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REGULAR ARTICLE

Biological nitrification inhibition in sorghum: the role of sorgoleone production

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Abstract
Background and aims Nitrification and denitrification are the two most important processes that contribute to greenhouse gas emission and inefficient use of nitrogen. Suppressing soil nitrification through the release of nitrification inhibitors from roots is a plant function, and termed "Biological Nitrification Inhibition (BNI)". We report here the role and contribution of sorgoleone release to sorghum-BNI function.
Methods Three sorghum genotypes (Hybridsorgo, IS41245 and GDLP 34-5-5-3) were evaluated for their capacity to release sorgoleone, which has BNI-activity, in hydroponic and soil culture. Sorgoleone released was measured using HPLC; BNI-activity was determined using a luminescent recombinant *Nitrosomonas europaea* assay.
Results Sorgoleone production and BNI-activity release by roots are closely associated (1 μg of sorgoleone is equivalent to 1 ATU activity in assay). Purified sorgoleone inhibited *Nitrosomonas* activity and suppressed soil nitrification. Sorghum genotypes release varying quantity of sorgoleone; GDLP 34-5-5-3 and Hybridsorgo showed higher capacity for both sorgoleone release and BNI-activity than did IS41245. In soil culture, GDLP 34-5-5-3 released higher quantity of sorgoleone into the rhizosphere, which had higher BNI-activity, and suppressed soil nitrification to a greater extent than did by IS41245.
Conclusions These results demonstrate genetic differences for sorgoleone release and its functional link with BNI-capacity; there is potential for genetic improvement of sorghum BNI-capacity and deployment of this in low-nitrifying sorghum production systems.

Keywords Biological nitrification inhibition (BNI) · BNI-capacity · Genotypic variation · Hydrophobic-BNIs · Nitrification inhibition · Sorghum · Sorgoleone

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Introduction

Nitrification is biological oxidation of ammonia to nitrate, and is an important process in the soil nitrogen (N) cycle. The oxidation of ammonia to nitrite is performed by two groups of organisms, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Treusch et al. 2005; Walker et al. 2010; Shen et al. 2012). In soil, the most studied AOB belong to the genera *Nitrosomonas* and *Nitrosococcus* (Chain et al. 2003; Klotz et al. 2006). The second step of nitrification,

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where nitrite is oxidized to nitrate is carried out mainly by bacteria of the genus *Nitrobacter* (Starkenburg et al. 2006; Hayatsu et al. 2008).

In terrestrial ecosystems, nitrification plays a key role in the N-cycle. A rapid conversion of the relatively immobile NH_4^+ to highly mobile NO_3^- leads to decreased nitrogen-use efficiency (NUE) and poses hazards to environmental quality (Subbarao et al. 2013b). The NUE for global cereal production is 33 %; and the unaccounted 67 % N represents an annual loss of US $16.7 billion (Raun and Johnson 1999). Economic loss apart, the unaccounted N poses serious hazards to environmental quality from NO_3^- pollution of surface and ground waters, and through the emissions of N_2O and NO. The loss of N by NO_3^- leaching is expected to reach 61.5 Tg N y^{-1} by 2050 (Schlesinger 2009). Globally, agricultural systems contribute nearly 30 % of nitric oxide (NO) and 70 % of nitrous oxide (N_2O) emission to the atmosphere (Bremner and Blackmer 1978; Smith et al. 1997; Hofstra and Bouwman 2005). Under natural ecosystems, nitrification can potentially lead to N starvation, which in turn forces plants to develop strategies to protect available N from loss (Subbarao et al. 2009, 2013a). Some plants have developed mechanisms to suppress soil nitrifier activity, a plant function termed as "biological nitrification inhibition (BNI)". This has been well characterized in *Brachiaria* pasture grasses (Ishikawa et al. 2003; Jones 2013; Subbarao et al. 2006, 2009).

Sorghum is the fifth most widely cultivated cereal grain crop globally; and is grown around the world, largely in drought-prone and less-developed regions of the arid and semi-arid tropics (Smith and Frederiksen 2000; Subudhi and Nguyen 2000). Sorghum is also widely used as a cover crop in North America, mainly in the USA (Weston 1996). Sorghum is known to release sorgoleone, an oily brownish exudate that contain lipid benzoquinones, which constitutes ≥90 % of the hydrophobic component of sorghum root exudates (Netzly and Butler 1986; Czarnota et al. 2003). Growing conditions including nutrient supply, the presence of growth hormones (e.g. auxins), and exposure to light may influence sorgoleone release. Further, factors that alter root hair development can potentially influence sorgoleone synthesis and release from root hairs (Czarnota et al. 2001, 2003; Yang et al. 2004; Dayan 2006; Uddin et al. 2010). Sorgoleone exudation has been investigated mostly from the allelopathic perspective for its capacity to suppress weeds (Alsaadawi et al. 1985). Recent reports indicate that sorghum roots release two distinct categories of BNIs, hydrophilic BNIs and hydrophobic BNIs. Sorgoleone is the major component of hydrophobic-BNI-activity (Subbarao et al. 2013a). The present study aims to contribute to our understanding of the relationship between sorgoleone released and hydrophobic-BNI-activity released from sorghum roots; and characterize the genetic differences in the BNI-capacity of selected sorghum cultivars.

Materials and methods

Sorgoleone analysis

Sorgoleone release from sorghum roots was quantified by analyzing the acidified dichloromethane (DCM) soluble fraction of root exudates using HPLC, as described earlier (Subbarao et al. 2013a). Briefly, the growing roots were excised from sorghum seedlings and dipped into acidified-dichloromethane (DCM) (1 % acetic acid:DCM v/v) for 1 min. The root-DCM wash was filtered and evaporated to dryness using a rotary-evaporator at 35 ° C; the residue was re-extracted with 10 ml methanol and evaporated again. Then, the residue was transferred to an eppendorf tube, filtered through a 0.20 μm syringe membrane filter; and the filtrate was evaporated to dryness. Residues were dissolved in 500 μl acetonitrile and filtered again through a 0.20 μm syringe membrane filter to remove any insoluble compounds that might interfere with HPLC analysis.

