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Acidification in Rhizospheric Soil of Field-Grown Sorghum Decreases Nitrification Activity

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Abstract

To date, most studies on biological nitrification inhibition (BNI) in sorghum have been performed with plants grown in hydroponic systems. However, the current study was conducted to determine whether or not sorghum inhibits nitrification in fields of Alfisols, and clarify the mechanism that results in inhibition of soil nitrification in the field. Nitrification activity in the rhizosphere of sorghum (*Sorghum bicolor* (L.) Moench) i.e. soil attached to its roots within a few millimeters was measured and compared with those in adjacent bulk soil. Sweet sorghum (6 varieties) and grain sorghum (3 varieties) were cultivated in 4 Alfisol fields in a semi-arid tropical region of India during the 2010 or 2011 rainy seasons. Soil samples were collected three times during the growing season. Nitrification activity in the rhizospheric soil was significantly lower than that in the bulk soil during 8 out of 12 samplings while the pH (H_2O, 1:2) of the rhizospheric soil was significantly lower than that of the bulk soil in 10 out of 12 samplings. Acidification of the soil by sulfuric acid decreased the nitrification activity to a comparable extent, as emerged in the rhizospheric soils. Our results indicate that acidification of soil around roots would be one of the main causes of nitrification inhibition by sorghum in the field.

Discipline: Soils, fertilizers and plant nutrition
Additional key words: Alfisol, biological nitrification inhibition, soil pH

Introduction

The ability of plants to release inhibitory compounds from roots to regulate/control soil nitrification is termed 'biological nitrification inhibition (BNI)'. The existence of the BNI in tropical pastures (*Brachiaria humidicola*), sorghum (*Sorghum bicolor* (L.) Moench) and wild wheat (*Leymus racemosus*) has been reported (Subbarao et al. 2007, 2009a, 2009b, 2012, Zakir et al. 2008). The BNI can potentially improve N uptake and N use efficiency (Subbarao et al. 2012). Evaluation of tropical forage grasses, cereal, and legume crops has indicated significant diversity in the BNI capacity (Subbarao et al. 2007). Among these plants, *Brachiaria spp*. performed the highest BNI capacity, while substantial genotypic variation was detected in BNI capacity within *B. humidicola*. Forage grasses of *B. humidicola* and *B. decumbens*, which are highly adapted to the low-N production environments of South American savannas (Miles et al. 2004, Rao et al. 1996), showed the highest BNI capacity among the tropical grasses tested (Subbarao et al. 2007, 2009a). Most of those works, however, were performed with plants grown in hydroponic systems, which is the only way to collect root exudate. Because soil conditions are so complicated to show strong evidence of the BNI, the BNI has never been experimentally proven in soil-plant systems.

It has been already shown that soil pH is the major factor regulating the nitrification process in soil. Sahrawat (2008) reported that the optimum soil pH for nitrification was around 8.5. Reducing the soil pH to a sub-optimal

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value results in reduced nitrification. Plant roots mediate pH changes in the rhizospheric soil via several processes (El-Sjatmawi & Makhadmeh 2001, Hinsinger et al. 2003) and the nitrification of ammonium fertilizer has been recognized as a major contributor toward acidification of agricultural soils (Yanai et al. 2000, Summer & Moble 2003).

Subbarao *et al.* (2009b) reported that sorgoleone (2-hydorxy-5-methoxy-3-[(z, z)-8', 11',14'-pentadecatriene]-p-benzoquinone) exuded from sorghum roots has a strong inhibitory effect on ammonia-oxidizing bacteria (*Nitrosomonas sp.*), and contributes significantly to the BNI function in sorghum.

Sweet sorghum has the ability to produce considerable carbohydrates like sugarcane, and can be used for multiple purposes such as human food, animal feed and bio-fuel (Rao et al. 2009). BNI in sweet sorghum has not been studied like that in grain sorghum.

Alfisol is a typical soil in semi-arid and sub-humid tropics, which covers about 10% of South America and 20% of Africa. In Asia, Alfisols are found in the dry zone of Sri Lanka and India (Kosaki 2001) and cover more than 20%, where sorghum is one of the major food crops.

