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Developing indicators and characterizing direct and residual effects of biological nitrification inhibition (BNI) by the tropical forage grass *Brachiaria humidicola*

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List of abbreviations

%	percent
α	alpha
a.k.a.	also known as
AMO	ammonium monooxygenase
*amo*A	bacterial and archaeal ammonia monooxygenase-α subunit
AOA	ammonium oxidizing archaea
AOB	ammonium oxidizing bacteria
ATP	adenosine triphosphate
Bh	*Brachiaria humidicola*
Bh	Previous *Brachiaria humidicola* pasture coverted into maize-cropping system
BMZ	Federal Ministry of Economic Cooperation and Development
BNI	biological nitrification inhibition
°C	degrees Celsius
C	carbon
C_2H_2	acetylene
Ca	calcium
$CaSO_4$	calcium sulphate
CEC	cation exchange capacity
CIAT	*Centro International de Agricultura Tropical*
CO_2	carbon dioxide
CORPOICA	*Corporación Colombiana de Investigación Agropecuaria*
cp.	Compare
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Cu	copper
cv.	cultivar
$\delta^{15}N$	^{15}N natural abundance (‰)
DAS	days after sowing
DANF	days after nitrogen fertilization
DCD	dicyandiamide
DMPP	3,4-dimethylpyrazole phosphate

DNA	deoxyribonucleic acid
e.g.	for instance (abbreviation for Latin "*exempli gratia*")
EMBRAPA	*Empresa Brasileira de Pesquisa Agropecuaria*
et al.	and others (abbreviation for Latin "*et alia*")
Fe	iron
g	grams
GHG	greenhouse gas
h	hour
ha^{-1}	per hectare
HAO	hydroxylamine oxidoreductase
HCL	hydrochloride
i.a.	among other things (abbreviation for Latin "*inter alia*")
IPCC	Intergovernmental Panel on Climate Change
K	potassium
kg	kilogram
KNO_2	potassium nitrite
KNO_3	potassium nitrate
L	liter
µmol	micromol
masl	meters above sea level
Mg	magnesium
min	minutes
mol	SI base unit symbol for mole
Mn	manganese
Mo	molybdenum
ηm	nanometer
ηmol	nanomol
N_2O	nitrous oxide
N	nitrogen
NADH	nicotine amide adenine dinucleotide
NH_3	ammonia

NH_4^+	ammonium
N_{min}	mineral nitrogen
N_{org}	organic nitrogen
NO_3^-	nitrate
NO_x	nitrous oxides
*nos*Z	nitrous oxide reductase genes
NR	nitrate reductase
NRA	nitrate reductase activity
NRs	nitrification rates
NUE	nitrogen use efficiency
O_2	oxygen
P	phosphorus
RCBD	randomized complete block design
rpm	rounds per minute
SE	standard error of the mean
SNI	synthetic nitrification inhibitor
spp.	species (plural)
sp.	species (singular)
UHOH	University of Hohenheim
vs.	versus
Zn	zink

Table of Contents

1. General Introduction

1.1 Nitrification and denitrification

One of the most important macronutrients for plant growth is nitrogen (N), which is mainly taken up by plant roots in its inorganic forms ammonium (NH_4^+) and nitrate (NO_3^-). NH_4^+ derives by the breakdown of organic matter in the soil by microbes (mineralization) or by organic or mineral fertilizer application. NO_3^- is the product of a soil metabolic microbial process called nitrification, where mainly bacteria and archaea under aerobic conditions oxidize NH_4^+ to NO_3^-. Nitrification represents one of the key microbial processes and is one of the rate-limiting steps of the nitrogen cycle (Skiba et al. 2011). The oxidative transformation of NH_4^+ is a two-step reaction mainly driven through aerobic, chemolithoautotrophic microbes. The first conversion step, the oxidation of NH_4^+ to nitrite (NO_2^-), is conducted by ammonia-oxidizers such as the common bacteria *Nitrosomonas* sp. (Belser 1979; Ferguson et al. 2007; Sahrawat 2008), whereas the second step is carried out by nitrite-oxidizing bacteria as, e.g. *Nitrobacter* sp. Under both oxidation steps the released energy is used by microbes for their metabolism. The reduction of NH_4^+ (or ammonia [NH_3] under high pH conditions) facilitated by the enzyme ammonium monooxygenase (AMO) is described by the following chemical reaction (Arp & Stein 2003):

$$2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 2\ H_2O + 4\ H^+ \quad (1)$$

