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Biological nitrification inhibition (BNI) activity in sorghum: Potential role for enhancing nitrogen-use efficiency (NUE)

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Nitrification and denitrification are the primary drivers for generating reactive-N (NO_3^-, N_2O and NO) the two processes of N-cycle, largely responsible for soil-N losses, resulting poor N-recovery and low-NUE in agricultural systems. Suppressing soil-nitrifier activity facilitates retention of soil mineral-N as ammonium, leads to better utilization of N in situations where nitrification is followed by N losses via leaching and/or denitrification. Soils in the WCS (West Central Sahelian zone of Africa) where sorghum is predominantly grown, are of light-textured sandy-loams with acidic (ph 5.0 to 6.0). Alfisols in India and Ultisols in South America are also of light-textured and acidic, where most of the sorghum grown globally. Nitrogen mineralized from SOM (soil organic matter) or from inorganic fertilizers is quickly nitrified and lost through leaching.

Production and release of nitrification inhibitors from plant roots to suppress soil-nitrifier activity and nitrification is termed 'Biological nitrification inhibition' (BNI). Sorghum, the fourth largest food-feed crop in the world, is one of the most nitrogen-efficient among staple food crops and is adapted to low-N production environments, unlike maize or wheat that are adapted to high-N input systems. It is hypothesized that the BNI function of sorghum-root systems contributes to adaptation to low-N environments. Sorghum roots release two categories of BNIs: hydrophilic (water-soluble)- and hydrophobic (water-insoluble)-BNIs. Sakuranetin (ED_{80} 0.6 ?M) and methyl 3-(4-hydroxyphenyl) propionate (MHPP) (ED_{50} 100 ?M) are part of the hydrophilic-BNIs that contribute only a small fraction of the hydrophilic-BNI-activity release from sorghum roots. Sorgoleone appears as the major component of the hydrophobic-BNI activity (ED_{80} 1 ?M) and accounts for about 80% of the inhibitory effect. Hydrophobic-BNIs account for nearly 70% of the total BNI activity (i.e. hydrophilic + hydrophobic) released from sorghum roots. Release of BNI activity is a highly regulated function in sorghum. The presence of NH_4^+ and rhizosphere pH in acidic range (pH of $\leq$5.0) seem critical conditions that stimulate and sustain BNI release from sorghum roots. It is thus likely that BNI function would be effective and optimally expressed in light-textured (e.g., Alfisols and other light-textured soils) acidic-pH soils. Significant and substantial genetic variation for sorgoleone release and BNI-capacity has been found in sorghum genetic stocks. Several high- and low- sorgoleone genetic stocks have been identified. Efforts are currently underway to identify genetic markers using RIL (recombinant inbred lines) populations

developed from high- and low-sorgoleone release genetic stocks. Development of high-sorgoleone release genetic stocks/cultivars can improve BNI-capacity in sorghum, a pre-requisite to develop low-nitrifying, low-N_2O emitting sorghum-based production systems that utilize N more efficiently, thus environmental-friendly and sustainable.

Why controlling nitrification is critical to reduce N losses and to improve NUE in sorghum

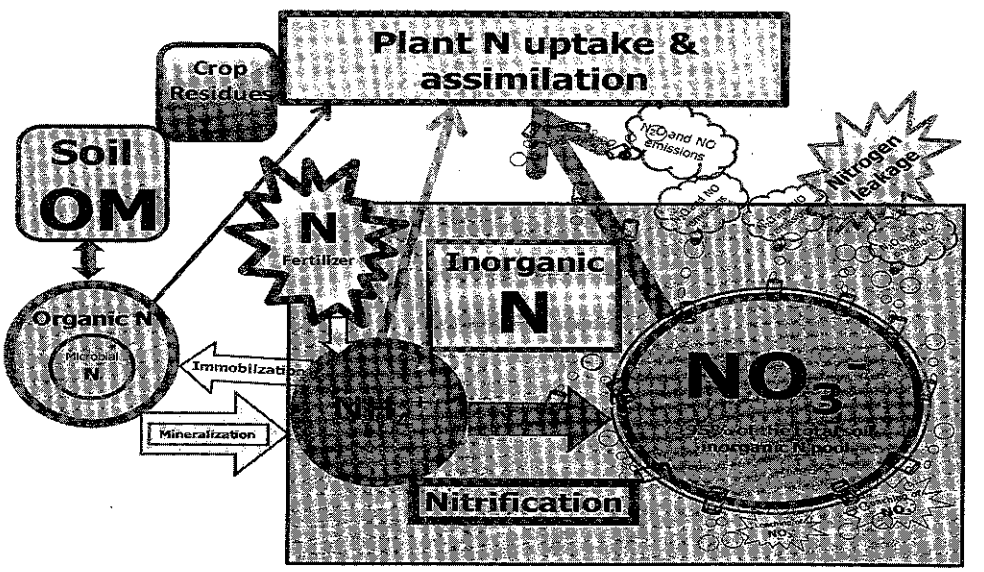
Sorghum is mostly grown on light-textured soils (such as Alfisols in India or sandy-loams in West and Central Africa and Ultisols in South America) and the crop is adapted to low-N input production environments. Soil organic matter (SOM) mineralization is often the only N source in most sorghum growing regions in world, especially in Africa where this is the staple. Nitrification and denitrification processes in soil are largely responsible for N losses, resulting in nearly 70% of the fertilizer-N or mineralized-N is lost from agricultural systems and contribute to low-NUE (Subbarao *et al.* 2013b) (Fig. 1).