The objective of this study is to obtain evidence of the BNI in sorghum in fields of Alfisols, and clarify the mechanism involved in inhibiting soil nitrification in the field.

Materials and Methods

Evaluation of BNI in soil from sorghum fields

Sweet sorghum and grain sorghum cultivars were grown in experimental Alfisol fields of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru (17.53 °N, 78.28 °E) during the rainy seasons of 2010 and 2011. The soil pH (H_2O, 1:2) of the surface layer (0-15 cm) prior to sowing was 8.1, 7.7, 7.4 and 5.6 for the Alfisol 1, 2, 3 and 4 fields, respectively. Alfisol 1 and 3 had been used for sorghum, pearl millet, groundnut and pigeonpea cultivation with irrigation every year in the previous decade, while the Alfisol 2 and 4 had been left fallow for most of the previous decade. Alfisol 2 was located at a bottom of a gentle slope while Alfisol 4 was located halfway up the slope.

Three grain sorghum cultivars (CSH 16, PVK 801 and HTJH 3201) and two sweet sorghum varieties (CSH 22SS and NTJ 2) were grown in two experimental fields of Alfisols (Alfisol 1 and 2) in ICRISAT during the rainy season of 2010. These sorghum varieties are recommended to farmers in the semi-arid tropical region of India by ICRISAT. The experimental set-up involved a randomized block design with three replications. The seeds were sown on the rows on June 22 in Alfisol 1 and July 2 in Alfisol 2 just after applying the basal fertilizer (urea: 30 kg N ha^{-1}, TSP: 40 kg P_2O_5 ha^{-1}, gypsum: 200 kg ha^{-1}, Boron: 0.475 kg ha^{-1} and $ZnSO_4$: 50 kg ha^{-1}). Urea (30 kg N ha^{-1} for each application) was top-dressed into the furrow along the row, one and two months after sowing (Table 1).

Six cultivars of sweet sorghum (CSH 22SS, NTJ 2, 675x700, ICSV 25263, ICSV 25274, ICSV 93046) were grown in two experimental fields (Alfisol 3 and Alfisol 4) in ICRISAT during the 2011 rainy season. The experimental design, method and application rate of fertilizers were unchanged from 2010 and the seeds were sown in rows on June 21 and 27 in Alfisol 3 and 4, respectively.

Soil was sampled from fields three or four times during the growing season. Three plants with roots and soil from the surface layer (0-20 cm) were collected from each plot. The plants were cut at ground level in the field and soil blocks with the roots (about 20 × 20 × 15 cm, width, length and depth, respectively) were brought to a pre-treatment room. The soil samples were separated into two parts, one

Table 1. Schedule of seeding, nitrogen fertilization and soil sampling (days after sowing)

	Alfisol 1	Alfisol 2	Alfisol 3	Alfisol 4
Ivents	Days after sowing			
Basal fertilizer application and sowing seeds	0	0	0	0
Top dressing (1st)	34	28	34	35
Soil sampling (1st)	41	38	41	42
Top dressing (2nd)	62	59	62	63
Soil sampling (2nd)	69	66	69	70
Soil sampling (3rd)	111	108	98	98
Soil sampling (for the supplemental experiment)	–	–	111	105

The sowing dates were June 22, 2010, July 1, 2010, June 21, 2011 and June 27, 2011 for Alfisols 1, 2, 3 and 4, respectively.

of which attached to the roots within a few millimeters (rhizospheric soil) and the other soil (bulk soil). After removing the bulk soil, the roots were shaken in the air and soil still attached to the roots was deemed rhizospheric soil. The rhizospheric soil and bulk soil from all three plants in the same plot were well mixed to estimate nitrification activity. Soil moisture and pH (H_2O, 1:2) for all soil samples was determined.

