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SHORT COMMUNICATION

The importance of phyllosphere microbial populations in nitrogen cycling in the Chaco semi-arid woodland

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In tropical rain forest, the interface between leaf surfaces and the atmosphere is a fundamental pathway for nutrient cycling (particulary nitrogen), possibly even more important than the soil–plant interface (Parker 1994, Silver *et al.* 1996). Most important nutrient exchanges in the phyllosphere–atmosphere interface are mediated by microbial populations. For example, some authors have considered that nitrogen fixation in the phyllosphere is the main mechanism for nitrogen gain in humid tropical ecosystems, because of the substantial nutrient demand resulting from a high plant productivity and the constraint imposed by the generally low nitrogen availability in soil (Ruinen 1974, Salati *et al.* 1982, Silver *et al.* 1996).

In contrast, it is usually assumed that development of phyllosphere microorganisms in arid and semi-arid regions is severely limited because of low humidity and high insolation on the leaf surface (Farnsworth *et al.* 1995, Puente & Bashan 1994). However, phyllosphere microorganisms may develop adaptations to direct exposure to environmental stress factors such as UV radiation and low water availability, including development of protective pigments and a mucoid matrix (Beattie & Lindow 1995, Bjorklof *et al.* 2000, Sundin & Jacobs 1999). Moreover, leaf surface characteristics (e.g. absorbing trichomes) in some aridity-adapted plants may favour water retention, providing adequate humidity for microorganisms (Freiberg 1999, Nandi & Sen 1981, Stone *et al.* 1996).

Accordingly, the potential for nitrogen fixing by the phyllosphere microorganisms in plants from arid regions deserves a more detailed analysis. Of special interest are those tropical semi-arid environments where there is a seasonal alternation of dry and wet periods, particularly when the wet season coincides with the warm period (e.g. woodlands and savannas in the subtropical semi-arid belt, Bucher 1982).

The vast, semi-arid Chaco ecoregion that extends over a significant portion of Argentina, Bolivia, and Paraguay provides a good opportunity for testing this possibility. During the Chaco's wet–warm season, a positive water balance together with adequate temperature may provide suitable environmental conditions for development of phyllosphere populations, including nitrogen-fixing groups. Despite the Chaco's geographical extent and ecological significance, no information is available either on the presence of phyllosphere microbes in the vegetation or on its nitrogen-fixing capacity. In this paper we report a survey of functional microbial groups involved in nutrient cycling and nitrogen fixation in phyllosphere populations of five dominant woody species from a Chaco semi-arid woodland in Córdoba, Argentina.

Our study was conducted at the Chancaní Provincial Reserve, Córdoba, Argentina (31°24′S, 65°33′W, 300 m asl). Annual rainfall averages 450 mm, concentrated during the summer (October–March). In the dry winter season (April–September) the water balance is negative, resulting in a soil moisture deficit. Mean temperature is 24 °C during the warmest month (January) and 10 °C during the coldest month (July). The local soils (Ustifluvent molico, USDA Soil Taxonomy) are of alluvial origin. Vegetation at the Reserve is the typical Chaco xerophyllous woodland. Woody species are mostly semi-deciduous (Bucher 1982).

We sampled leaves from the following dominant woody species: *Aspidosperma quebracho-blanco* Schlecht.

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(Apocynaceae), *Ziziphus mistol* Griseb. (Rhamnaceae), *Celtis pallida* Torrey (Ulmaceae), *Prosopis flexuosa* DC. (Fabaceae), and *Larrea divaricata* Cav. (Zygophyllaceae). For each species, leaves were obtained from three adult individuals selected randomly in a 1-ha sector of the Reserve. For each individual, we collected approximately 200 g of fresh leaves at a height of 1.50–2.50 m every 3 mo through 1 y from February 2001. The collected material was kept at 4 °C until processing (within 48 h).