Controlling nitrification have major implications for the retention of soil mineral-N as ammonium, increase in N-uptake and improved NUE in production systems (Subbarao *et al.* 2006; 2012; 2013b,c). Alfisols, Ultisols, and sandy-loams (soil pH range from 5.0 to 6.0) are the soil types where sorghum is predominantly grown. Nitrogen mineralized nitrifies rapidly, and the nitrate formed lost through leaching in these soil environments. Controlling nitrification will facilitate retention of soil-N in NH_4^+-form, which is available for extended periods for uptake by plants during the growing seasons and this may aid in improving NUE (Subbarao *et al.* 2013b,c).

Sorghum BNI function

Among the staple food crops, sorghum seems to have the strongest BNI-capacity, i.e. nitrification inhibitor production and release from root systems, but relatively weaker than tropical *Brachiaria* pasture grasses such as *B. humidicola* (Subbarao *et al.* 2013b). Sorghum roots release two categories of nitrification inhibitors – hydrophobic-BNIs and

Fig. 1. The nitrogen cycle in a typical agricultural system (i.e. upland aerobic soil) dominated by the nitrification pathway in which >95% of the N flows through, and NO_3^- remains the major inorganic N form for absorption and assimilation by plants (adapted from Subbarao et al. 2013b).



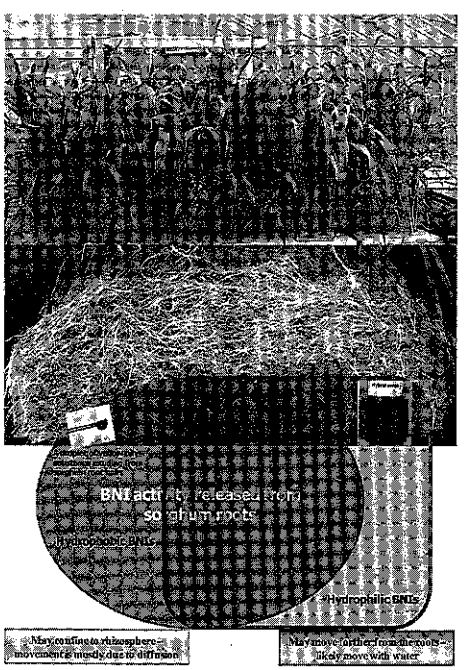
hydrophilic-BNIs; together they contribute to nitrification inhibition in soil-root environments (Fig. 2) (Subbarao *et al.* 2013a).

Due to their differential mobility and solubility in water, it is postulated that hydrophobic-BNIs may remain close to the root as they could be strongly adsorbed on the soil particles, increasing their persistence; their movement in soil is likely to be *via* diffusion across the concentration gradient and are likely to be confined to the rhizosphere (Dayan *et al.* 2010). In contrast, the hydrophilic-BNIs may move away from the point of release due to their solubility in water, and this may improve their capacity to control nitrification beyond the rhizosphere. The distribution of hydrophobic- and hydrophilic-BNIs in the rhizosphere, however, likely differ and may have a complementary functional roles, such as differential inhibitory effects on AOB (ammonia oxidizing bacteria, *Nitrosomonas*) *vs.* AOA (ammonia oxidizing archea) (Subbarao *et al.* 2013a).