To estimate nitrification activity, the soil samples were taken into centrifuge tubes (each equivalent to 5 g of dry soil) and distilled water was added, followed by the addition of ammonium sulfate solution to samples held in tubes at supply 20 mg kg^{-1} as N. The final soil moisture was adjusted to make it equivalent to field capacity [0.24 as moisture/dry soil (w/w) (Sahrawat 1984)]. The samples were then sealed by Parafilm and incubated at 25°C. Ammonium and nitrate-N were extracted using 2 mol l^{-1} KCl solution at zero (before applying ammonium sulfate), one, two and three days respectively after incubation, and the extracts were kept in a cold room until analysis. Ammonium and nitrate concentrations in the soil extracts were determined by an auto-analyzer (SKALAR, Netherlands). Nitrate + nitrite accumulation rates (mg N per kg dry soil per day) were calculated from the change in the nitrate + nitrite concentrations during the incubation (i.e.: the regression line slope) and regarded as nitrification activity of the soil samples.

The effects of soil type (i.e. the rhizospheric or bulk soils), sorghum varieties and sampling times on the nitrification activity and soil pH were tested by analyzing variance (ANOVA). To evaluate the BNI activity of sorghum (sweet and grain sorghum) cultivars on the nitrification activity in rhizospheric soil, a paired t-test was conducted for the nitrification rates and soil pH between the rhizospheric and bulk soils for five (in the 2010 season) or six (in the 2011 season) varieties overall. The correlations between the nitrification rates and soil pH were then tested for the samples collected during each sampling.

Estimation of the nitrification inhibition rate

The nitrification inhibition rates in the rhizospheric soils to the bulk soils were calculated by the following formula:

Nitrification inhibition rate (%) = {1− (Nitrification activity in the rhizospheric soil / Nitrification activity in the bulk soil)} × 100(%) (1)

Effect of soil pH modification on nitrification activity

An experiment was conducted to evaluate how the acidification of rhizospheric soil affected nitrification activity. Soil was sampled from the bare (−N) plots and CSH 22SS plots in the Alfisol 3 and 4 fields at 111 and 105 days after sowing (DAS) respectively, in 2011. The sampled soil from the CSH 22SS plot was separated into rhizospheric and bulk soils as mentioned above and the pH of bulk and bare soils (−N) from Alfisol 3 was modified by sulfuric acid solution with three different strengths (0, 6.9×10^{-5} and 1.4×10^{-4} mol kg^{-1} dry soil). Similarly, the pH of bulk and bare soils (−N) from Alfisol 4 was modified by sulfuric acid solution with three different strengths (0, 6.0×10^{-5} and 1.2×10^{-4} mol kg^{-1} dry soil). Sulfuric acid was not added to the rhizospheric soils from both Alfisol 3 and 4. For all these bulk, bare and rhizospheric soils, ammonium sulfate (20 mg kg^{-1} as N) was added. The moisture contents were adjusted to 0.24 as moisture/dry soil (w/w). The samples were incubated as mentioned above and their nitrification activity was measured.

Results

The soil type (rhizospheric or bulk soils) significantly affected the nitrification activity for Alfisol 1, 3 and 4 (Table 2). Nitrification activities in rhizospheric soils were lower than those in bulk soils during eight out of 12 sampling times (Fig. 1), while ammonium N declined at a higher rate in the rhizospheric soil than the bulk soil in most sampling cases (Fig. 2), which showed that nitrification was inhibited in the hizospheric soils. There was a tendency for nitrification activities to decline in later growth stages except for Alfisol 3. Nitrification activities in the low pH Alfisol (Alfisol 4) were lower than those in Alfisols with higher pH.

The sorghum varieties significantly affected the nitrification activity for Alfisols 1, 3 and 4 (Table 2). In Tukey's multiple comparison test, nitrification activities did not differ significantly among the samples for all 12 sampling times, although the soil type significantly affected the soil pH for Alfisols 3 and 4 (Table 3). It was shown that the pH of the rhizospheric soils were lower than that of the bulk soils collected from the Alfisols fields in ten out of 12 sampling times (Fig. 3).

The nitrification activities and soil pH showed a significant positive correlation for Alfisols 3 and 4 except for Alfisol 3 at 98 days after sowing (Fig. 4), while the nitrification activity and soil pH had significant positive correlation for Alfisols 1 and 2 collected during the mid and late growth stages in the four fields.