An aliquot (10 μl in acetonitrile) from the sample was injected into the isocratic flow (2 mL min^{-1}) of the eluent (55 % acetonitrile and 45 % of 0.5 % formic acid), and sorgoleone was detected at 280 nm using a spectrophotometer. Identification and quantification of sorgoleone was done by comparison with the retention time and peak area of a known standard.

BNI estimation

BNI activity in an aliquot of processed sample was determined using a "nitrification inhibition bioassay" that employs recombinant luminescent *Nitrosomonas europaea* (Subbarao et al. 2006). A sub-sample of the acetonitrile-soluble fraction prepared for sorgoleone analysis by HPLC was taken and evaporated to dryness. Then, the residue was dissolved in dimethyl sulfoxide (DMSO); and 1 μl of this aliquot was used for the

determination of BNI-activity in the bioassay. BNI-activity is expressed in units defined in terms of the action of a standard inhibitor, allylthiourea (AT), where the inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ is defined as one AT unit (ATU) of activity (Subbarao et al. 2006).

Experiment 1: genetic differences in sorghum genotypes in hydroponics

Three sorghum genotypes (Hybridsorgo, IS41245 and GDLP 34-5-5-3) were grown in a walk-in growth chamber (25 ° C, 14/10 h light/dark period, average photosynthetic photon flux of 300 μmol m^{-2} s^{-1}) for 7 days. Seeds were soaked in aerated germination solution (200 μM $CaSO_4$) for 24 h before sowing them into folded filter paper (MN 710, MACHEREY-NAGEL GmbH & Co. KG, Germany) supported by hard plates with one end touching the bottom of the seedling growth box supplied with the germination solution. This system allowed the seedlings to be continuously supplied with germination solution via capillary movement. Boxes were supplied daily with the germination solution to keep filter papers moist. Sixty seedlings were germinated per replicate.

At 7 days after sowing (DAS), 40 seedlings were selected, their roots excised and dipped in 40 mL acidified DCM (1 % v/v DCM:acetic acid) for 1 min, and the product from this is subsequently referred to as root-DCM wash. Root-DCM wash was filtered and further processed for determining sorgoleone content and BNI-activity as described above. Roots were placed in a ventilated drier (65 °C) prior to gravimetric dry weight determination. The experiment was repeated three times, each time with three replicates each to confirm the reproducibility of the results. In this experiment, we focused on two sorghum genotypes (IS41245 and GDLP 34-5-5-3), which have been used as parents of a bi-parental mapping population to identify genetic markers for sorgoleone, as well as host plant resistance grain molds in a tan-plant, white-pericarp, nonpigmented-testa, and agronomically elite genetic background as part of on-going research by ICRISAT and JIRCAS (G.V. Subbarao, S.P. Deshpande, R. Sharma and C.T. Hash, personal communication). The variety Hybridsorgo is grown in Japan. It is used as a reference variety, and also as a model system for sorghum BNI characterization.

Another experiment was conducted to determine the genetic differences in sorgoleone release and BNI-capacity of IS41245 and GDLP 34-5-5-3, as affected by plant age, to gain additional information on the agronomic value of these characters in these cultivars. Thus, plants were grown using the same growth boxes described previously, with small modifications that allowed plants to grow for 25 DAS by replacing the germination solution (200 μM $CaSO_4$) with full strength nutrient solution. Thirty seeds were germinated per replicate and allowed to grow until 25 DAS. At harvest, ten seedlings were selected per treatment and roots were excised and dipped into 40 mL of acidified-DCM solution for 1 min. Root-DCM wash was processed for determining its sorgoleone content and BNI-activity as described earlier. Root dry weight was recorded. Four replicates were used for each treatment.

Experiment 2: genetic differences in sorghum genotypes in soil culture

Two sorghum genotypes (IS41245 and GDLP 34-5-5-3) were grown in pots filled with soil [(volcanic ash soil, Typic Hapludands (pH H_2O 6.0, clay 54.8 %, silt 26.3 %, sand 18.9 %, total carbon=29.2 mg g^{-1} soil; total *N*=2.5 mg g^{-1} soil; C/N ratio of 11.7 and CEC= 119.1 me/100 g)] collected from the JIRCAS [(Japan International Research Center for Agricultural Sciences) experimental farm in Tsukuba, Japan]. Plants were grown for 120 days and four replicates for each genotype were used, while bare-soil was included as control treatment. Two kg air-dried soil was filled in each pot and 700 ml of full strength nutrient solution was added to ensure sufficient supply of all nutrients. Then seven seeds were sown in each pot. After germination, seedlings were thinned to five seedlings per pot. When seedlings showed chlorosis (at around 30 DAS), the pots were irrigated with full strength nutrient solution instead of water. This helped seedlings to maintain normal growth for 120 days.

At 120 DAS, plants were removed from the pots and shaken lightly to remove loosely adhering soil particles. Then soil strongly attached to plant roots from a given pot was collected as a "rhizosphere soil" sample by thorough shaking. After carefully removing the rhizosphere soil, each root sample along with remaining rhizoplane soil was transferred into a beaker, and immersed in acidified-DCM for 1 min. The slurry was then filtered through ash-free filter paper (ADVANTEC 4A, Toyo Roshi Kaisha Ltd., Japan), and the filtrate evaporated for quantitiative determination of sorgoleone content and BNI-activity.

