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Root extracts of *Bracchiaria humidicola* and *Saccharum spontaneum* to increase N use by sugarcane

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ABSTRACT: Retaining the mineral N in the form of NH_4^+ in the soil for a lengthy period is desirable for reducing losses. Furthermore, there is evidence that sugarcane prefers NH_4^+-N in place of NO_3^--N. This study aimed firstly, to evaluate the potential of root extracts of *Bracchiaria humidicola* and *Saccharum spontaneum,* in contrast with the DCD (Dicyandiamide) inhibitor, to increase absorption of N by plants fertilized with ammonium sulfate, and secondly, to quantify the emission of N_2O fluxes with the use of this inhibitor. The experiment was developed in a glasshouse in an entirely randomized design where four treatments were applied: AS) ammonium sulfate (control); AS+DCD) ammonium sulfate associated with dicyandiamide; AS+BCH) ammonium sulfate associated with root extracts of *Brachiaria humidicola*; and AS+SCS) ammonium sulfate associated with root extracts of *Saccharum spontaneum*. Differences were observed in biomass production in plants 45 and 60 days after fertilization (DAF) and 15 and 60 days in biomass accumulation of roots. The application of AS associated with DCD synthetic inhibitor kept NO_3^--N values low throughout the evaluation period, while in other treatments the concentration increased right up to the second evaluation 15 DAF. Sugarcane plants did not benefit from the increased presence of ammoniacal N promoted by DCD. The use of DCD reduced the average flux of N_2O during the evaluation period compared to plants receiving AS treatments only, which was not observed when root extracts of *B. humidicola* and *S. spontaneum* were used.
Keywords: ammonium, nitrate, dicyandiamide, nitrification, nitrous oxide

Introduction

One of the most controversial issues in sugarcane crop management is nitrogen fertilization. Regardless of the dose of N-fertilizer used on this crop, the uptake efficiency of the plant in N provided by fertilizers is almost always lower than 50 % (Cantarella, 2007). For this reason, countless studies have focused on finding strategies to improve the efficiency of fertilizers that will yield a higher economic payback and reduce environmental impact. The losses of N in the sugarcane fields occur through volatilization of NH_3, leaching of nitric forms, and denitrification of the N-fertilizer to N_2 or N_2O (Cantarella, 2007). When added to the soil, under aerobic conditions, NH_4^+ from ammoniacal fertilizers can be rapidly nitrified (production of NO_3^-), N_2O is also a by-product of nitrification. NO_3^- from nitrification, or from nitrate fertilizers, is subject to leaching losses by water drainage, or denitrification under conditions of low oxygen supply (Pierzynski et al., 2000). In view of this, it is desirable to have N in the ammoniacal form, which may be adsorbed by soil negative charges. One of the strategies used to retain N as NH_4^+ after its application is the use of nitrification inhibitors. Dicyandiamide (DCD), which has bacteriostatic action and inhibits the first stage of nitrification, has been the product most often used for this purpose (Zaman et al., 2009). On the other hand, there is evidence that inhibition on nitrification occurs naturally in agricultural or uncultivated soils, since some plants produce compounds that have inhibitory action of nitrification, of which Brachiaria is one of the most common species (Subbarao et al., 2007). Countless studies have related cultivation or occurrence of *Brachiaria* sp. to the predominance of N in ammoniacal form (Carmo et al., 2005; Moro et al., 2013; Fernandes et al., 2011) It is possible that the direct ancestor of cultivated sugarcane, such as *Saccharum spontaneum*, has a similar mechanism, since sugarcane seems to prefer to absorb N as NH_4^+ instead of NO_3^- (Robinson et al., 2011). Confirmation of this information would be valuable to the genetic improvement of sugarcane, and may contribute to the understanding of the limited response of sugarcane to the application of N on certain occasions.

This study aimed to evaluate the potential of root extracts of *Bracchiaria humidicola* and *Saccharum spontaneum*, in contrast with DCD to increase the absorption of N by plants fertilized with ammonium sulfate and to quantify the emission of N_2O fluxes with the use of this inhibitor.

Materials and Methods

Characterization and experimental design

The experiment was developed in a glasshouse in Campinas-SP, Brazil (22°48'09'' S, 47°03'11'' O) during 96 days. Sugarcane bud planting took place on 29 Jan 2014; the last evaluation was made on 06 May 2014. The

experimental design was entirely randomized with four treatments and four replications. For the destructive evaluation, five sets of pots were installed for the five periods of evaluation. The treatments were: AS ammonium sulfate (300 mg kg^{-1} of N) (control); AS + DCD) ammonium sulfate + dicyandiamide (5 % of N dose); AS + BCH) ammonium sulfate + root extract of *Brachiaria humidicola*; AS + SCS) ammonium sulfate + root extract of *Saccharum spontaneum*. The amount of root extracts was 100 mL per pot. The N was applied exclusively as NH_4^+.