Microbial counts from each sample were made on a suspension of 10 g of fresh leaves in 100 ml saline solution (9‰) shaken vigorously for 5 min. Microbial functional groups were estimated by counting using the most probable number (MPN) and agar plate methods. For the MPN method (three replicates), we used mineral salt basal medium containing: K_2HPO_4 (1.0 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}) with selective substrate for each functional group as follows: ammonifiers, mineral media plus asparagine; nitrifiers, mineral media plus ammonium sulphate; and cellulose-degrading bacteria mineral media plus cellulose strips (Lorch *et al.* 1995). For agar plate methods we used NFB medium for N-fixing bacteria (Döbereiner 1995). The media pH was 7. Samples were incubated at 28 °C for 5–21 d, depending on the functional group. Results are given as $\log_{10}$ counts g^{-1} fresh leaves. Nitrogenase activity in all samples was determined using the acetylene reduction technique (Alef 1995), incubating 1 g of fresh leaves for 4 h. A 3:1 ratio for C_2H_4: fixed N_2 was assumed for all fixation estimates (Werner 1995).

Seasonal differences in microbial functional groups and nitrogen fixed were analysed through a repeated-measures ANOVA. We tested normality using the Kolmogorov–Smirnov test, and variance homogeneity through the Bartlett test. Means were compared using the least significant difference test (LSD) ($P \leq 0.05$). Data on daily rainfall were obtained from the meteorological station located in the Reserve.

Leaves of the studied plant species support a rich and diverse community of micro-organisms (Figure 1). Sampled plant species showed a similar phyllosphere microbial abundance except *Larrea divaricata* that had significantly lower values (Figure 1) (ammonifiers, P = 0.048; cellulose-degrading bacteria, P = 0.017). Nitrifiers were absent in all species at all sampling dates. Ammonifiers were the most abundant group for all species and dates (Figure 1a). The studied species showed a similar seasonal pattern with a minimum in August (dry-cold season) except *Larrea divaricata*. The observed differences may be related to each species' phenology, particularly leaf renewal.

Cellulose-degrading bacteria (Figure 1b) showed significant differences in abundance between species (P = 0.017) and dates (P = 0.028) without revealing a well-defined pattern. Nitrogen-fixing organisms had similar abundance (range = 4.41–4.71 $\log_{10}$ g^{-1} fresh leaves) in all species (P = 0.883) and seasons (P = 0.711). The amount of nitrogen fixed (Figure 2) changed significantly through the year (P = 0.001). For all species, levels were highest in November and lowest in February. *Aspidosperma quebracho-blanco* was the species with the highest values in all seasons. We found no significant correlation between N-fixer abundance and nitrogen fixed (R = 0.019, P = 0.940).