For testing nitrification potential in the rhizosphere of the sorghum plants, an incubation experiment was set up using the rhizosphere-soil and providing NH_4^+ as the nitrogen source. Each replication of each treatment in this experiment was conducted in a set of identical bottles in which the soil samples were incubated. Two control soils (i.e. soils collected from bare soils i.e. plant-free pots) were also prepared with- and without DCD (dicyandiamide, a commercial nitrification inhibitor, at 25 ppm). Two g air-dried rhizosphere-soil or control-soil samples were supplied with treatment solution of 720 μL of 28.63 mM $(NH_4)_2SO_4$, giving it an equivalent level of 200 μg N g^{-1} soil, and moisture level of 60 % WFPS (water-filled pore space) which is optimum for nitrification process in this soil (Mosier et al. 1996). The mouth of each bottle was sealed with parafilm with a needle head hole to maintain good aeration and constant moisture level. After recording final weights, bottles were placed inside a temperature- and humidity-controlled incubator (Bench-top type temperature and humidity chamber, ESPEC Corporation, Japan), at 25 ° C and 85 % humidity. Soils were sampled to determine nitrification potential, using four replicates of bottles for each pre-determined incubation time (0, 15 and 30 days incubation duration). Thus, for each designated incubation time, a set of bottles were removed from the incubator and weighed for confirmation of any moisture loss. Inorganic-N in the soil at the end of incubation period was determined by shaking soil with 20 mL of 2 M kCl for 30 min and filtering the slurry through Whatman 2 filter paper (Whatman International Ltd., England). NH_4^+ and NO_3^- concentration in the filtrate was measured using an autoAnalyzer (Anon 1974). Soil nitrification is calculated using the following formula: $[NO_3^-/(NH_4^+ + NO_3^-)]$.

Experiment 3: field study with sorghum genotypes GDLP 34-5-5-3 and IS41245

The two sorghum bi-parental mapping population parental inbred line genetic stocks (IS41245 and GDLP 34-5-5-3) were sown into a short-day length environment during the third week of August to the first week of September in an Alfisol field at the ICRISAT-Patancheru Research Farm, annually during 2008 to 2011. Day-lengths following completion of the brief juvenile growth phase of these genetic stocks in late September were short enough to induce floral initiation; and these entries reached 50 % flowering within a 2-week period in mid-November. These two genetic stocks were sown in three replications arranged in a randomized complete block design, in 4-row plots of 2-m length, with 60-cm spacing between rows. Plots were over-sown and thinned to a uniform stand, retaining an average within-row spacing of 20 cm between plants. Unplanted control plots treated in the same way, were also included; and soil samples are collected for determining nitrification rates. A total of 100 kg N ha^{-1} as NH_4SO_4 fertilizer was applied in three splits: 30 kg N ha^{-1} basal application, followed by two side-dressings each at 30 DAS and 60 DAS at the rate of 35 kg N ha^{-1}. Rhizosphere soil samples were collected destructively in late November from ten competitive plants from the central portion of the two central rows of each plot shortly after panicle emergence. Plants were uprooted using a crowbar with the bulk soil (within 30-cm depth) attached; all the bulk soil was removed by shaking the attached plant roots followed by cutting off the shoot. The roots with rhizosphere soil were kept in a plastic bag and shaken for several minutes to obtain rhizosphere soil, which was air-dried and used for testing nitrification potential using soil incubation studies as described above; and sorgoleone content of each soil sub-sample was measured.

For determining sorgoleone concentration in the rhizosphere soil, two g air-dried rhizosphere soil was placed in conical flasks, and 100 ml of 80 % acetonitrile was added to the soil sample. The flasks were then placed into a shaker for 2 h; following shaking, the slurry was filtered through ash free filter paper (ADVANTEC 4A, Toyo Roshi Kaisha Ltd., Japan) and evaporated using rotary evaporator. The residue was then dissolved using 10 ml methanol, and the contents evaporated. The residue was transferred to an eppendorf tube and filtered through a 0.20-μm syringe membrane filter, and the filtrate then evaporated. Finally, the residue obtained was dissolved in 500 μL acetonitrile and filtered once again using syringe membrane to remove particles that can interfere with HPLC analysis. Sorgoleone was determined by injecting an aliquot of the re-filtered acetonitrile solution, as described above.

Experiment 4: using purified sorgoleone to determine its inhibitory effect on soil nitrification

An additional soil incubation experiment was conducted by adding purified sorgoleone to the control-soil sample

to obtain the following treatments: 0, 10, 20, 30, 40 and 100 μg sorgoleone g^{-1} soil. Details of the method used for mixing and preparing soils with BNI compounds for soil incubation experiments were described earlier (Subbarao et al. 2008); other details of the soil incubation method used were similar to that described above, except that the soil incubation temperature was 20C instead of 25C, and the incubation period was 60 days.

Results

Experiment 1: genetic differences in sorghum genotypes in hydroponics

Sorghum genotypes showed comparable growth in seedling growth boxes supplied with nutrients via capillary movement. Visual observations of root-DCM wash color intensity indicate differences in sorghum genotypes tested; and this was confirmed by the HPLC chromatographic peak size. GDLP 34-5-5-3 produced peak sizes similar to that of 2.834 mg mL^{-1} pure sorgoleone standard, while IS41245 showed close to half the size to this standard (data not shown). Quantification of sorgoleone levels in the root DCM-wash by HPLC analysis confirmed the visual observation of genetic differences between the genotypes; GDLP 34-5-5-3 and Hybridsorgo released more sorgoleone than did IS41245 (Fig. 1). There was a similar trend in the total BNI-activity; GDLP 34-5-5-3 and Hybridsorgo had similar levels and these were higher than that of IS41245 (Fig. 1). Specific sorgoleone concentration (μg sorgoleone g^{-1} root DM) and specific BNI-activity (ATU g^{-1} root DM) results were similar to those for total sorgoleone exudation and BNI-activity, as the root biomass produced by the three sorghum genotypes were similar (data not shown).

There was a strong relationship (r^2=0.95) between total sorgoleone level in root-DCM wash and BNI-activity of the three sorghum genotypes tested (Fig. 2). Sorgoleone is therefore the predominant hydrophobic compound with BNI-activity that is present in sorghum root-DCM wash; and this sorgoleone plays an overwhelmingly predominant role in BNI-activity of the three sorghum genotypes studied. In addition, the genetic differences in quantitative sorgoleone release and BNI-capacity between IS41245 and GDLP 34-5-5-3 widens with plant age. As 7 days old seedlings, GDLP 34-5-5-3 showed about double the sorgoleone release and BNI-activity of IS41245, while by 25 days, these differences grew to nearly 4-fold (Table 1).

