Original article

Contribution of nitrogen-fixing organisms to the N budget in Trachypogon savannas

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Abstract

Trachypogon savannas in Venezuela are mainly used for extensive cattle raising. These savannas are currently affected with man-made or natural fires. During fires, 24% of the nitrogen (N) necessary for primary production is lost through volatilisation. More is lost by leaching and/or erosion. Since those losses are not compensated for by N input through precipitation, N balance in these savannas depends on biological mechanisms. In this study we explore the possible forms of biological N fixation, in particular the cyanobacterial activity from soil microbial crusts, and the contribution of grass rhizosphere microorganisms. Determinations were made by using, in situ, the method of acetylene reduction as an estimate of nitrogenase activity (NA). N_2 fixation due to NA in the soil–plant system is 13.7 and 7.8 kg ha^{-1} year^{-1} for the burned and protected plots, respectively. Even considering the lowest fixation values by microbial crusts, they could provide 6% of the N needed for annual production of the vegetation of the savanna under fire, and 9% in the protected savanna. These amounts of N_2 sustained the productivity of the vegetation experiencing periodical fires.

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1. Introduction

Trachypogon savannas occupy an area of more than 500,000 km^2 in the "llanos" of Colombia and Venezuela. These savannas with a markedly seasonal rainfall pattern represent a biome characterised by low plant production in well-weathered nutrient-depleted soils, occasionally with a hardpan [12]. Periodical bush fires result in the presence of a permanent plant cover, mainly represented by perennial grasses associated with

some shrubs and arborescent pyrophytes scattered throughout the landscape.

Those savannas are currently used for extensive and semi-extensive cattle raising, taking advantage of the natural pastures. Fire becomes a fundamental management tool used to eliminate highly lignified plants, inedible by the cattle, and also to stimulate re-growth of grasses. Yearly fires volatilise 24% of the N required for the net primary production of savanna herbaceous vegetation [8]. N volatilised by fires and nitrogen losses due to leaching and erosion are not compensated for by N input due to precipitation [8,19,20]. This situation could cause a progressive reduction in the potential pro-

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ductive capacity of savannas in the absence of alternative mechanisms of N input, apart from precipitation [1]. Consequently N budgets must be balanced by N biological fixation.

In general, N-fixation can occur in these savannas from three different sources: first from organisms located in the rhizosphere, rhizoplane and endorhizosphere of grasses [9]; second from microbial crusts on the soil surface formed by cyanobacteria [13]; third from rhizobium–legume symbiosis [14]. In a comprehensive study of rhizobium–legume systems in Trachypogon savannas, Barrios and González [6] found that 86% of 127 species examined formed nodules. In the study, most of the nodules seemed inactive due to the poor soil conditions. More recently, Izaguirre-Mayoral et al. [14], working with 16 native legumes from the same savannas, asserted that nodulated species showed reduced N fixation, as compared to cultivated legumes, probably due to the low nutritional status of these soils. According to Pereira [23], tropical species show a low specificity to rhizobium strains compared with that of temperate species, resulting in a less efficient system. Furthermore, rhizobium–legume associations have little importance in the plant canopy as the legume biomass is small compared to the grass biomass [7,19]. Therefore the other two mechanisms of N-fixation mentioned above seem to be more relevant for the nitrogen economy of Trachypogon savannas. In fact, the ability for N fixation of a considerable number of tropical microorganisms as isolates or in synergistic associations has been well documented [21,24,29].

Cyanobacteria are able to colonise a broad range of environments due to their versatile metabolism [26]. In the tropics they play an important role in the nitrogen economy, fixing between 3 and 30 kg N ha^{-1} year^{-1} [13,27]; thus, Stewart et al. have reported for a herbaceous soil–plant system in Nigeria that the input is as high as 38 kg N ha^{-1} year^{-1} [27]. Information about the amount of N fixed by cyanobacteria is very scarce for neotropical savannas. Nonetheless, in Brazil, Döberenier and Days [9] estimated, using intact soil–plant columns (*Paspalum notatum*), considerable N fixation (78 to 239 kg N ha^{-1} year^{-1}).