The average nitrification inhibition rates of the three measurements ranged from −19 to 27% and were not consistent among the used varieties (Table 4). The nitrification inhibition rates were approximately 10-20% in most of the soil samples. The nitrification inhibition rates during the third sampling were exceeded those in the first and second

Table 2. Nitrification activities in soil samples (mgN kg^{-1} day^{-1})

Soil Type¶	Variety	Alfisol1 41 DAS	Alfisol1 69 DAS	Alfisol1 111 DAS	Alfisol2 38 DAS	Alfisol2 66 DAS	Alfisol2 108 DAS
Rhizospheric soil	CSH22SS	10.6	8.1	3.7	10.3	6.8	2.7
	NTJ2	8.6	7.1	3.3	9.7	9.6	4.2
	CSH16	9.3	7.4	4.3	12.2	7.8	4.3
	PVK801	11.6	7.9	4.5	14.8	10.8	6.2
	HTJH3201	9.0	8.6	5.0	13.1	9.6	4.8
Bulk soil	CSH22SS	10.4	8.2	5.4	10.4	7.7	5.8
	NTJ2	10.8	7.8	4.9	9.6	10.7	6.9
	CSH16	10.4	80	4.5	11.4	8.8	6.3
	PVK801	13.1	9.1	5.5	10.2	8.8	6.5
	HTJH3201	11.5	8.9	5.6	10.4	9.5	5.1
ANOVA							
A (soil type)			**			NS	
B (Variety)			*			NS	
C (Sampling timing)			***			***	
A×B			NS			NS	
A×C			NS			*	
B×C			NS			NS	
A×B×C			NS			NS	

Soil Type¶	Variety	Alfisol3 41 DAS	Alfisol3 69 DAS	Alfisol3 111 DAS	Alfisol4 38 DAS	Alfisol4 66 DAS	Alfisol4 108 DAS
Rhizospheric soil	CSH22SS	6.1	5.3	6.3	1.9	2.0	1.4
	NTJ-2	5.6	4.9	5.9	3.3	2.2	1.9
	675X700	5.3	4.2	5.7	3.2	2.4	1.7
	ICSV25263	5.0	4.2	5.6	2.9	2.4	1.7
	ICSV25274	5.2	4.8	6.1	3.0	2.6	1.5
	ICSV93046	5.5	5.3	6.1	3.1	2.4	2.5
Bulk soil	CSH22SS	6.0	5.1	5.5	2.1	2.5	2.0
	NTJ-2	6.6	6.0	6.1	2.9	3.7	2.0
	675X700	6.0	5.7	5.4	3.3	3.7	2.4
	ICSV25263	5.4	5.5	5.7	3.2	3.5	2.5
	ICSV25274	6.3	5.4	6.5	3.2	3.3	2.1
	ICSV93046	7.0	6.9	6.3	3.7	3.9	3.0
ANOVA							
A (soil type)			**			***	
B (Variety)			*			**	
C (Sampling timing)			**			***	
A×B			NS			NS	
A×C			NS			*	
B×C			NS			NS	
A×B×C			NS			NS	

¶: Rhizospheric soil or bulk soil. *, ** and *** show significances of $p<0.05$, $p<0.01$ and $p<0.001$, respectively. NS: Not significant.