Each experimental unit consisted of one pot (6 L) without holes in the base, filled with 5.5 kg of air-dried soil sifted using a 2-mm mesh sieve removed from Psamments (Soil Survey Staff, 2010). The soil was removed from the surface layer (0-20 cm), an area cultivated with sugarcane for over 30 years using a system of prior burning of the crop, but having adopted mechanized harvest with preservation of residues on the soil over the past eight years. The physic and chemical characteristic of the soil are shown on Table 1.

Soil pH correction and the increase of calcium and magnesium contents were effected by the application of dolomitic lime (PRNT 90 %) to reach 70 % of base saturation of the cation exchange capacity (CEC), as described by Raij et al. (2001). After liming, the soil was incubated for 16 days, with moisture adjusted to 70 % of the maximum capacity of water retention.

The seedlings of the RB915156 variety of sugarcane (mini sets with a 25-mm-long bud) were taken from ripe stalks of plants cultivated in a nursery. The extracted buds were germinated and cultivated for three weeks in inert vermiculite deprived of nutrients. The plants were transferred to a plastic pot with supplementation of phosphorus (50 mg kg^{-1} of soil), together with 67 % of the recommended dose of potassium (118 mg K kg^{-1}) and 50 % of the need of micronutrients (3 mg Zn kg^{-1}; 1 mg B kg^{-1}; 0.5 mg Cu kg^{-1}; 5 mg Fe kg^{-1}; and 4 mg Mn kg^{-1}), the treatments were applied 20 days after transposition of the stocks. The remaining doses of potassium and micronutrients were applied 40 days after transplantation of the young plants.

Root extracts were obtained according to the methodology described by Gopalakrishnan et al., (2009), in which 400 grams of fresh roots of *Brachiaria humidicola* or *Sacharrum spontaneum* were washed in running water, set in a container, slightly macerated using a spatula and placed in 2 L of distilled water for 36 h under 20 ºC to transfer the extracts to the solution. Polar metabolites were extracted from 100 µL of root extracts of *Brachiaria humidicola* and *Sacharrum spontaneum*, using the methodology described by Giavalisco et al., (2011). After extraction, the samples were derivatized (Lisec et al., 2006) and analyzed using a GC-MS equipment. The parameters for acquisition of chromatograms were identical to those described by Weckwerth et al., (2004). Chromatograms were exported from the ChromaTOF software (version 3.25) to the R 2.12.2 software. Detection of peaks, alignment of retention times, and searches in libraries were done using the TargetSearch package of the Bioconductor (Cuadros-Inostroza et al., 2009). Metabolites were quantified by peak intensity of a selected mass, which was later, using an internal pattern (sorbitol C13), transformed by base-2 logarithm. Forty-five polar metabolites were found in the extracts of *B. humidicola* and *S. spontaneum*, but in concentrations very similar in both extracts (Table 2). After this period, the solution was filtered through analytical filter paper and kept chilled (2.5 ºC) up to the time of application. The plants of *B. humidicola* were obtained from an area where this species has grown for over 25 years, located in the municipality of Bebedouro-SP. The roots of *S. spontaneum* were collected at the breeding station in Camamu-BA.

Evaluations

Five evaluations were made to measure the availability of inorganic NO_3^--N and NH_4^+-N in the soil 7, 15, 30, 45, and 60 days after fertilization (DAF). The samples, once collected, were stored forthwith in a freezer to reduce microbiological activity, in order to prevent changes in the contents of NH_4^+-N and NO_3^--N in the soil.

For extraction of the inorganic forms of N, a sample of five grams of moist soil and 25 mL of solution of KCl 2 mol L^{-1} were shaken in an orbital shaker for one hour, then filtered through a blue ribbon analytical filter paper. Later, the inorganic N was determined via flow injection analysis (FIA).

The NH_4^+-N was analyzed in alkaline medium passing through a hydrophobic membrane (PTFE), with the flow directed to a conductivity cell (Reis et al., 1997). The forms of nitric N (NO_3^--N + NO_2^--N) were simultaneously determined by spectrophotometric method, with the reduction of nitrate to nitrite by a copperized cadmium column, followed by reaction of nitrate with sulfanilamide in an acidic medium forming an azo compound (Giné et al., 1980). The results of N mineral were corrected and expressed in a mass of oven-dried soil, after drying of subsamples at 105 ºC.

Table 1 – Chemical and physical characteristics of the soil used in experiment.