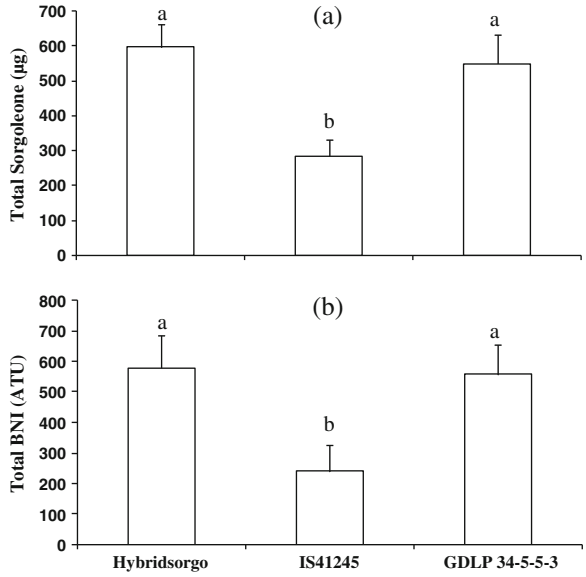

Fig. 1 Total amount of sorgoleone released (**a**) and BNI-activity (**b**) measured from three sorghum genotypes using 7-day old seedlings (40 seedlings per treatment). Values show bulked data of two experiments (n=6) and bars represent means ± SE. *Bars* carrying different letters are significantly different at P=0.05 Duncan's one-way ANOVA test

Experiments 2: genetic differences in sorghum genotypes cultivated in soil medium

Experiments were conducted in soil to check whether the observed genetic differences in sorgoleone release and BNI-capacity under hydroponic condition also occur in soil culture. Sorgoleone levels measured in the

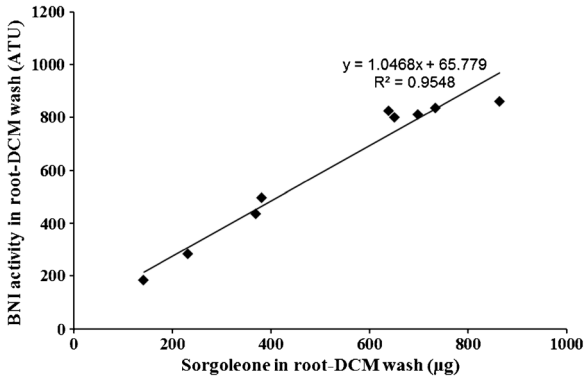

Fig. 2 Relationship between total sorgoleone concentration (μg) and BNI-activity (ATU) in root-DCM wash of three sorghum genotypes. Data bulked from one experiment with three replicates for each genotype (nine replicates)

Table 1 Differences between IS41245 and GDLP 34-5-5-3 as measured by specific sorgoleone concentration (μg g^{-1} root DW) and specific BNI-activity (ATU g^{-1} root DW) in 7-day seedlings and 25-day old plants

Genotype	Specific sorgoleone (μg g^{-1} root)		Specific BNI (ATU g^{-1} root)	
	7 days	25 days	7 days	25 days
IS41245	1,633±219[c]	1,460±72[c]	1,282±428[c]	1,599±284[bc]
GDLP 34-5-5-3	2,426±178[b]	5,049±401[a]	2,460±201[b]	4,260±393[a]

Values represent means ± SE of six replicates for 7-day treatments and means ± SE of four replicates for 25-day treatments. Each replicate of each genotype for the 7-day treatment contained 40 seedlings, while the 25-day treatment had ten plants per genotype per replicate. Means followed by the same letter are not significantly different at $P=0.05$ in Duncan's one-way ANOVA test

rhizoplane of IS41245 and GDLP 34-5-5-3 genotypes grown in pots filled with soil showed the presence of measureable levels of sorgoleone, with higher concentration of sorgoleone and higher BNI-activity in rhizoplane soil of GDLP 34-5-5-3 than in that of IS41245 (Fig. 3). This was reflected in both total sorgoleone (μg per pot) and total BNI-activity (ATU per pot) (Fig. 3). Further, soil nitrification in the bare-soil control treatment showed complete nitrification of NH_4^+ (100 % during the 30-day incubation period), which was substantially greater than that in rhizosphere-soil from pots in which sorghum was grown (Fig. 4). This indicates that sorghum plants released one or more BNI-active compounds from their roots. In addition, the genetic differences in sorgoleone level measured in the rhizoplane soil of GDLP 34-5-5-3 and IS41245 were accompanied by comparable differences in soil nitrification potential, i.e., higher nitrification rate in IS41245 (88 % for the 30-day incubation period) compared to that in GDLP 34-5-5-3 (60 % during the 30-day incubation period) (Fig. 4). Nitrification inhibition in the rhizosphere of GDLP 34-5-5-3 seems to have physiological and agronomic significance as it shows nitrification inhibition close to that shown by the known synthetic inhibitor (DCD) treatment, which showed 40 % nitrification during the 30 days incubation period (Fig. 4).