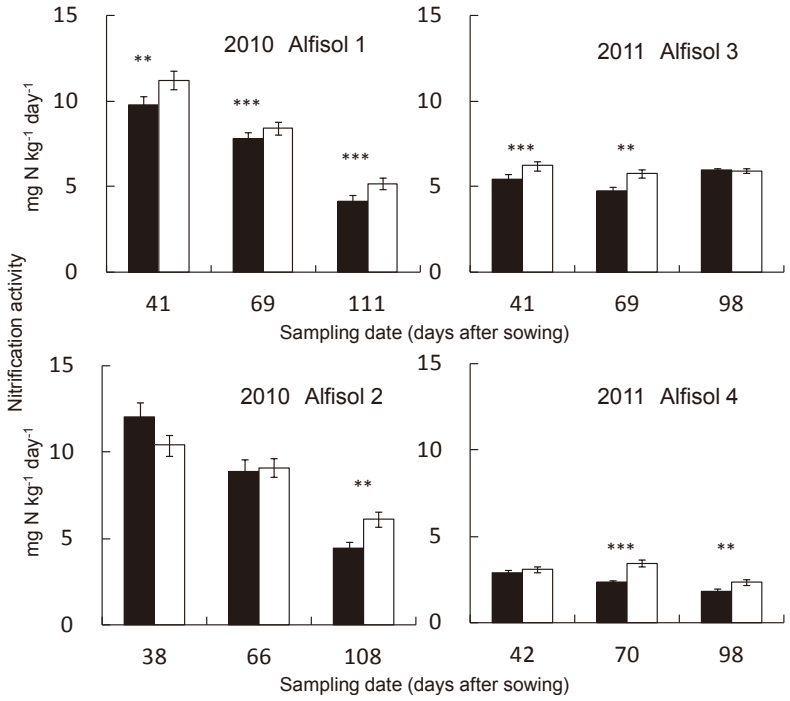

Fig. 1. Average nitrification activities in the rhizospheric and bulk soils in each sampling

Black and white bars indicate rhizospheric and bulk soils respectively. Vertical bars mean standard error. (n=15 for Alfisols 1 & 2 and n=18 for Alfisols 3 & 4). ** and *** show significant differences $p<0.01$ and $p<0.001$, respectively.

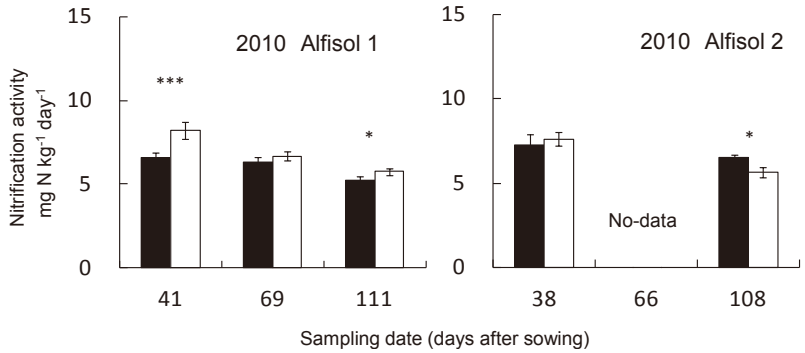

Fig. 2. Average rate of decline in ammonium in the rhizospheric and bulk soil in each sampling
Black and white bars indicate rhizospheric and bulk soils respectively. Vertical bars mean standard error (n=15). ** and *** show significant differences $p<0.01$ and $p<0.001$, respectively. Data for the Alfisol 2 (66 DAS) and Alfisols 3 and 4 were not available because the KCl used for the extraction was contaminated by ammonium.

Table 3. Results of analysis of variance to evaluate the effects of rhizospheric or bulk × variety × timing of the sampling on soil pH

	Alfisol 1	Alfisol 2	Alfisol 3	Alfisol 4
A (Rh or Bulk)	NS	NS	***	**
B (Variety)	NS	*	NS	NS
C (Timing)	*	**	***	*
A×B	NS	NS	NS	NS
A×C	NS	NS	*	NS
B×C	NS	NS	NS	NS
A×B×C	NS	NS	NS	NS

*, ** and *** show significances of $p<0.05$, $p<0.01$ and $p<0.001$, respectively. NS: Not significant.