Depth	pH	T.O.C.	Al	NO_3^--N	NH_4^+-N	P	S	K	Ca	Mg	CEC	V	Sand	Silt	Clay
cm		g dm^{-3}		mg dm^{-3}				$mmol_c$ dm^{-3}				%	g kg^{-1}		
0 - 20	5.2*	8	<1	8.96	0.0	14	4	0.5	7	3	23.2	45	952**	23	25

*Analysis performed according to the methodology of Raij (2001); **Analysis performed according to the methodology of Embrapa (2013). T.O.C. = Total organic carbon; CEC = Cation exchange capacity.

Table 2 – Polar metabolites identified in the extracts of *B. humidicola* (BCH) and *S. spontaneum* (SCS).

Met ID	*BCH extract* Log 2	*SCS extract* Log 2	*Met ID*	*BCH extract* Log 2	*SCS extract* Log 2
2-Hydroxypyridine	14.51	14.85	Glutamate	10.36	11.19
Acetate	14.93	15.53	C5H10O5 [Ribulose|Xylulose]	9.89	7.59
Glycerol	15.93	16.60	(r|x) Putrescine	16.14	15.93
Isoleucine	NA	12.72	(r|x) Dodecanoate	13.42	13.44
Glycine	12.99	13.91	Phenylalanine	9.15	10.70
Orthophosphate	15.12	14.07	Ribonate	10.62	10.05
Benzoate	13.60	14.20	4-Hydroxybenzoate	13.93	12.56
Succinate	11.36	10.84	Arabinono-1.4-lactone	10.28	7.74
(r|z) Riboflavin	17.50	15.90	2-Aminoadipat	10.69	NA
Phenylacetate	8.89	7.63	(r|x) 1.5-Diaminopentane	15.67	12.34
Nicotinate	11.37	9.18	Suberate	9.80	9.71
b-Alanine	12.17	10.46	Ornithine	11.03	11.68
Erythrose	11.66	8.99	Tridecanoate	9.96	10.01
C4H10O4 [Erythritol|Threitol]	12.10	10.74	Quinate	10.24	10.01
Citramalate	9.70	7.95	C6H12O6 [Mannose|Allose]	17.10	17.58
Decanoate	12.23	12.72	Citrate	14.53	9.41
Malate	10.93	9.73	Glucose	NA	11.48
4-Aminobutanoate	13.97	10.64	Putrescine	10.94	9.84
Threonate	8.40	7.63	Tetradecanoate	14.97	15.55
Deoxyribose	11.00	10.06	myo-Inositol	16.03	9.25
Adipate	11.92	NA	4-Coumarate	11.56	11.53
1.3-Diaminopropane	13.33	11.14	Heptadecanoate	13.71	14.30
3-Hydroxybenzoate	9.98	11.12	Sucrose	12.54	12.35
5-Oxoproline	15.69	15.23			

In each evaluation, the following characteristics were also determined: *plant height* (H), measured from the base of the stalk up to the insertion of the +1 leaf; *plant diameter* (DIA), measured at the medium third of the plants; and dry biomass of plants and roots, including concentration of N in these two parts of the plant after processing. For this, the samples of the plant and roots were dried in a forced-air ventilation oven at 65 °C, with subsamples submitted to sulfuric digestion for the determination of total N using the Kjeldahl method (Nelson and Sommers, 1973).

N_2O emission was evaluated during the entire experimental period. For this we developed sampling chambers that were positioned inside each pot of the last group of pots to be dismounted 60 days after the application of the treatments. The structure of the chambers was in two parts: a PVC base with 5-cm in diameter and 15-cm high, and a lid made with 5-cm-diameter PVC caps. The lid was perforated and a butyl rubber plug (22.5 mm) was perfectly adjusted to prevent any gas leak. The chambers had a minimum volume of approximately 0.2 dm³. The bases were fixed to the soil next to the sugarcane plant and remained open, except during sampling. Samples for analysis of N_2O fluxes were taken daily during the first month of evaluation, and every other day during the second month. For this, the bases were closed by a perfectly adjusted lid, preventing any leak, and a sample of gas (20 mL) accumulated after 30 minutes was removed through the plug and stored in Exetainer flasks (12 mL). In order to determine the concentration of N_2O in the environment at the time of closing the chamber (*t0*), four samples of air from the glasshouse were taken, simultaneously with the plot samples. N_2O concentration was analyzed by gas chromatography (Chromatograph Model GC 2014), with determination of N_2O concentration by an electron capture detector (ECD) operating at 325 °C. Further details of the gas chromatography system may be found in the work by Vargas et al. (2014).