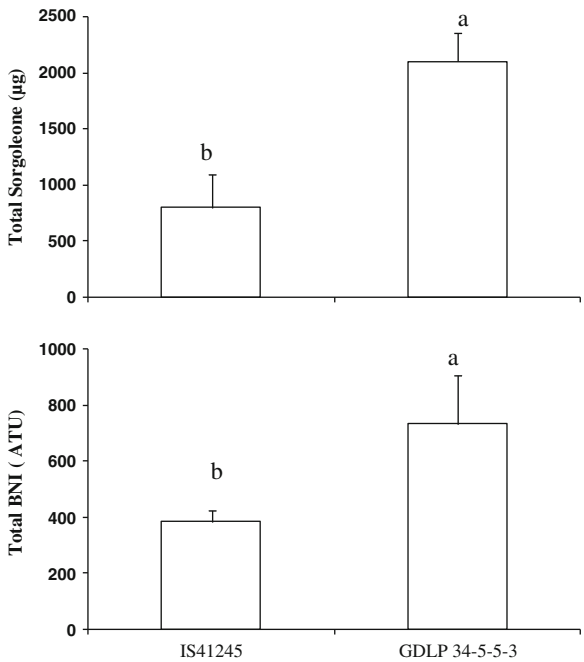

Fig. 3 Measured total sorgoleone concentration (μg per pot) and BNI-activity (ATU per pot) on the rhizoplane of two sorghum genotypes grown on pots filled with soil. *Bars* represent means of four replicates ±SE. *Bars* carrying different letters are significantly different at *P=0.05* Duncan's one-way ANOVA test

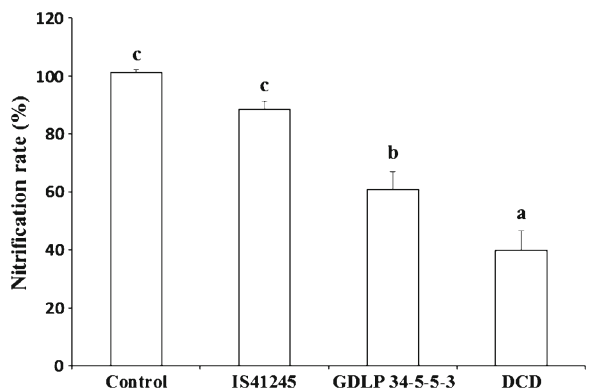

Fig. 4 Nitrification rate at 30-day incubation period along with NH_4^+ inoculation of rhizosphere soil collected from two sorghum genotypes (IS41245 and GDLP 34-5-5-3) grown up to heading stage in potted soil. Control pots were included with bare soil without plants but handled the same way like pots with plants. As positive control, soils taken from control treatments were also incubated with DCD addition at 25 ppm (a known synthetic inhibitor) as a reference. *Bars* represent four replicates ±SE. *Bars* denoted with different letters are significantly different at $P=0.05$ Duncan's one-way ANOVA test

Experiment 3. Field study with sorghum genotypes GDLP 34-5-5-3 and IS41245

In agreement to the results obtained from the greenhouse experiments, rhizosphere soil taken after panicle emergence from a field grown sorghum genotypes IS41245 and GDLP 34-5-5-3 showed that sorgoleone levels in the rhizosphere of GDLP 34-5-5-3 were higher than those of IS41245 (Fig. 5). This was also reflected in the inhibition of nitrification as the nitrification rate was significantly higher in soil collected from the rhizosphere of IS41245 than GDLP 34-5-5-3 (Fig. 6).

Experiment 4. Use of purified sogoleone to determine its effect on soil nitrification

The soil incubation experiment with purified sorgoleone demonstrates a clear relationship between the amount of sorgoleone applied to the soil and the inhibition of nitrate formation from ammonium; this was further confirmed by observation of higher NH_4^+ concentrations in soil to which higher levels of sorgoleone were applied (Fig. 7).

Discussion

Nitrification has been studied for decades due to its adverse influence on nitrogen use efficiency and environmental quality. To reduce such undesirable effects of nitrification, synthetic nitrification inhibitors were developed and proposed for use in agricultural production systems (Subbarao et al. 2006, 2013b). Existence of nitrification inhibitors of plant origin has been reported (Moore and Waid 1971; Lata et al. 1999, 2004). BNI-activity in sorghum has recently been reported (Subbarao et al. 2007a, b, 2013a; Zakir et al. 2008; Jones 2013). A highly sensitive bioassay using recombinant luminescent *Nitrosomonas europaea* has been developed that can detect and quantify the amount of nitrification inhibitors produced by plants (Subbarao et al. 2006). However, most of the research to date on the BNI function in sorghum roots has focused on the hydrophilic fraction of their root exudates

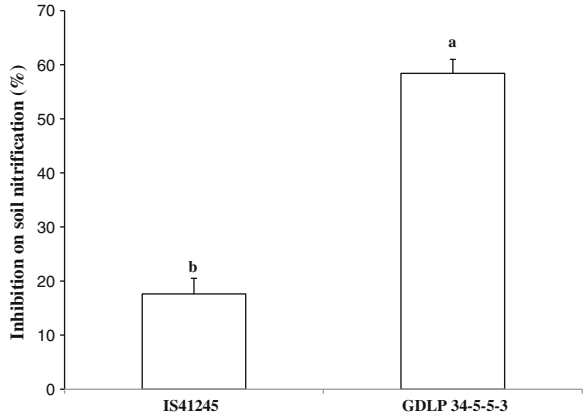

Fig. 6 Inhibition of nitrification in soils collected from the rhizosphere of two sorghum genotypes (GDLP 34-5-5-3 and IS41245) grown under field conditions until panicle emergence. Soils were incubated for 60 days at 20 ° C. *Bars* represent mean values of three replicates ±SE. *Bars* carrying different letters are significantly different at $P=0.05$ Duncan's one-way ANOVA test

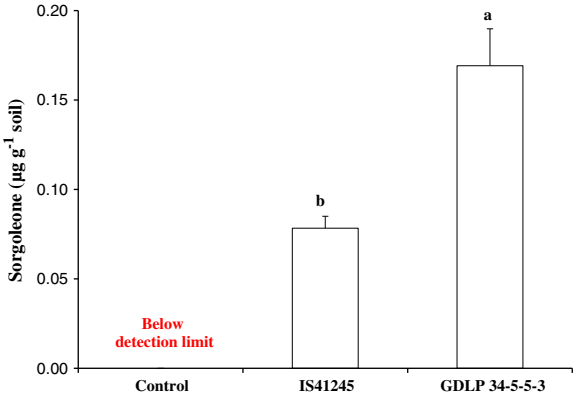

Fig. 5 Sorgoleone concentration of rhizosphere soil samples collected from field grown sorghum genotypes (IS41245 and GDLP 34-5-5-3) at panicle emergence. *Bars* represent means of three replicates ±SE. *Bars* carrying different letters are significantly different at $P=0.05$ Duncan's one-way ANOVA test