The following is a preliminary study of the nitrogen economy in Trachypogon savannas that takes into account the soil–plant system and microbial crusts on the topsoil and the role of biological fixation in making good the reduction in N budgets due to seasonal fires. We consider the role of fire, edaphic conditions and climatic factors in cyanobacterial N-fixation.

2. Materials and methods

The work was carried out at Estación Experimental La Iguana (EELI), Guárico State, in central Venezuela. The climate is characterised by a rainy season that lasts approximately 6 months (from May to October) with an annual rainfall of 1300 mm. The mean annual temperature is 27,9 °C. The soil of the study area was classified as Quartzisamments, sandy, acid, with a 0–3% slope [17]. An experimental plot of 1 ha was evenly divided into two sub-plots, one of which was burned at the end of the dry season (April of the first year and February of the second year). The other half of the plot had been protected from fire for 2 years previous to sampling. Large herbivores were excluded from the experimental plot.

2.1. Nitrogenase assay

NA was determined in situ for both soil–plant and the microbial crust systems by using the acetylene reduction method of Hardy and Holsten [11] which assumes that NA values are indicators of N_2 fixation. Since NA is strongly affected by soil water content, the moisture contents of soil and microbial crusts were gravimetrically determined on each sampling date (from the weight difference between wet and dry samples).

2.2. Determination of biological N_2 fixation on microbial crusts

Determination of biological N_2 fixation by microbial crusts was carried out using 15 crust samples per sub-plot (burned and protected). Crust samples were collected by using small metallic cylinders (3.3 cm diameter and 2.5 cm height). The collected samples were immediately placed in glass bottles with a 15–20% acetylene concentration. After 24 h field incubation, 5 ml of the reaction mixture was transferred into vacuum sealed test tubes. In the laboratory 1 ml of the diluted reaction mixture was injected into a gas chromatograph (3700 Varian®). The chromatograph had an ionising hydrogen flame detector and a stainless steel column (183 cm × 3.2 mm) containing Poropax N® (80–100 nets) that was able to separate the acetylene–ethylene mixture at 90 °C. Nitrogen was used as a carrier gas with a flux of 33 ml min^{-1}. Gas separation was measured in a register, 9176 Varian®. Retention time for ethylene and acetylene was 58.8 and 82.2 s, respectively.

Cyanobacteria activity was tested by using the Bristol's solution for cyanobacteria [2]. Most probable number (MPN) method done for microbial crusts in EELI reported values of 1.2×10^4 ufc.

The mean values of NA obtained from the acetylene method are indicators of N_2 fixation. Mean NA values were transformed into inputs to biological N_2 fixation using the relation 3:1 (C_2H_2/N_2) as suggested by Hardy and Holsten [11].

2.3. Determination of % of microbial crust cover

Microbial crust cover was determined monthly in 15 quadrates of 1 m^2 in each plot, the surface coverage of microbial crust being visually determined and copied onto a sheet of paper, after which a planimeter was used to measure the area of crust. The mean %age surface cover obtained for the fifteen quadrates corresponds to the microbial crust area.

Monthly nitrogen inputs were estimated using the NA values and the estimated area of microbial crust cover. NA for the months that were not sampled was calculated using the regression line relating monthly precipitation and NA.

2.4. Determination of biological N_2 fixation in the soil–plant system

In situ biological N_2 fixation of the soil–plant system was evaluated according to the methodology of Balandreau and Dommergues [4] and Patriquin and Denike [22]. In each plot (burned and protected), seven cylinders (30 cm diameter and 25 cm height) were buried 20 cm deep in the soil at randomly chosen positions. Cylinders enclosed the plants found at each sampling point. A plastic bag was tightly secured to the buried cylinder using elastic bands, creating a closed atmosphere in the system and covering the aerial part of the enclosed plants. A control cylinder was buried in an area without vegetation or microbial crusts between the two treatments in order to measure NA in the bare soil. Acetylene was generated in situ by adding water to 15 g of CaC_2 in a beaker previously placed in the system. Water addition and gas sampling were carried out through a rubber stopper located in the buried cylinder at soil surface level.