The gas flux (µmol mol h^{-1}) was calculated as a linear increment/decrease in concentration inside each chamber during the collection of samples, as suggested by Livingston and Hutchinson, (1995). The fluxes were corrected according to the temperature and atmospheric pressure inside the glasshouse, using the following equation (Jantalia et al., 2008):

$$f = \frac{CC_{t30} - C_{t10}}{\Delta t} * \frac{V}{A} * \frac{m}{V_m}$$

where: f is the flux of each gas; $\frac{\Delta C}{\Delta t}$ is the variation of concentration of each gas inside the chambers during the time they remained closed (30 minutes); V and A are, respectively, the volume and the area of the chamber; m is the molar mass of each gas, and Vm is the molar volume of N_2O at the sampling temperature. The fluxes were expressed in µg N m^{-2} d^{-1}. Estimates of the cumulative emission of each gas were made via linear interpolation between the daily fluxes of adjacent sampling dates, considering the interval between samplings.

Soil moisture was adjusted on a daily basis to 70 % of the maximum capacity of retention by weighing. The pots where the chambers were installed for sampling N_2O emissions were also weighed, and the moisture at the base of the chamber was corrected according to its area, with the use of a syringe. This operation was always carried out after collection of the gases. During the experimental period, the relative humidity of the air and the temperature inside the glasshouse were recorded hourly using a data logger. The mean temperature during the experimental period was 24 °C and the relative humidity 80 %.

Analyses of variance were performed to determine treatment effects. All comparisons were made at the 0.05 probability level except N_2O fluxes which used a probability level of 0.1, unless otherwise stated using the Tukey test.

Results

Biometric parameters and biomass and N accumulation in plants

The application of ammonium sulfate (AS) associated with nitrification inhibitors, dicyandiamide (DCD) and root extracts of *Brachiaria humidicola* (BCH), and of *Saccharum spontaneum* (SCS), did not increase the height nor diameter of the plants at most periods of (Table 3). 15 and 30 DAF, the application of BCH with ammonium sulfate had an effect superior to the control (AS) in relation to height; 45 DAF, the control plants showed smaller diameters than the plants treated with the two root extracts, no different from plants that received DCD as a nitrification inhibitor.

There was a difference ($p < 0.05$) between the treatments for biomass accumulation by the plants 45 and 60 DAF, and for biomass accumulation in the roots 15 and 60 DAF (Figure 1). As regards biomass accumulation, the highest yields was obtained with AS + BCH and AS + SCS 45 DAF. In turn, 60 DAF, the the biomass AS+SCS treatment was higher (Figure 1). The treatment with the highest root yield was AS + BCH, superior to both AS and AS + DCD 15 DAF, and superior to the AS + DCD at 60 DAF.

At 7 DAF, the concentration of N in the plant was lower in the AS + BCH and AS + SCS treatments. The application of DCD together with N increased the N content in the plant and in the roots to the same degree as at the beginning of the period (Table 4). However, the greatest concentration of nitrogen in plant and roots did not result in further accumulation of this nutrient, due to the lower accumulation of biomass obtained when the commercial nitrification inhibitor was used.

The concentration of N in the plant was reduced during the experimental period, except for the evaluation made 30 DAF. Contrarily, the concentration of N in the roots increased throughout this same period (Table 4). The general mean concentration of N in the plant was 40.9; 38.2; 45.7; 37.1 and 34.3 g kg^{-1} and in the roots was 24.6; 30.6; 34.9; 31.0 and 39.5 g kg^{-1} for the evaluations performed 7, 15, 30, 45, and 60 DAF, respectively.

Availability of NH_4^+-N and NO_3^--N in the soil

At 7 DAF, a higher peak of 250 mg NH_4^+-N kg^{-1} of soil relative to the application of 300 mg N kg^{-1} the soil was recorded for all treatments (Figure 2). Later we

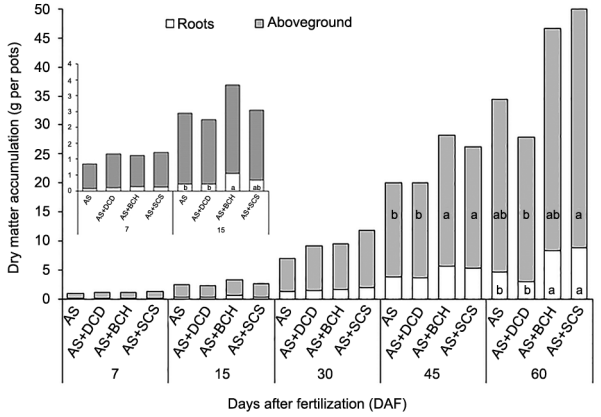

Figure 1 – Dry matter accumulation in plant and roots related the ammonium sulfate (AS) application with nitrification inhibitor dicyandiamide (DCD), and root extracts of *B. humidicola* (BCH), and root extracts of *S. spontaneum* (SCS).