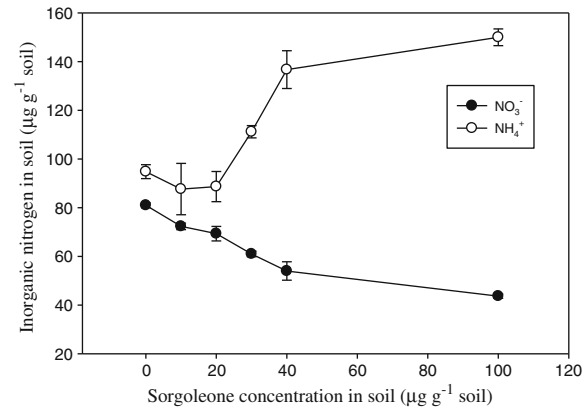

Fig. 7 Concentration of inorganic N (NO_3^- and NH_4^+) in soil samples incubated after adding different concentrations of sorgoleone (0, 10, 20, 30, 40, and 100 µg g^{-1} soil) for 60 days. Values represent means of three replicates ±SE

(Zakir et al. 2008; Subbarao et al. 2013a). In contrast, the present study was aimed at characterizing the BNI function of the hydrophobic-fraction of sorghum root exudates, with a special emphasis on assessing the relationship between sorgoleone release and BNI-activity.

Sorghum is grown globally, particularly in the semi-arid regions of Africa, Asia, and the Latin Americas (Subudhi and Nguyen 2000). Nitrate leaching is considered as one of the main causes of N fertilizer loss in tropical soils, especially in those with lighter texture (Allison 1966); and in intensified agricultural production systems (Hadas et al. 1999). One of the strategies to reduce N loss by leaching following nitrification is to suppress soil nitrification and so to keep N in the NH_4^+ form for an extended period, allowing uptake by growing crop for an extended period of time (Subbarao et al. 2009, 2013b). Recently, sorghum root exudates have been reported to possess BNI-activity (Zakir et al. 2008; Subbarao et al. 2007a, b, 2013a). Results of the present study with hydroponically-grown plants and soil-grown plants confirm these earlier results and supplement them by providing additional insights on BNI-activity of the hydrophobic component of sorghum root exudates (Fig. 1).

Sorgoleone is the main component of the hydrophobic part of sorghum root exudates (Czarnota et al. 2003; Subbarao et al. 2013a). In agreement with the results from these reports, HPLC chromatogram of an aliquot from sorghum root exudates (i.e. root-DCM wash) showed only one major peak that corresponds to sorgoleone, based on comparative evaluation of retention time with that of a purified sorgoleone standard (data not shown). Interestingly, BNI measurement of the same aliquot shows activity similar to that of sorgoleone in the sample, confirming that sorgoleone is the sole hydrophobic compound responsible for BNI function in the small number of sorghum genotypes assessed to date (Fig. 1). The protocol used in the current study allowed us to detect considerably higher levels of sorgoleone (up to 5,000 μg g^{-1} root DW) and BNI-activity (up to 4,000 ATU g^{-1} root DW) than had been detected in previous studies, which could largely be due to the evaluations being made during early seedling growth stages. Earlier reports of BNI-activity from sorghum suggest that BNI-activity (both hydrophobic and hydrophilic) levels of about 25 ATU g^{-1} root DW can be detected in 50 to 100 days old plants Subbarao et al. 2013a).

The organic compounds released from plant roots are known to differ both quantitatively and qualitatively (Bertin et al. 2003). Genotypic differences were reported for sorgoleone release based on some preliminary evaluations performed at seedling growth stages (i.e. 7 days) (Czarnota et al. 2003). There were distinct differences between the sorghum genotypes tested in this study (Figs. 1, 3 and 5; Table 1). In the hydroponic culture, roots of GDLP 34-5-5-3 and Hybridsorgo released markedly higher levels of sorgoleone (≈ 600 μg) than did IS41245 (≈ 300 μg) (Fig. 1). These differences in sorgoleone release were accompanied by similar observed differences in the BNI-activity level (Fig. 1). In most instances, traits that can be easily quantified under hydroponic condition are difficult to quantify in soil culture; however, from a chemical ecology perspective, such traits must be expressed under soil-culture conditions to have any physiological significance. For this reason, the presence of genetic differences was also assessed under soil-culture conditions, in both greenhouse and field environments. Analysis of the rhizosphere-soil of these sorghum genotypes taken from controlled greenhouse conditions and from the field confirmes the existence and persistence of genetic differences in sorgoleone release. Despite all of the technical limitations faced in extracting sorgoleone from the soil, clear differences in sorgoleone concentration in the rhizospheres of GDLP 34-5-5-3 and IS41245 were detected under both greenhouse and field conditions (Figs. 3 and 5).

Purified sorgoleone showed inhibitory effects on *Nitrosomonas* activity in the bioassay, and the strength of the inhibitory effect was linear to the sorgoleone concentration (in aconcentration range of up to 13 μM) (Subbarao et al. 2013a). This allows the prediction that the genetic differences in sorgoleone release under both hydroponic- and soil-culture will result in inherent genetic differences in BNI-capacity that persists in the soil. Measurement of BNI-activity in the rhizoplane (root-DCM wash) of IS41245 and GDLP 34-5-5-3 plants grown in soil-culture in the greenhouse showed differences between the two sorghum genotypes; GDLP 34-5-5-3 produced two-fold greater BNI-activity than did IS41245 (Fig. 3). This was also reflected in reduced soil nitrification potential in the rhizosphere soil of GDLP 34-5-5-3 than in that of IS41245, and particularly evident relative to the bare-soil control (Figs. 4 and 6). These inhibitory effects on soil nitrification can be reasonably stable, often lasting

>30 days (from the results presented here and also from Subbarao et al. 2013a), but may subsequently lose its effectiveness as sorgoleone is mineralized (Gimsing et al. 2009).