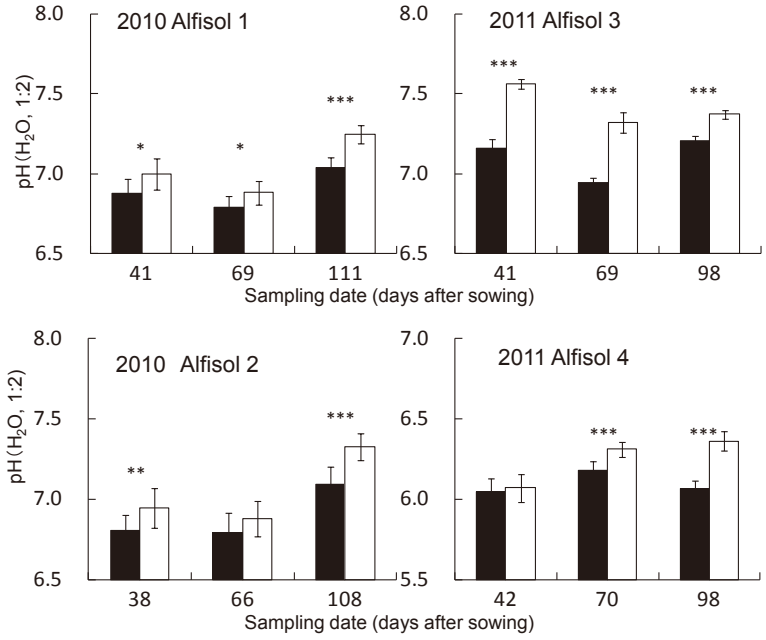

Fig. 3. Average pH (H_2O, 1:2) of the rhizospheric and bulk soils in each sampling
Black and white bars indicate rhizospheric and bulk soils respectively. Vertical bars mean standard error (n=15 for Alfisols 1 & 2 and n=18 for Alfisols 3 & 4). *, ** and *** show significant differences $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

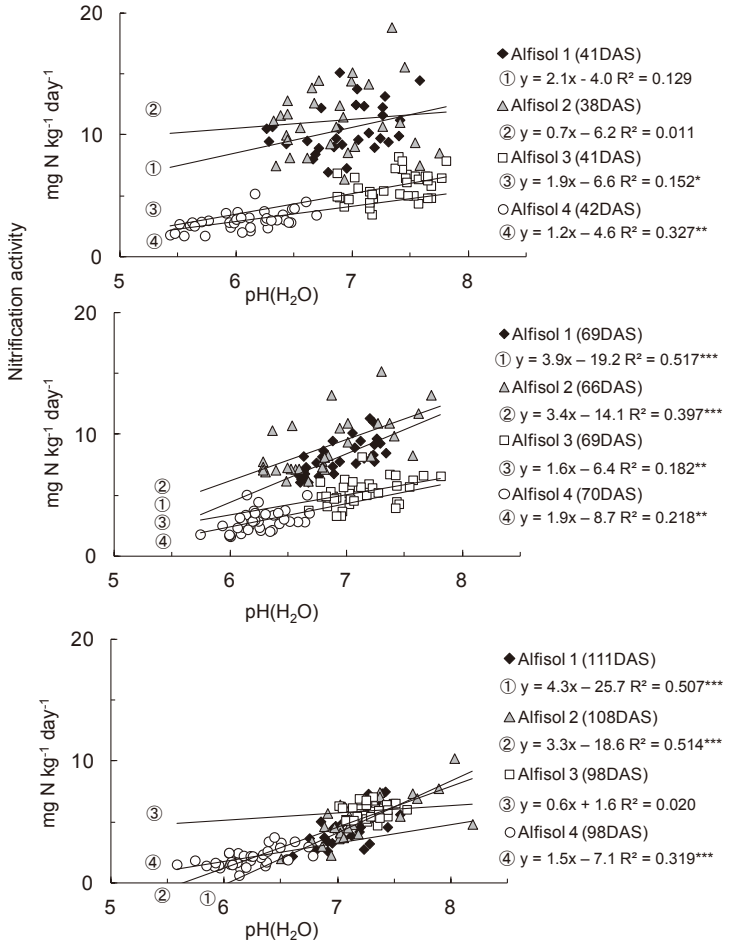

Fig. 4. Relationship between nitrification activity and soil pH (H_2O, 1:2) of rhizosphere soil

*, ** and *** show significant positive correlation $p<0.05$, $p<0.01$ and $p<0.001$, respectively. The upper, center and lower graphs indicate samples collected for the first, second and last time for each field, respectively.