The production and release of hydrophilic- and hydrophobic- BNIs appears to be of similar in magnitude across the growth phases of sorghum. Based on BNI release rates observed at various growth phases, it is estimated that about 5640 ATU of hydrophilic-BNI activity can be released per plant; an equal amount of hydrophobic-BNI activity can be released. Together (i.e. hydrophobic- + hydrophobic- BNIs) 11280 ATU of BNI activity can be released per sorghum plant during its growing period, which is equivalent to application of 6768 □g of nitrapyrin (a widely used synthetic nitrification inhibitor) (Subbarao *et al.* 2013a).

Two active components of hydrophilic-BNI activity have been identified - Methyl 3-(4-hydroxyphenyl) propionate (MHPP) (ED_{50} 100 □M) and sakuranetin (ED_{80} 0.6 □M); however their contribution to the hydrophilic-BNI activity is <20%, and the chemical identity of the major components is yet to be established (Zakir *et al.* 2008; Subbarao

Fig. 2. Hydrophobic- and hydrophilic- nitrification inhibitors (BNIs) released from sorghum roots and its significance to BNI function (source: Subbarao et al. 2013a)



et al. 2013a). In contrast, the major component of hydrophobic-BNI activity is identified as sorgoleone (ED_{80} 1.0 ?M, which accounts for 80% of inhibition (Subbarao *et al.* 2013a). The mode of inhibition of these BNI components (i.e. MHPP, sakuranetin and sorgoleone) vary; MHPP inhibits *Nitrosomonas* activity by blocking AMO (ammonia monooxygenase) enzymatic pathway; sakuranetin and sorgoleone blocks both the enzymatic pathways, AMO and HAO (hydroxylaminooxidoreductase) (Subbarao *et al.* 2013a); BNI function in sorghum is clearly due to a cocktail of inhibitors with diverse mode of action on *Nitrosomonas*, and the suppressive effect on soil-nitrifier activity is likely to be relatively stable. This is in contrast to the synthetic nitrification inhibitors such as nitrapyrin, DCD and DMPP inhibits *Nitrosomonas* activity primarily by blocking AMO function.

The BNIs release requires the presence of NH_4^+ in the root environment and the stimulatory effect of NH_4^+ lasts for 24 h (Zhu *et al.* 2012; Subbarao *et al.* 2013a). Rhizosphere pH has a major effect on the release of BNIs from sorghum roots. Threshold rhizosphere pH should be <5.0; at higher pH (i.e. >5.0) hydrophilic-BNIs release is severely affected and nearly 80% decline was observed at a rhizosphere pH of 7.0 or higher (Subbarao *et al.* 2013a). Thus, heavy black soils (i.e. Vertisols), which generally have soil pH of >7.0 and large buffering capacity (Burford and Sahrawat, 1989), resists acidification of the rhizosphere pH; sorghum grown on such soils might not release BNIs and hence such soil types might not be suitable for the expression of BNI function. Light-textured soils (such as Alfisols, sandy-loams and Ultisols), where sorghum is predominantly grown in Asia, West Africa and South America, with a low-buffering capacity and moderate acidity (pH ≤5.0) are likely to be better suited for the expression and exploitation of the BNI function in sorghum (Zhu *et al.* 2012; Subbarao *et al.* 2013a).

The hydrophilic-BNIs from sorghum roots are moderately effective in suppressing soil nitrification. A minimum inhibitory activity of about 10 ATU g^{-1} soil is needed to suppress nitrification by about 40%, and further increases in the concentration of BNI in soil did not improve inhibitory effects on nitrification (Subbarao *et al.* 2013a). The inhibitory effect on soil nitrification is stable in the temperature range of 20ºC to 30ºC. In contrast, dicyandiamide (DCD), the most commonly used synthetic nitrification inhibitor is more effective at 20ºC than at 30ºC. Soil –physical, -chemical and –biological characteristics modulate the effectiveness of BNIs (Goring 1962a,b; Sahrawat 1996; Subbarao *et al.* 2013a). The isolated BNI compounds, MHPP and sakuranetin show different trends in inhibiting nitrification in soil as compared to their performance in culture-bioassay. MHPP showed a relatively weak inhibitory activity in the culture-bioassay, but showed a moderate and stable inhibitory effect on soil nitrification. In contrast, sakuranetin showed strong inhibitory activity in the culture-bioassay, but its inhibitory function was lost in the soil-assay (Subbarao *et al.* 2013a). Sakuranetin released from sorghum roots thus may not contribute to its potential BNI-capacity, showing that not all compounds with BNI-activity detected in an *in vitro* culture-bioassay are effective in the soil. It is likely that the ratio of functional:non-functional components with BNI-activity may vary among sorghum germplasm; thus it is prudent to select genetic stocks that not only have high potential to release BNIs, but the released BNIs should be effective and functionally stable in the soil for controlling nitrifier activity (Subbarao *et al.* 2013a).