Systems were set up between 06:00 and 07:00 h and samples were collected every 2 hours until 18:00 h. A final sample was taken at 08:00 h the following day. Five millilitres of gas samples was transferred to test tubes and processed as in 2.1. Ethylene production in

a given time period was expressed as NA per cylinder or as NA per gram of root. At any season AN values are important just during day-time (a significant decline in AN activity was observed at dark hours), therefore daily AN means were obtained from values registered in diurnal hours.

The mean values of NA obtained from the acetylene method for the seven cylinders were converted into inputs to biological N_2 fixation [11].

2.5. Statistical analysis

To establish significant differences of coverage and NA of the microbial crusts and the soil–plant system between the protected and burned plots, the Mann–Whitney U-test was used [25]. Regression equations of microbial crusts against soil moisture content and monthly precipitation were calculated after checking variable requirements for the test. Kendall's τ-test of correlation range was applied to determine the relationship between NA in the soil–plant system, soil moisture and monthly precipitation [25].

3. Results and discussion

3.1. Microbial crust coverage and soil and crust moisture content

Precipitation is closely correlated with soil and microbial crust moisture content in both burned and protected treatments (Tables 1 and 2B). The correlation was also significant with microbial crust cover in the protected savanna but not for the burned treatment (Table 2B). The mean values of the microbial crust cover in the protected savanna (11.8%) is more than for the burned savanna (9.9%). Similar results throughout the experimental period were found for soil and microbial crust moisture content (Table 1), which were always higher in the protected savanna than in the burned plot.

3.2. Microbial crust system

Moisture content in the microbial crusts strongly influences NA activity, which is limited to the rainy season (Fig. 1). Furthermore, sampling was done on the surface soil where N-fixing bacteria are most active. During the dry season no NA was recorded, even after 24 hours of crust re-wetting. This could indicate that cyanobacteria are very active and responsible for N fixation during the wet season, whereas between rainy

Table 1
Monthly precipitation (mm), soil and microbial crusts water content (%), and surface coverage of microbial crusts (%), throughout the sampling period in protected (PS) and burned (BS) savanna

Sampling days	Monthly precipitation (mm)	Soil water content (%)		Microbial crust water content (%)		Microbial crust cover (%)	
		PS	BS	PS	BS	PS	BS
0	208.0	6.3 ± 0.2	5.3 ± 0.6	8.1	n.d.[a]	13.1 ± 2.5	14.9 ± 2.4
35	289.7	7.5 ± 2.1	6.5 ± 2.1	11.1	7.5	16.8 ± 3.2	10.8 ± 1.3
65	322.4	5.4 ± 1.5	4.7 ± 0.9	8.0	4.1	12.0 ± 1.8	7.9 ± 1.0
106	118.7	4.3 ± 1.9	5.3 ± 0.8	1.1	0.6	12.8 ± 2.5	15.3 ± 3.7
160	32.5	2.2 ± 0.2	1.8 ± 0.04	0.4	0.3	7.1 ± 1.8	4.1 ± 1.0
238	0.0	0.6 ± 0.1	0.5 ± 0.03	0.1	0.2	9.1 ± 2.4	6.7 ± 1.1