Table 4. Nitrification inhibition (%) in rhizospheric soil of sorghum varieties

	Alfisol 1				Alfisol 2			
	41DAS	69DAS	111DAS	Avg.	38DAS	66DAS	108DAS	Avg.
CSH22SS	-3	2	33	11	17	11	53	27
NTJ2	20	9	32	20	-17	10	35	9
CSH16	10	7	6	8	6	9	30	15
PVK801	10	13	17	13	-36	-24	2	-19
HTJH3201	19	3	11	11	-31	-1	5	-9

	Alfisol 3				Alfisol 4			
	41DAS	69DAS	98DAS	Avg.	42DAS	70DAS	98DAS	Avg.
CSH22SS	-3	-8	-14	-8	10	19	27	19
NTJ-2	16	19	4	13	-15	38	4	9
675X700	13	27	-7	11	3	34	27	21
ICSV25263	7	22	2	10	10	33	27	24
ICSV25274	19	12	6	12	6	21	25	17
ICSV93046	22	22	4	16	9	32	10	17

samplings in 2010, but this trend was not observed in 2011 to the same degree as in the rhizospheric soils.

It was shown that acidifying the soil by applying sulfuric acid decreased the nitrification activity to the same degree as observed in rhizospheric soils (Fig. 5).

Discussion

The nitrification activity and soil pH showed a positive correlation on nine of the 12 sampling occasions (Fig. 4). In addition, the rhizospheric soil samples showed significantly lower nitrification activity and soil pH than the bulk soil samples in 8 and 10 of the 12 sampling times, respectively (Fig. 1 and 3), which suggests that the lower nitrification activity in the rhizospheric soil in the sorghum field would be due to acidification. This assumption is supported by the decline in nitrification activity alongside a drop in soil pH by adding sulfuric acid (Fig. 5). The slopes of the regression lines in Fig. 4 were almost comparable of those in Fig. 5. Although we cannot exclude the possibility that other mechanisms were working, it could be concluded that the acidification of rhizospheric soil would play a significant role in inhibiting nitrification by sorghums in Alfisol fields.

Analyzed data of the nitrification activities and soil pH seemed to be scattered around the linear regression line for the four experimental fields during the third sampling time (bottom graph in Fig. 4). In Alfisols 1 and 2, the nitrification activities of the soil samples collected during the first and second sampling times exceeded those at the third sampling time (Fig. 1 and 4). We suspect that the nitrification was accelerated in these samples due to highly available ammonium (ammonia). Some samples collected from Alfisols 1 and 2 at the first and second top-dressing contained higher ammonium and nitrite + nitrate concentrations than those collected at the third sampling time (data not shown). It is known that nitrification becomes active after nitrogen fertilizers such as urea and ammonium sulfate are applied (Sahrawat 2008). In addition, we suspect that the availability of ammonium (ammonia) was widely uneven in the field for 1 week or more after the urea applications. Under such conditions, nitrification might be partially accelerated and the unevenly accelerated nitrification might disturb the relation between nitrification and soil pH. The nitrification activity and soil pH showed no significant correlation in Alfisols 1 and 2 during the first sampling. Despite significant correlation between nitrification activity and soil pH in Alfisols 1 and 2 during the second sampling, the dispersion of the correlation exceeded that for the third sampling. The nitrification activity probably returned to a stable and uniform condition by the time of the third sampling i.e. more than 1 month after the second top-dressing. For the samples in Alfisols 3 and 4, no such tendency emerged. We cannot find a plausible explanation for these differences among Alfisols 1 & 2 and 3 & 4, respectively.