Table 3 – Height (H) and diameter (DIA) of the plants related the ammonium sulfate (AS) application with nitrification inhibitor dicyandiamide (DCD) and extracts of *B. humidicola* (BCH) and *S. spontaneum* (SCS).

TRAT	Days after fertilization (DAF)											
	0		7		15		30		45		60	
	H	DIA	H	DIA	H	DIA	H	DIA	H	DIA	H	DIA
AS	10.5	3.5	10.8	3.1	15.8 bc	5.1	15.7 b	6.0	26.5	8.1 b	23.2	10.3
AS+DCD	10.1	4.0	12.3	3.6	13.1 ab	4.6	21.2 a	7.3	24.7	8.9 ab	23.7	10.5
AS+ BCH	10.3	2.7	11.4	3.6	17.0 a	4.9	20.9 a	7.6	23.8	10.3 a	24.0	10.4
AS+SCS	10.9	4.4	11.4	3.8	12.4 c	5.3	21.7 a	7.7	25.4	10.0 a	26.7	11.6
LSD	1.9	1.3	2.8	1.4	2.9	0.7	3.9	1.5	5.1	1.4	3.7	1.2
$p > 0.05$	ns	ns	ns	ns	*	ns	*	ns	ns	*	ns	ns

AS = Ammonium sulfate; DCD = dicyandiamide; BCH = Root extracts of *Brachiaria humidicola*; SCS = Root extracts of *Saccharum spontaneum*; H = Height of plants (cm); DIA = diameter of plants (mm); Means with the same lowercase letter in columns do not differ according to Tukey test $p < 0.05$; ns: not significant; LSD = least significant difference; *$p < 0.05$ = significant at 5 % by Tukey test.

verified a linear decrease in concentration of NH_4^+-N, this reduction occurring due to the nitrification of part of the N and also the increase in N consumption by the plants. In the final period of evaluation, the NH_4^+ content in the soil in the pots that received the AS + DCD treatment was almost twofold higher than the content in the control, indicating a more gradual oxidation of NH_4^+ to NO_3^-, evidencing inhibition in the process of nitrification of NH_4^+ from the fertilizer. Such inhibition was not detected with the use of root extracts of *Brachiaria humidicola* and *Saccharum spontaneum*, since for both treatments the contents of NH_4^+ were similar to the control during the entire evaluation period. The contents of NO_3^- were variable: higher contents were confirmed in the intermediate period for the treatments AS + BCH and AS + SCS (30 DAF), reaching the peak 45 DAF in the AS. At the end of the experimental period, the contents were similar for both.

N_2O Fluxes

In spite of the experiment having been conducted in a semi-controlled environment, the N_2O fluxes had a temporal oscillation as a function of the daily variation in temperature. Negative fluxes, indicating N_2O consumption by the soil, were also recorded. At 60 days after the evaluation that followed the application of the nitrogen fertilizer, associated or not with DCD and to the root extracts, the N_2O fluxes oscillated between < 0.1 to 3.4; 0.1 and 1.4; < 0.1 to 3.4; and < 0.1 to 1.9 mg N_2O-N

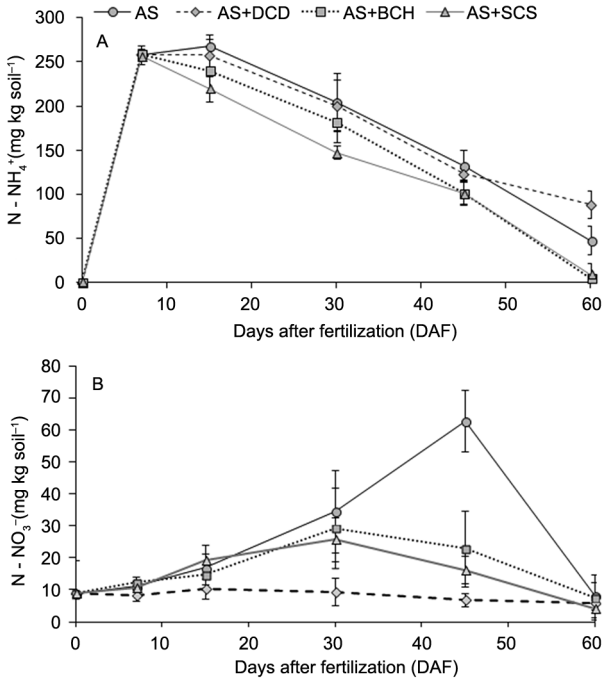

Figure 2 – Availability of NH_4^+-N (A) and availability NO_3^--N (B) in the soil during the experimental period as a function the ammonium sulfate (AS) application with nitrification inhibitor dicyandiamide (DCD), and root extracts of *B. humidicola* (BCH), and root extracts of *S. spontaneum* (SCS).