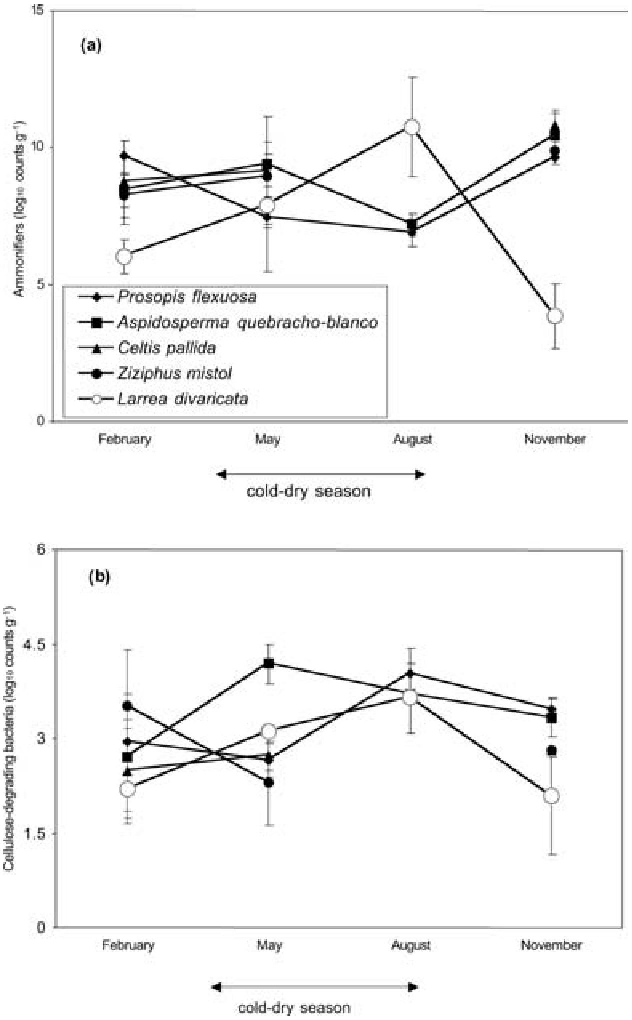

Figure 1. Seasonal variation in numbers of functional microbial groups in phyllosphere of the five plant species studied (a) ammonifiers, (b) cellulose-degrading bacteria. Error bars correspond to 1 SD.

Dominant woody plants in the Western Chaco had significant phyllosphere microbial populations despite the semi-arid conditions. If we estimate total heterotrophic bacteria by adding our values corresponding to each functional bacterial group, our results (10^8 g^{-1} of fresh leaf) are in the upper range of values found in the literature for different species and ecosystems, which range 10^3–10^8 g^{-1} of fresh leaf (Jensen 1974, Nandi & Sen 1981, Werner 1995). Our relatively high values

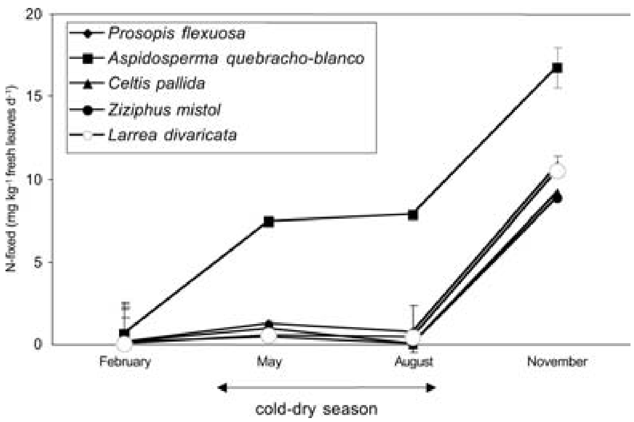

Figure 2. Seasonal variation of N-fixed (mg N kg^{-1} fresh leaves d^{-1}) in the phyllosphere of the five plant species studied. Error bars correspond to 1 SD.

may be due to methodological reasons. We used specific media for each functional group, whereas figures in the literature correspond to counts in general heterotrophic culture media, which cannot provide optimal nutritional conditions for all microbial groups (Lorch *et al.* 1995).

Total absence of nitrifiers despite a high abundance of ammonifiers may be explained by the lack of available ammonia due to active absorption by the plant (Salisbury & Ross 1992). Nitrifiers were found, however, in soybean phyllosphere, particularly in senescent leaves (Arias *et al.* 1999). At the same time, we found nitrifiers in fresh litter from the same plant species in the same area (Torres *et al.* in press). These facts suggest that ammonia becomes available in either leaves of plants that have alternative sources of nitrogen (such as root nodules in soybean) or in dead leaves.

Lack of a well-defined seasonal pattern in the remaining functional groups, a common characteristic of phyllospheric populations (Jacques & Morris 1995), may be related to the physiological characteristics of each functional group and the phenology of the host plants. For example, we found a summer decrease in those functional groups that lack resistant forms (endospores or cysts) such as cellulose-degrading bacteria, which cannot survive leaf desiccation. In our case, leaf desiccation at the moment of sampling was expected because vegetation had been exposed to the high temperatures that characterize this season and an 11-d period since the last rain.

In relation to the influence of plant phenology, we found that ammonifiers increased in November when plants produce new leaves, with the exception of *Larrea divaricata* that grows mostly in the cold–dry season (Barbour *et al.* 1978), which may be due to a higher exudation rate by young leaf tissues (Mercier & Lindow 2000, Werner 1995). The semi-deciduous phenological pattern of Chaco plants may allow recolonization of new leaves from active microorganisms on old leaves, which explains the observed high phyllosphere spring populations. In contrast, in temperate deciduous vegetation, latent microbial populations in leaf buds recolonize the new leaf surfaces each spring (Jensen 1974).

