazotrophic species both in the soil and in the rhizosphere (Kononkov, Umarov, and Mirchink, 1980), was the only species among these isolated capable of high nitrogen-fixing activity in pure culture.

All other strains of bacteria isolated did not exhibit nitrogen-fixing activity in pure culture yet they have been able to grow repeatedly in a nitrogen-free medium. Their lack of activity could depend on specific needs that cannot always be satisfied in pure culture. Indeed, for some types of *Bacillus* a strict anaerobiosis is necessary; for other genera it has been found that long periods of lab culture provoke a strong reduction in nitrogenase activity. In brief, for many bacterial species the best conditions for an active nitrogen fixation are not yet well known.

The occurrence of microorganisms known to be typical of aquatic environments (*Rahnella aquatica*, *Vibrio fluvialis*, *Aeromonas hydrophyla*) exclusively on atmospheric species is significant. This apparent anomaly could be related to the highly accentuated development of the wings of the absorbing trichomes which may result in a consistently high level of humidity in the phyllosphere.

A nitrogen-fixing microflora on the leaves of the *Tillandsia*, in addition to confirming our previous observations (Favilli et al., 1975), is of potentially great importance for the nutrition of these plants, if seen in relation to the absorbing role of the foliar trichomes. Moreover, the nitrogen-fixing microorganisms also contribute to the nutrition of other phyllospheric microorganisms (yeasts, fungi) which constitute a considerable proportion of the

TABLE 3. Bacterial species isolated from *Tillandsia* leaves

<i>Tillandsia</i> species	Bacterial species <sup>a</sup>
<i>T. deppeana</i>	* <i>Bacillus megatherium</i> , <i>Bacillus licheniformis</i> , <i>Bacillus subtilis</i>
<i>T. gymnobotria</i>	<i>Bacillus licheniformis</i> , <i>Vibrio vulnificus</i> , <i>Agrobacterium radiobacter</i>
<i>T. imperialis</i>	* <i>Bacillus megatherium</i> , <i>Pseudomonas cepacia</i>
<i>T. juncea</i>	<i>Bacillus brevis</i> , <i>Bacillus subtilis</i>
<i>T. andrieuxii</i>	<i>Bacillus subtilis</i>
<i>T. bartramii</i>	* <i>Bacillus megatherium</i>
<i>T. macdougallii</i>	<i>Bacillus</i> spp.
<i>T. schiedeana</i>	* <i>Bacillus megatherium</i> , <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> , <i>Pseudomonas cepacia</i>
<i>T. albida</i>	<i>Aeromonas hydrophyla</i> , <i>Pseudomonas paucimobilis</i>
<i>T. circinnatoides</i>	<i>Pseudomonas maltophilia</i> , <i>Vibrio fluvialis</i> , <i>Xanthomonas maltophilia</i>
<i>T. recurvata</i>	<i>Pseudomonas oryzo-habitans</i> , <i>Rahnella aquatilis</i> , <i>Erwinia</i> spp.
<i>T. usneoides</i>	* <i>Bacillus megatherium</i>

<sup>a</sup> The strains with asterisks exhibited nitrogen-fixing activity in axenic culture.

degradable organic matter. The materials made available by the degradation of this organic matter are transported via the trichomes to the leaf interior. The role of this organic matter is especially important in the tankless species, i.e., those that lack the microhabitat from which the *Tillandsia* living in a humid habitat obtain the majority of their nutrients. In conclusion, our investigations indicate that nitrogen fixation in the *Tillandsia* phyllosphere occurs only under specific conditions, probably because the majority of the nitrogen-fixing bacteria living on the leaves are soil or water inhabitants, not well adapted to a foliar environment. Nonetheless, the questions relating to nitrogen fixation in *Tillandsia* phyllosphere still remain open and require further detailed investigations.

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