Table 4 – N content and N accumulation in the plant and roots during the experimental period related the ammonium sulfate (AS) application with nitrification inhibitor dicyandiamide (DCD), and root extracts of *B. humidicola* (BCH), and root extracts of *S. spontaneum* (SCS).

Treatments	Days after fertilization (DAF)									
	7	15	30	45	60	7	15	30	45	60
	N content					N Accumulation				
	g kg^{-1}					mg per pot				
	N in plant									
AS	45.9 a	40.5	47.9	36.7	34.8 b	35.2	90.2	278.8	602.0	1037.6
AS+DCD	44.3 ab	37.9	48.9	37.8	40.0 a	46.1	75.7	353.1	613.2	1003.2
AS+ BCH	35.5 b	37.5	46.4	36.7	31.2 b	34.9	104.4	369.7	825.7	1164.0
AS+SCS	38.1 b	36.9	39.8	37.2	31.3 b	41.6	80.0	390.3	780.6	1273.5
LSD	10.2	5.6	19.1	6.2	6.1	12.3	33.3	223	199.2	438.0
CV	15.6	30.1	26.1	10.5	11.2	19.5	23.7	40.0	17.6	24.8
$p > 0.05$	*	ns	ns	ns	*	ns	ns	ns	ns	ns
	N in roots									
AS	22.9	21.3	36.9	34.0	43.5 ab	1.97	4.5 b	44.8	129.2	195.1 bc
AS+DCD	19.9	32.9	35.7	30.5	45.3 a	2.29	8.1 b	47.4	108.3	130.5 c
AS+ BCH	23.8	34.9	36.7	27.2	37.0 ab	3.52	19.7 a	59.8	155.6	294.4 a
AS+SCS	20.6	33.2	30.4	32.6	33.2 b	2.45	12.3 ab	55.6	155.1	276.6 ab
LSD	6.7	12.7	10.0	11.7	11.2	2.25	9.0	35.6	86.1	94.4
CV	19.2	25.9	17.9	23.64	17.7	55.2	50.7	42.8	39.3	26.3
$p > 0.05$	ns	ns	ns	ns	*	ns	*	ns	ns	*

AS: Ammonium sulfate; DCD: dicyandiamide; BCH: Root extracts of *B. humidicola*; SCS: Root extracts of *S. spontaneum*; LSD: least significant difference; CV: Coefficient of variation; Means with the same lowercase letter in columns do not differ according to Tukey test $p < 0.05$; ns: not significant; *$p < 0.05$ = significant at 5 % by Tukey test.

m^{-2} d^{-1}, respectively, in the AS, AS + DCD, AS + BCH, and AS + SCS treatments. Considering the accumulated losses of N as N_2O, the highest emission was observed in pots fertilized with ammonium sulfate only, and when the fertilizer was associated with the extract of *B. humidicola* (AS + BCH). In these cases, N_2O emission exceeded 60 µg m^{-2} in the last evaluation 60 DAF. On the other hand, the use of DCD associated with ammonium sulfate reduced the emission of N_2O by 57 % in relation to the use of that nitrogen fertilizer (Figure 3).

Discussion

Evaluating root exudates, Subbarao et al., (2007) found positive effects of sorghum and peanuts as inhibitors of nitrification; among forage grasses, the most intense inhibition of nitrification was found in *B. decumbens* and *B. humidicola*. Sugarcane was not included in their study. Since sugarcane seems to prefer absorption of NH_4^+-N, it is possible that its roots also produce compounds that preserve this chemical form of N in the soil.