Except for the absence of nitrifier organisms, the abundance and variety of functional groups in phyllosphere micro-organisms in Chaco leaves closely resembles microbial communities in fresh litter obtained from the same study area (Torres *et al.* in press). This similarity supports the hypothesis that the initial stages of leaf decomposition are dependent on the phyllospheric micro-organisms present in the living leaves (Jensen 1974).

With respect to nitrogen fixation by phyllosphere microbes, our results indicate that dominant Chaco woody plants have significant populations of N-fixing organisms in the phyllosphere. Their abundance is relatively low if compared with tropical plants (10^4 versus 10^7 cm^{-2} of leaf surface). However, it must be taken into consideration that counts in tropical plants include phototrophic N-fixing organisms such as cyanobacteria, in both free-living forms and lichens (Freiberg 1999, Ruinen 1974). The outstanding superiority of *Aspidosperma quebracho-blanco* (the dominant tree species in the area) over the other plants studied in terms of N-fixation rate is intriguing. Further research would be needed to elucidate the adaptive significance of this trait.

Contrary to what has been observed in other ecosystems (Freiberg 1998, Nandi & Sen 1981), we found no correlation between N-fixing populations and nitrogen fixation. This difference may result from changes in bacterial activity because of the microclimatic conditions on the leaf surface (presence of water film, etc.) (Freiberg 1998, Paul & Clark 1996), and the phenological condition of the host plant (Ruinen 1974). Presence of an adequate water film is related positively with rainfall and negatively with insolation. Therefore, the low N-fixing activity found in summer could be associated with the long dry period during the hot season, whereas the high values found in November may correlate with rainfall that took place only 1d before sampling. N-fixing activity in the Chaco was highest in November, coinciding with peak leaf production when nitrogen demand for growth is highest.

Our results suggest therefore that nitrogen fixation by phyllosphere microbial communities in the Chaco is highly variable, linked with daily and seasonal climatic variation. Accordingly, estimation of annual rates of nitrogen fixation based on a few observations may be subject to significant error (Freiberg 1998).

Despite these limitations, and for comparative purposes only, we estimated the amount of nitrogen fixed by *Aspidosperma quebracho-blanco* (per adult tree and ha^{-1}), considering its importance in terms of both the amount of nitrogen fixed per tree and tree density. We calculated nitrogen fixation by an adult tree (25 cm diameter at breast height, DBH) as the product of the amount of nitrogen fixed g^{-1} leaf d^{-1} (average of four measurements = 8.1 μg N), the leaf weight (0.7 g), and the number of leaves per tree (10 000) (ABA, unpubl. data). Total nitrogen fixation ha^{-1} y^{-1} was estimated multiplying nitrogen fixation per tree (2.1 g N y^{-1}) by adult tree density in the study area (56 ha^{-1}) (Rivera L, unpubl. data). The result was 1.10 kg N ha^{-1} y^{-1} of mature forest.

Available information on nitrogen fixation by the phyllosphere microbiota in world ecosystems is scarce as well as confused in terms of units and rates in which it is measured, making comparisons with values found in the Chaco very difficult. From the few cases where comparison is possible we found a wide range of values (0.003–62 kg N ha^{-1} y^{-1}). Values similar to our estimation were found in coffee plantations in Mexico (0.7–1.4 kg N ha^{-1} y^{-1}) and cotton plantations in India (1.6–3.2 kg N ha^{-1} y^{-1}) (Freiberg 1998, Roskoski 1982, Ruinen 1974). It must be noticed however that in these examples we compare crop monocultures with a single species within a natural community, which underestimates the total N-fixing potential for the Chaco forest.

In conclusion, our findings demonstrate that (1) phyllosphere microbial populations are abundant, and (2) nitrogen fixation on leaf surfaces may be a significant nitrogen source for the Chaco ecosystem. We expect that the same situation could be found in other similar tropical semi-arid savannas and woodlands.

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