[a] Not determined.
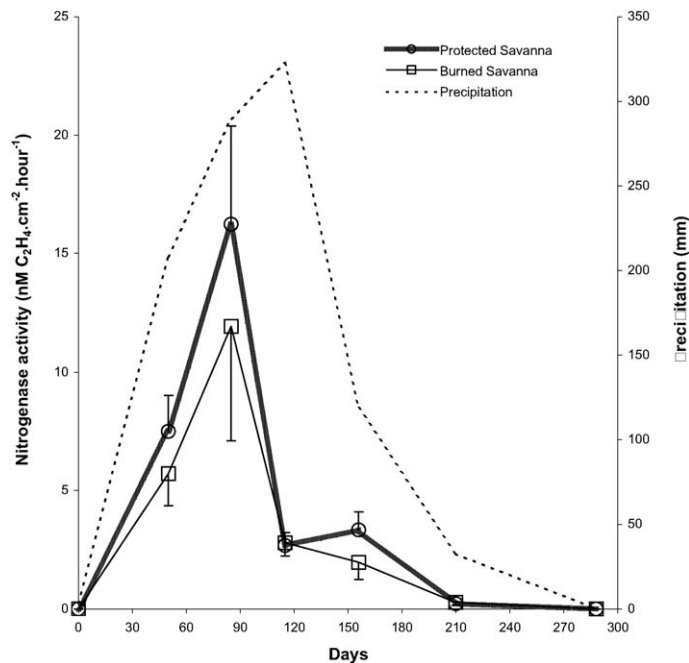

Fig. 1. NA (acetylene reduction) in nM $C_2H_4 cm^{-2} hour^{-1}$ of microbial crust system in burnt (†) and protected (O) savanna as a function of time (days) and cumulative monthly precipitation before sampling. Precipitation numeric data in Table 1.

seasons they are dormant due to the severe drying of the sandy soil [18]. It appears that the soil system needs a long period of continuous rehydration to restart N_2-fixation activity [10].

NA was not significantly different in the burned and protected plots (Fig. 1), except for day 160, at the end of the rainy season. The larger plant cover in the protected plot maintains, at a microclimatic level, a relative higher crust moisture content (Table 1) creating more favourable conditions for NA during the rainy season [27]. Regression coefficients between NA and monthly precipitation are lower than those relating NA and soil moisture, which in turn reflect the water content of the microbial crusts (Table 2A). By using the microbial

crust cover (Table 1) and NA measurements, N_2 fixation by microbial crusts can be estimated to be 2.4 and 4.0 kg ha^{-1} year^{-1} in the burned and the protected savanna, respectively. However, the contribution of N_2

Table 2A
Regression equations relating microbial crust NA (in nM C_2H_4 cm^{-2} hour^{-1}) with soil humidity and monthly precipitation in burned and protected plots

Variable (X)	Plot	R^2	Equation
Soil humidity	Protected	0.74	NA = 1.479$X^{1.050}$
Soil humidity	Burned	0.87*	NA = 1.0639$X^{0.919}$
Precipitation	Protected	0.73	NA = −0.557 + 0.042X
Precipitation	Burned	0.81*	NA = −0.500 + 0.033X

* Significant at $P < 0.05$.

Table 2B
Correlation coefficients between precipitation and microbial crust cover and soil water content in burned and protected plots

	Soil humidity		Crust water content		Crust cover	
	PS	BS	PS	BS	PS	BS
Precipitation	0.897*	0.827*	0.922*	0.866*	0.769*	0.3502[ns]
Soil water content PS	—	0.958*	0.913*	0.776*	0.884*	0.591[ns]
Crust cover PS	0.884*	0.899*	0.812*	0.839*	—	0.677[ns]

* Significant at $P < 0.05$.

fixing organisms found in these crusts to N-cycling could differ from the earlier estimates due to the close relationship between the NA and the microenvironmental conditions at the moment of sampling. In any case, fixation should be less than 13.5 kg ha^{-1} year^{-1}, a value which would be met if the maximum NA recorded was maintained during the 214 days that cyanobacteria remained active.