Earlier report shows up to 40 % reduction in nitrification following addition of hydrophilic BNIs collected from sorghum roots (at a concentration level of 10 ATU g^{-1} soil) and incubated for 30 days (Subbarao et al. 2013a). In the present study, inhibition of nitrification after 30 days incubation of rhizosphere soil from cultivar GDLP 34-5-5-3 (60 %) was close to that of a known synthetic chemical inhibitor (DCD, 40 %), while in cultivar IS41245 (88 %) and control treatments (bare soil control, 100 %), almost all of the NH_4^+ was converted to NO_3^- during the 30-day incubation (Fig. 4). This indicates a physiologically significant level of the BNI-active compound sorgoleone is released into the rhizosphere from sorghum roots, and that this has the potential to help keep fertilizer-N as NH_4^+ for a longer period of time. It is worth mentioning that the measured genetic differences in sorgoleone release and BNI activity at the seedling stage can continue to accumulate throughout the plant growth period and reach a significant level of physiological function (Czarnota et al. 2003). In hydroponic culture, the concentration of sorgoleone shows cumulative effects as the differences in sorgoleone accumulation and BNI-activity in root-DCM wash of GDLP 34-5-5-3 and IS41245 increased from one-fold higher at 7 days age to more than 4-fold higher at 25 days age; i.e. while the sorgoleone release and BNI-activity per g root of GDLP 34-5-5-3 increased significantly during this period, however, there was no change in the case of IS41245 (Table 1).

Interestingly, the close relationship between total sorgoleone released and BNI-activity in the root-DCM wash of all three sorghum genotypes studied (r^2=0.95) indicates that 1 μg of sorgoleone may correspond to 1 ATU BNI-activity (Fig. 2). Hence, the measurement of sorgoleone may predict the BNI-activity in the soil for a given sorghum genotype. Moreover, a close relationship between sorgoleone release and BNI-activity demonstrates that sorgoleone is the sole hydrophobic compound present in sorghum root exudates with the BNI-function. This was further shown in a soil incubation experiment that demonstrated a strong relationship between the amount of sorgoleone applied to the soil and inhibition of nitrate formation from ammonium (Fig.7). Thus sorgoleone release has an additional biological function as an inhibitor of nitrifying bacteria apart from its reported herbicidal function (Nimbal et al. 1996a, b; Czarnota et al. 2001; Hejl and Koster 2004; Dayan et al. 2009).

Conclusion

We conclude that sorgoleone is the sole compound present in the hydrophobic fraction of sorghum root exudate that possesses BNI-function. The relationships between sorgoleone released and BNI-activity are linear,, i.e. 1 μg sorgoleone has 1 ATU BNI activity. Moreover, there are genetic differences in agronomically elite sorghum cultivars for the release of sorgoleone and BNI-capacity that can be exploited by breeding programs to develop improved cultivars for use in more sustainable and eco-friendly crop production systems than those presently prevailing in most areas where this crop is grown globally.

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References

Allison FE (1966) The fate of nitrogen applied to soils. Adv Agron 18:219–258

Alsaadawi IS, Al-Uqaili JK, Alrubeaa AJ, Al-Hadithy SM (1985) Allelopathic suppression of weed and nitrification by selected cultivars of *Sorghum bicolor*(L.)Moench. J Chem Ecol 12: 209–218

Anon (1974) Technicon autoanalyzer II. Technicon Industrial Systems, Tarrytown

Bertin C, Yang X, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. Plant Soil 256:67–83

Bremner JM, Blackmer AM (1978) Nitrous oxide: emission from soils during nitrification and denitrification of fertilizer nitrogen. Science 199:295–296

Chain P, Lamerdin J, Larimer F, Regala W, Lao V, Land M, Hauser L, Hooper A, Klotz M, Norton J et al (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonaseuropaea*. J Bacteriol 185:2759–2773

Czarnota MA, Paul RN, Dayan FE, Nimbal CI, Leslie A, Weston LA (2001) Mode of action, localization of production, chemical nature, and activity of sorgoleone: a potent PSII inhibitor in *Sorghum* spp. root exudates. Weed Technol 15:813–825

Czarnota MA, Rimando AM, Weston LA (2003) Evaluation of seven sorghum (*Sorghum* sp.) accessions. J Chem Ecol 29: 2073–2083

Dayan FE (2006) Factors modulating the levels of the allelochemical sorgoleone in *Sorghum bicolor*. Planta 224:339–346

Dayan FE, Howell J, Weidenhamer JD (2009) Dynamic root exudation of sorgoleone and its in plant mechanism of action. J Exp Bot 60:2107–2117

Gimsing AL, Baelum J, Dayan FE, Locke M, Sejero LH, Jacobsen CS (2009) Mineralization of the allelochemical sorgoleone in soil. Chemosphere 76:1041–1047

Hadas A, Hadas A, Sagiv B, Haruvy N (1999) Agricultural practices, soil fertility management modes and resultant nitrogen leaching rates under semi-arid conditions. Agric Water Manag 42:81–95

Hayatsu M, Tago K, Saito M (2008) Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci Plant Nutr 54:33–45

Hejl AM, Koster KL (2004) The allelochemical sorgoleone inhibits root H^+-ATPase and water uptake. J Chem Ecol 30: 2181–2191

Hofstra N, Bouwman AF (2005) Denitrification in agricultural soils: summarizing published data and estimating global annual rates. Nutr Cycl Agroecosyst 72:267–278

Ishikawa T, Subbarao GV, Ito O, Okada K (2003) Suppression of nitrification and nitrous oxide emission by the tropical grass *Brachiaria humidicola*. Plant Soil 255:413–419