Functional link between BNI function and nitrogen use efficiency

Nitrogen-use efficiency ($NUE_{agronomic}$ = grain yield per unit of applied-N) is a function of both intrinsic-NUE ($NUE_{intrinsic}$ = dry matter produced per unit of N absorbed), harvest index (HI) and N uptake. $NUE_{intrinsic}$ of a plant is physiologically conserved function (Glass 2003), thus not easy to manipulate genetically. Improvements in $NUE_{agronomic}$ can therefore only come from improvements in crop N uptake (Finzi *et al.* 2007), which is largely related to

recovery of mineralized-N or N-fertilizer (Subbarao *et al.* 2013b). Consequently, BNI function can positively influence $NUE_{agronomic}$ by improving N recovery by reducing N losses associated with nitrification and denitrification. Recent modeling studies support this hypothesis, and the results are in accord with the actual results from *in situ* measurements in savanna systems indicating that grasses that inhibit nitrification exhibit a 2-fold greater productivity in above-ground biomass than those that lack such ability (Lata *et al.* 1999; Boudsocq *et al.* 2009; 2012).

Potential for genetic improvement of BNI function to improve NUE in sorghum production systems

Based on BNI-activity release rates observed at various growth stages, it is estimated that sorghum plants can release hydrophilic-BNI activity of about 5640 ATU $plant^{-1}$ during the 130 d growing period and at least similar amounts of hydrophobic-BNI during this period. Thus, in total (i.e. hydrophobic + hydrophilic) BNI activity released could reach close to 1200 ATU $plant^{-1}$ during the growing phase of sorghum (Subbarao *et al.* 2013a).

Substantial genetic variability is detected for sorgoleone release from breeders' genetic stocks and ranged from 0 (IS 720) to 150 μg sorgoleone g^{-1} root dry wt (PVK 801). RIL (recombinant inbred lines) mapping populations were developed from parental lines differ in sorgoleone release capacity from roots [(high-sorgoleone parent) PVK 801 x 296B (low-sorgoleone producing parent)]. These RIL populations were genotyped for several thousands of SNP markers to generate saturated mapping. Currently this RIL population is undergoing sorgoleone-phenotyping, which will facilitate combining this phenotyping data with the available genotyping data to identify genetic markers for sorgoleone release trait. Such MAS (marker-assisted selection) based breeding approaches are critical to integrate physiological traits such as sorgoleone release into main-stream breeding programs to improve BNI-capacity in sorghum. Development of such genetic stocks and sorghum varieties are integral part of genetic strategies to improve BNI-capacity of sorghum root systems to control nitrification to improve NUE.

There seems an association between acid-tolerance and BNI-capacity in sorghum. Based on recent evaluations of acid-tolerant and acid-sensitive genetic stocks for BNI-capacity, it appears that many acid-tolerant genetic stocks also showed higher-BNI capacity compared to acid-sensitive genetic stocks. Also, preliminary findings suggest that some of the sweet-sorghum lines showed significant BNI-capacity in their root systems. Some perennial wild sorghums indicate that *S. plumosum* may have significantly higher BNI-capacity than cultivated sorghums, suggesting the need for a comprehensive evaluation of sorghum germplasm for BNI-function/capacity to identify genetic stocks with higher levels of BNI-capacity to initiate breeding programs to improve this trait (i.e. BNI-capacity).

Concluding remarks

It has been established that sorghum has relatively strong BNI-capacity. Initial results also indicate genetic differences in BNI-activity among sorghum cultivars. The challenge lies with our ability to exploit these genetic differences in BNI function to design sorghum genetic stocks with high-BNI capacity integrate this trait with high-yield potential to develop the next-generation sorghum cultivars to improve NUE through suppression of nitrification.

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