3.3. Soil–plant system

During the sampling period, only *Trachypogon plumosus* and *Paspalum carinatum* were always present in the cylinder and plastic bag system placed at random, due to the local high densities of both species. Other plant species were found at very low frequencies, such as the legumes *Phaseolus linearis* and *Cassia hispidula*, both with small nodules (less than 5 mm), pink in the inside and testing positive for NA. Nevertheless, in those cylinders containing legumes, NA was within the same range as the cylinders that only contained grasses. Thus, considering the limitations of the method used with a coefficient of variation of 20–30% (Fig. 2), it can be concluded that N$_2$ fixation mediated by legume symbionts is relatively low as compared to free living organisms associated with the grass roots. On the other hand, in the same place (Estación Experimental La Iguana), *Vigna unguiculata* plants cultivated with fertiliser addition, and with a high natural nodulation, analysed for NA showed a NA 27 and 58 times higher than that obtained in the protected and burned plots, respectively. In the case of this legume, low natural available P in soil can alter nodulation and dinitrogen fixation [30].

NA in the soil–plant system had two peaks in both plots: the first at the time of maximum precipitation and the second in the last month of sampling (Fig. 2). The first peak coincides with the flowering of *T. plumosus* while the second peak occurs in the middle of the dry season. In the burned plot the second NA peak is much higher than that of the rainy season. That peak on the burned plot was recorded 3 weeks after the annual fire,

which could suggest: a stimulation effect of fire (or fire-produced ashes) on NA. In fact, Jorgensen and Wells (1971) found increases in N$_2$ fixation up to 10 times higher when controlled fires were applied to pine forest as compared to unburned controls. The drastic increase in NA was explained in that experiment by the effect of ashes on increasing pH and nutrient availability (especially phosphorus) in the soil surface.

During the dry season, the low water availability in savannas could be a major limiting factor for microbiological activity. The latter effect was observed on savannas of the Ivory Coast where a considerable decrease in the ability for acetylene reduction was recorded when 10-day cumulative precipitation was below 30 mm [5]. A measure of soil moisture and green regrowth in the burned plot indicated that: soil moisture continued to be very low in the surface (0.5% at 238 days, Table 1), whereas green regrowth and root biomass reached 11 and 100 g m^{-2}, respectively [7]. The latter results confirm that after fire there is an intensive decomposition process that facilitates microenvironmental conditions for N-fixation [15].

In the protected plot, the increase of NA in the dry season, even though it is lower than the peak recorded in the rainy season, indicates the effect of rhizosphere conditions (moisture content, exudates, pH) on associated organisms. It must be emphasised that in this plot the percentage of nitrogen in the root increases precisely during the rainy season, whereas underground biomass decreases in both plots [7]. A possible redistribution of assimilates and root decomposition, which could be taking place in spite of low soil moisture (below 1.8%, which corresponds to more than 15 bar), generates a root microenvironment that allows diazotrophic activity [16], although this work does not indicate the relative importance of each of these variables on recorded NA peaks. It is obvious that environmental factors, plant internal metabolism rhythms or both may simultaneously give rise to the observed peaks.

Monthly fluctuation of NA in the burned plot is positively correlated with monthly precipitation and soil moisture (Kendall's τ, $\alpha = 0.05$), whereas the same cor-
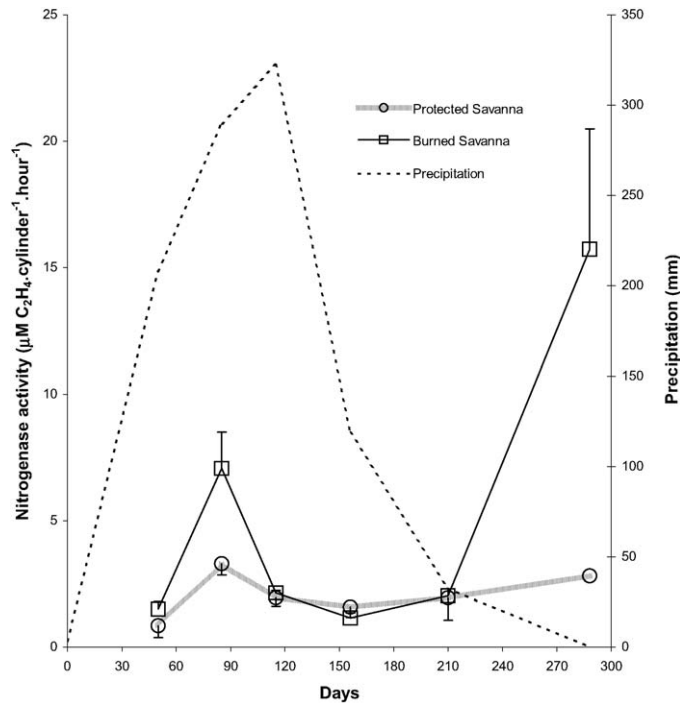
Fig. 2. NA (acetylene reduction) in □M C_2H_4cylinder^{-1} hour^{-1} of soil plant system in burnt (†) and protected (O) savanna in function of time (days) and cumulated monthly precipitation before sampling. Precipitation numeric data in Table 1.