Jones N (2013) Grass gets greener. Nature 501:291

Klotz MG, Arp DJ, Chain PS, El-Sheikh AF, Hauser LJ, Hommes NG, Larimer FW, Malfatti SA, Norton JM, Poret-Peterson AT et al (2006) Complete genome sequence of the marine, chemolitho-autotrophic, ammonia oxidizing bacterium *Nitrosococcusoceani* ATCC 19707. Appl Environ Microbiol 72:6299–6315

Lata JC, Durand J, Lensi R, Abbadie L (1999) Stable coexistence of contrasted nitrification statuses in a wet tropical savanna system. Fun Ecol 13:762–763

Lata JC, Degrange V, Raynaud X, Maron PA, Lensi R, Abbadie L (2004) Grass populations control nitrification in savanna soils. Fun Ecol 18:605–611

Moore DRE, Waid JS (1971) The influence of washing of living roots on nitrification. Soil Biol Biochem 3:69–83

Mosier AR, Duxbury JM, Freney JR, Heinemeyer O, Minami K (1996) Nitrous oxide emissions from agricultural fields: assessment, measurement, and mitigation. Plant Soil 181:95–108

Netzly DH, Butler LG (1986) Roots of sorghum exude hydrophobic droplets containing biologically active components. Crop Sci 26:775–778

Nimbal CI, Pedersen JF, Yerkes CN, Weston LA, Weller SC (1996a) Phytotoxicity and distribution of sorgoleone in grain sorghum germplasm. J Agric Food Chem 44:1343–1347

Nimbal CI, Yerkes CN, Weston LA, Weller SC (1996b) Herbicidal activity and site of action of the natural product sorgoleone. Pestic Biochem Physiol 54:73–83

Raun WR, Johnson GV (1999) Improving nitrogen use efficiency for cereal production. Agron J 91:357–363

Schlesinger WH (2009) On the fate of anthropogenic nitrogen. PNAS (USA) 106:203–208

Shen JP, Zhang LM, Di HJ, He JZ (2012) A review of ammonia-oxidizing bacteria and archaea in Chinese soils. Front Microbiol 3:1–7

Smith CW, Frederiksen RA (2000) Sorghum: origin, history, technology, and production. Wiley, New York

Smith KA, McTaggart IP, Tsuruta H (1997) Emissions of N_2O and NO associated with nitrogen fertilization in intensive agriculture and the potential for mitigation. Soil Use Manag 13:296–304

Starkenburg SR, Chain PS, Sayavedra-Soto LA, Hauser L, Land ML, Larimer FW, Malfatti SA, Klotz MG, Bottomley PJ, Arp DJ et al (2006) Genome sequence of the chemo-litho autotrophic nitrite-oxidizing bacterium *Nitrobacterwinogradskyi* Nb-255. Appl Environ Microbiol 72:2050–2063

Subbarao GV, Ishikawa T, Ito O, Nakahara K, Wang HY, Berry WL (2006) A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with *Brachiaria humidicola*. Plant Soil 288:101–112

Subbarao GV, Rondon M, Ito O, Ishikawa T, Rao IM, Nakahara K, Lascano C, Berry WL (2007a) Biological nitrification inhibition (BNI) - is it a widespread phenomenon? Plant Soil 294: 5–18

Subbarao GV, Wang HY, Ito O, Nakahara K, Berry WL (2007b) NH_4^+ triggers the synthesis and release of biological nitrification inhibition compounds in *Brachiaria humidicola* roots. Plant Soil 290:245–257

Subbarao GV, Nakahara K, Ishikawa T, Yoshihashi T, Ito O, Ono H, Ohnishi-Kameyama M, Yoshida M, Kawano N, Berry WL (2008) Free fatty acids from the pasture grass *Brachiaria humidicola* and one of their methyl esters as inhibitors of nitrification. Plant Soil 313:89–99

Subbarao GV, Nakahara K, Hurtado MP, Ono H, Moreta DE, Salcedo AF, Yoshihashi AT, Ishikawa T, Ishitani M, Ohnishi-Kameyama M, Yoshida M, Rondon M, Rao IM, Lascano CE, Berry WL, Ito O (2009) Evidence for biological nitrification inhibition in *Brachiaria* pastures. PNAS (USA) 106:17302–17307

Subbarao GV, Nakahara K, Ishikawa T, Ono H, Yoshida M, Yoshihashi T, Zhu Y, Zakir HAKM, Deshpande SP et al (2013a) Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366:243–259

Subbarao GV, Sahrawat KL, Nakahara K, Rao IM, Ishitani M, Hash CT, Kishii M, Bonnett DG, Berry WL, Lata JC (2013b) A paradigm shift towards low-nitrifying production systems: the role of biological nitrification inhibition (BNI). Ann Bot 112:297–316

Subudhi PK, Nguyen HT (2000) Linkage group alignment of sorghum RFLP maps using a RIL mapping population. Genome 43:240–249

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP, Schleper C (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Env Microbiol 7:1985–1995

Uddin MR, Park KW, Kim YK, Park SU, Pyon JY (2010) Enhancing sorgoleone levels in grain sorghum root exudates. J Chem Ecol 36:914–922

Walker CB, de la Torre JR, Klotz MG, Urakawa H, Pinel N, Arp DJ, Brochier-Armanet C, Chain PSG, Chan PP, Gollabgir A, Hemp J, Huegler M, Karr EA, Koenneke M, Shin M, Lawton TJ, Lowe T, Martens-Habbena W, Sayavedra-Soto LA, Lang D, Sievert SM, Rosenzweig AC, Manning G, Stahl DA (2010) Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proc Natl Acad Sci USA 107(19):8818–8823

Weston LA (1996) Utilization of allelopathy for weed management in agroecosystems. Agron J 88:860–866

Yang X, Owens TG, Scheffler BE, Weston LA (2004) Manipulation of root hair development and sorgoleone production in sorghum seedlings. J Chem Ecol 30:199–213

Zakir H, Subbarao GV, Pearse SJ et al (2008) Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). New Phytol 180:442–451