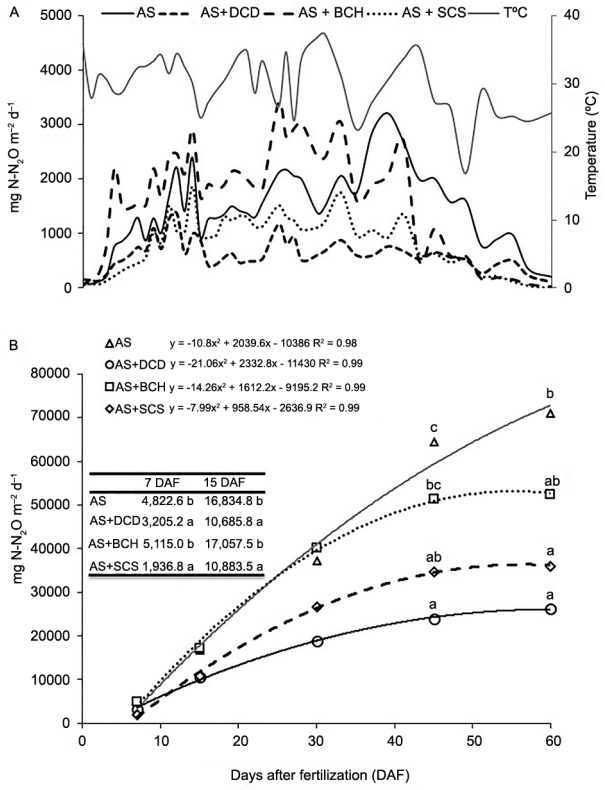

Figure 3 – N_2O daily fluxes (A) and cumulative N_2O emission (B) as a function the ammonium sulfate (AS) application with nitrification inhibitor dicyandiamide (DCD), and root extracts of *B. humidicola* (BCH), and root extracts of *S. spontaneum* (SCS). *Means with the same lowercase letter in columns and the same evaluation do not differ according to Tukey test $p < 0.1$.

S. spontaneum, a species that forms the sugarcane hybrids currently cultivated, contributes mainly to provide rusticity and an abundant root system (Matsuoka and Garcia, 2011), and may be a possible source of this characteristic. Largely cultivated in the Brazilian savannas, pastures of *B. humidicola* and *B. decumbens* are highly adapted to conditions of low availability of N. In this sense, as reported by Subbarao et al., (2007), it is possible that species better adapted to the Brazilian savannas are provided with mechanisms to conserve and use N efficiently, the main factor limiting growth and yield (Lata et al., 2004).

Subbarao et al. (2009) were able to isolate an effective nitrification inhibitor (brachialactone) from the root exudates of *B. humidicola*. Gopalakrishnan et al., (2009) also reported a 29 % reduction in nitrification with an application of 182 mg $(NH_4)_2SO_4$-N kg^{-1} of soil associated with the application of exudates of *B. humidicola*.

Although this study was not designed to measure the inhibiting effect of nitrification of *B. humidicola* and *S. spontaneum*, the content of NO_3^--N in the soil 45 days after application of ammonium sulfate containing DCD or extracts of the two species of grasses was lower than that observed in the control treatment, only with the nitrogen fertilizer (Figure 2). However, the inhibiting effect, if any, was smaller than that of DCD, which maintained the contents of nitrate in the soil at about 10 mg N kg^{-1} during the entire period, while extracts of roots of grasses were not so effective in the samples of soil taken 15 and 30 days after fertilization (Figure 2).

The data on N_2O emission (Figure 3) may also be indirect indicators of nitrification inhibition. The emissions were lower ($p < 0.05$) in the treatment with DCD (Figure 3). Several studies indicate that nitrification inhibitors result in reduction of N_2O emission (Snyder et al., 2009; Snyder et al., 2014), including in sugarcane (Soares et al., 2015). Nevertheless, in the present study, some reduction of N_2O was observed with the treatment containing extract of *S. sponteneum*, but not with *B. humidicola* (Figure 3). Accordingly, the evidence that the extracts of roots of these grasses led to reductions in nitrification in this study may be considered preliminary and not conclusive. Subbarao et al., (2009) indicated that production of nitrification inhibitor compounds by roots is stimulated when plants grow in a medium containing ammoniacal N. The pasture from where the plants of *B. humidicola* were obtained had no fertilizer, and there are no records of the management practices in adopted the area from which the roots of *S. spontaneum* were removed. Therefore, it is possible that the production conditions of these grasses had not been ideal for the formation of nitrification inhibitors.

Other studies conducted in Brazil also indicate that the effects of brachiarias were relatively little or almost insignificant in terms of nitrification reduction. Moro et al., (2013) did not find effects of prior cultivation of brachiarias, including *B. humidicola*, on the accumulation of NH_4^+ in soil in comparison with the use of

DCD. Otherwise, Fernandes et al., (2011) observed that the content of nitrate in soil fertilized with ammonium sulfate was lower when *B. brizantha*, *B. ruziziensis* and *B. decumbens* had been previously cultivated, as compared to the control treatment, without plants.

Since the soil used in the experiment was obtained from a sugarcane plantation, it is possible that the microbiota responsible for nitrification had been sufficiently active, since at the time of the application of treatments, the quantity of NO_3^--N was about 9 mg NO_3^--N kg^{-1} of soil, and the availability of NH_4^+-N was very low. In spite of the values of NO_3^--N 45 DAF in the AS + DCD treatment having been lower ($p < 0.05$) than the AS, the low values of NO_3^--N observed during the study are due to the effect of the nitrification inhibitor 45 DAF there was a peak of NO_3^--N in the soil in the AS treatment, indicating higher nitrification in the treatment without application of the inhibitor.