relation was lower and barely significant for the protected plot (Table 2A). NA was always higher in the burned plot than in the protected plot, although the differences are slightly smaller (Fig. 2).

N_2 fixation mediated by NA in the soil–plant system is 13.7 and 7.8 kg ha^{-1} year^{-1} for the burned and protected plots, respectively, Similar values were obtained in Loudetia savannas in the Ivory Coast (Africa) by using the same experimental design [1,3]. Our values were calculated assuming a 10-hour activity period, but they could be slightly higher if night fixation is considered. If recorded NA is in fact associated with grass roots, then the differences observed in NA in both plots during the dry season should be attributed more to differences in root biomass than to different fixation ability due to fire. Besides, the use of CaC_2 to generate acetylene could partially affect photosynthesis due to the formation of calcium hydroxide which acts as an efficient carbon dioxide trap. Using a short incubation period it might not be a problem, but with a longer incubation period the shortage of metabolic products, if occurring, could induce an underestimation of NA. Carbohydrate deprivation of nodules has been previously suggested as one of the factors of the acetylene-induce decline in experiments with removal of shoots [28]. Our plant-system, however, used intact plants.

4. Conclusion

The ability of photoautotrophic organisms and those associated with the *Trachypogon* roots to significantly contribute to nitrogen economy was clearly shown [6, 7]. The information obtained, however must be considered with regard to the limitations of acetylene reduction assay values and their conversion to N-fixing values as used by Hardy and Holsten (1977), particularly in field conditions, where a high coefficient of variation (CV) for AN was observed. CV values were around 20–25% and 20–40% for the microbial crusts in the protected and burned plots (Fig. 1), respectively, whereas for the plant-soil system the values were higher (Fig. 2), around 34–56% and 12–50 for the protected and burned plots, respectively.

The amount of N required for net primary production reached 43.6 and 43.0 kg ha^{-1} year^{-1} in the protected and burned plot, respectively (Table 3). Therefore, the lower fixation values for microbial crusts represent 6% of the N needed for annual plant production in the burned Trachypogon savanna and 9% in the protected savanna, according to their primary production levels.

N-fixation mediated by free-living organisms associated with grass roots can account for 43% of the N

Table 3
Main fluxes in N-budget for protected and burned savanna. Fluxes are presented in kg ha^{-1} year^{-1}. Adapted from Chacón et al. 1991

	Protected savanna	Burned savanna
Net primary production-N	43.6	43.0
N inputs		
Precipitation	6.2	6.2
N$_2$ fixation microbial crusts	4.0	2.4
N$_2$ fixation soil–plant system	7.8	13.7
N outputs		
Fire	0.0	8.2
Leaching	2.1	2.1

input in the protected, and 61% in the burned savanna (Table 3). Overall, out of the total amount of N required for the net primary plant production in the burned savanna, free living organisms could be contributing 37%, compared with 27% for protected savanna. In burned savanna such a contribution (16 kg ha^{-1} year^{-1}) exceeds the volatilisation loss (8 kg ha^{-1} year^{-1}, Table 3). The estimated amounts of dinitrogen fixation seem to be enough to maintain plant production under periodical fires, and in the conditions of this experiment.

Acknowledgments

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