Plants uptaking NH_4^+ release protons to the rhizosphere to counterbalance the corresponding excess of positive charges, thereby reducing the rhizosphere pH (Gahoonia et al. 1992, Imas et al. 1997). It can be predicted that lower nitrification activity in the rhizospheric soil due to such acidification may be observed not only in sorghum, but also other plant species. The reduced pH in rhizospheric soil might be due to organic acids exudated from the sorghum roots. Many plants, including sorghum, are known to release organic acids from their roots (Jones 1998, Schwab et al. 1983). Plants also release significant amounts of photosynthetically derived compounds into the

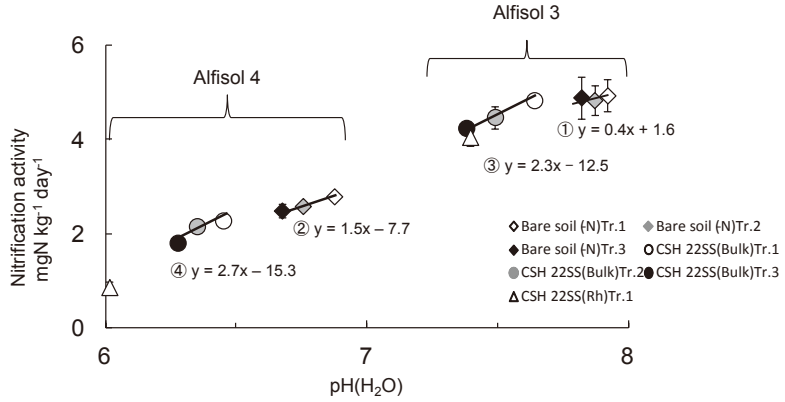

Fig. 5. Effects of soil pH modification on nitrification activity

The bulk and bare soils (−N) from Alfisol 3 were amended with sulfuric acid solution of three different strengths (0, 6.9×10^{-5} and 1.4×10^{-4} mol kg^{-1} dry soil). The bulk and bare soils (−N) from Alfisol 4 were also amended with sulfuric acid solution of three different strengths (0, 6.0×10^{-5} and 1.2×10^{-4} mol kg^{-1} dry soil) ① ② ③ ④ indicate the regression line for the Tr. 1, 2 and 3 of the bare soil of Alfisol 3, the bare soil of Alfisol 4, CSH22SS (Bulk) of Alfisol 3 and CSH22SS(Bulk) of Alfisol 4, respectively.

rhizosphere through root exudation and these compounds affect microbial abundance and activity in the rhizospheric soil compared to bulk soil (El-Shatnawi & Makhadmeh 2001, Shi et al. 2011). Subbarao *et al*. (2009b) reported that sorgoleone, a *p*-benzoquinone exuded from sorghum roots, has a strong inhibitory effect on ammonia-oxidizing bacteria (*Nitrosomonas sp.*) and hence concluded that th e compound contributes significantly to the BNI activity of sorghum. Considering the molecular structure, it is unlikely that exudation of sorgoleone decreases soil pH in the rhizosphere. Accordingly, it is expected that the inhibitory mechanism of nitrification by BNI compounds such as sorgoleone may be triggered not through acidification, but a more direct pathway. As Dayan (2006) reported that sorgoleone production increased with declining pH of hydroponic media, soil pH may affect BNI through root exudates. Since BNI theory has been developed in hydroponic systems with bioassays in laboratories, it has yet to be proven in soil-plant systems, particularly in fields where crops are cultivated. Although our study showed that acidification of soil would be a main driving force for nitrification inhibition in rhizosphere, root exudates such as sorgoleone also may enhance BNI simultaneously. Mainly due to technical difficulties in soil, which contains numerous and wide-ranging organic compounds, it remains unclear whether specific compounds exudated from plants inhibit nitrification in the soil-plant system. Further studies are necessary to clarify the contribution of root exudates to the BNI activity in the field.

The nitrification inhibition we detected in the sorghum field is considered rather moderate and can be observed only at a certain distance from the root surface i.e. the rhizospheric soil. It seems the BNI of the sweet sorghum detected in this study is so moderate that its effect in terms of improving nitrogen fertilizer efficiency would be limited. Many environmental factors such as aeration (oxygen), temperature, moisture and the abundance of ammonium ions affect nitrification in soil (Sahrawat 2008, Yuan et al. 2005). We must find plants with stronger BNI and/or conditions under which nitrification will work to favor nitrogen usage by crops in the field.

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