Even without causing an inhibitory effect in the nitrification process, the addition of root extracts of *B. humidicola* and *B. decumbens* to the nitrogen fertilizer enhanced sugarcane growth (Figure 1). This effect may be related to the presence of about 45 compounds in the extracts of BCH and SCH. The action of a number of these metabolites on the growth of roots and plant has already been proved. This was the case of the nonprotein gamma-aminobutyrate (GABA), whose exogenous application may influence the absorption of nitrate, stimulating root growth as observed in *Arabidopis thaliana* by Barbosa et al. (2000). Another example of positive effects on the growth of plants was reported by Stoyanova and Doncheva, (2002). These authors demonstrated that the presence of succinate promotes higher root growth by increasing the absorption of phosphorous. Considering that the solution of BCH and SCH had been extracted from root macerate, it is probable that it contained a significant quantity of phytohormones such as auxin (AIA). Synthesized in the apical meristem and translocated toward the root system where it promotes the growth of this tissue (Overvoorde et al., 2010).

The addition of DCD together with ammonium sulfate has caused a more gradual oxidation of NH_4^+ to NO_3^-, reducing nitrification. As a consequence, a reduction in the daily fluxes of N_2O (Figure 3) was verified, providing a reduction in accumulated N_2O emission equivalent to 57 % at the end of the experiment. In spite of there continuing to exist controversial information on the use of these additives under different agricultural systems, the effect of DCD has resulted in an effective strategy in the reduction of N_2O emission associated with the use of nitrogen fertilizers in sugarcane in Brazil, a country where, in view of the tropical climate, one could expect lower efficiency of these molecules. Vargas et al. (2014) demonstrated that the synergistic effect of keeping the straw of the sugarcane and nitrogen fertilization on the emission of N_2O was reduced by the use of DCD together with the nitrogen fertilizer by up to 70 %, depending on the quantity of straw on the soil. In a field experiment conducted over two years, Soares et al., (2015), found that reductions in N_2O emission as a function of the application of DCD together with urea in sugarcane may be in the range of 81-95 %. These authors have also verified that reapplication of DCD did not cause any loss of efficiency of DCD in its mitigating effect on N_2O emission.

Thus, this study confirms previous reports that the use of nitrification inhibitors is a tool which can reduce the environmental impact of nitrogen fertilization of sugarcane. Nitrification inhibitors can contribute to reductions in N_2O emissions, by delaying the nitrification process in the soil (Weiske et al., 2001; Chen et al., 2010), and by reducing the quantity of NO_3^- available to denitrification that occurs under conditions of low pressure of O_2. In this study, the losses of N_2O in both processes may have occurred simultaneously, but it is probable that losses caused by nitrification may be predominant, since there was no limitation of O_2 during the experiment, and the soil, formed almost totally of sand, had little or no sites with low O_2 pressure as a function of its macroporosity.

The root extracts of *B. humidicola* did not reduce N_2O emission when it was associated with ammonium sulfate in comparison with AS alone (Figure 3). It is possible that the method used to obtain the root extracts may have influenced the capacity of inhibiting nitrification and the N_2O emission. When performing a slight maceration of the root system, there is a release of soluble C applied to the soil together with the extract. The increase in availability of soluble C may have stimulated denitrification as it is a source of energy for microorganisms (Firestone and Davidson, 1989), even if the extracts had acted inhibiting nitrification and reducing the availability of NO_3^- in the soil. Under natural conditions in the brachiaria pastures, the brachialactone is released in the soil as a function of the availability of NH_4^+, and probably there is no increase in the concentration of soluble C as a function of this release. Although further studies may be required, Subbarao et al. (2007) observed that in the brachiaria pastures the population of nitrifiers is suppressed, directly influencing nitrification and the production of N_2O. The addition of DCD caused a reduction in nitrification, with no effect on the production of dry matter or accumulation of N in the sugarcane plants cultivated in pots. This indicates that, at least in the initial stages of cultivation, the predominance of N in the ammoniacal form brought no nutritional advantages to the plants. Accordingly, the data does not allow us to confirm the observations of Robinson et al., (2011) as regards preferential absorption of NH_4^+ by this species.

Conclusions

The application of root extracts of *Brachiaria humidicola* and *Saccharum spontaneum* together with ammonium sulfate increased initial growth in sugarcane,

but the effect on nitrification and N_2O emission from the soil was of low significance. The use of DCD together with the N-fertilizer inhibited nitrification of the N applied, and consequently, the emission of N_2O from the soil, without increasing initial sugarcane growth. The plants did not benefit from the increased presence of ammoniacal N promoted by DCD. The addition of grass extracts promoted an increase in yield of dry matter of sugarcane, probably due to the effect of existing growth promoting compounds in the extracts.

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