In the second reduction step, the enzyme hydroxylamine oxidoreductase (HAO) reduces NO_2^- to NO_3^-:

$$2\ NO_2^- + O_2 \rightarrow 2\ NO_3^- \quad (2)$$

Generally, nitrification depends on temperature, pH, water and O_2 availability and finally substrate (NH_4^+) availability (Sahrawat 2008). In this regard, alteration of this microbial process by temperature (Shammas 1986) has been reported with a maximum NH_4^+ oxidation rate at around 30°C (Borchardt 1966; Wild et al. 1976). Furthermore, nitrification is pH dependent, whereby an optimum pH for *Nitrosomonas* was reported as 8.3 to 8.8 and for *Nitrobacter* 7.7 to 9.3 (Meyerhof 1917; Hofman & Lees 1953; Engel & Alexander 1958). However, nitrification also exists in acidic soils with a pH below 5.5 caused by obligate acidophilic ammonia oxidizers archaea (AOA) such as *Candidatus* Nitrosotalea devanaterra (Lehtovirta-Morley et al. 2011, 2016) that also might have a higher optimum temperature

for ammonium oxidation (Ouyang et al. 2017). Total nitrification activity is related mainly to autotrophic nitrifiers activity, whereas heterotrophic nitrification by bacteria and fungi exists, but contributes to a smaller extent, with reported values between 7 – 19% (Barraclough & Puri 1995; De Boer & Kowalchuk 2001; Islam et al. 2006). Concerning the abundance and activity of ammonium oxidizing bacteria (AOB) and AOA and their respective importance on entire nitrification, studies of the last decade delivered rather contradicting results. In this respect AOB abundance and activity appeared to be positively correlated with N availability (topsoil vs. subsoil, N application) which could not be confirmed for AOA (Jiang 2011; Di et al. 2009, 2010). Other studies suggested that abundance rather than growth and activity of AOA and not AOB control nitrification in acidic soils (Leininger et al. 2006; Gubry-Rangin et al. 2010; Zhang et al. 2012).

From an agroecological point of few, nitrification is also an important process for plant N nutrition, since the much more mobile mineral N form NO_3^- can be taken up in larger quantities by plant roots via mass flow. NH_4^+, due to its cationic charge, is less mobile in the soil and mostly attached to negative charged particles as e.g., clay minerals, and is taken up by the plant via diffusion. However, before NO_3^- can be assimilated by the plant, it has to be first reduced which constitutes a higher cost in terms of energy for the plant. The two enzymes nitrate reductase and nitrite reductase are responsible for these two reduction steps. In contrast uptake of NH_4^+ maintain a faster assimilation of taken up mineral N. However, high NH_4^+ concentrations in the plant can be toxic to the plant (Britto & Kronzucker 2002). In this regard, mentioned advantages and disadvantages of plant uptake of either NH_4^+ or NO_3^- lead consequently to the adaption that most plants cover their N demand through uptake of both mineral N forms (Marschner 2011). On the other hands certain species tend to prefer either one of the mineral N forms, which is related to evolutionary developed specialization (Boudsocq et al. 2012; Britto & Kronzucker 2013).

From an environmental point of view, nitrification has been identified as the major pathway associated with N losses in both, natural and agricultural systems (Subbarao et al. 2006a, 2009). Due to its anionic charge and consequently its mobility in the soil matrix, NO_3^- is susceptible to be leached from the soil and contributes to pollution of water bodies. Besides N losses through NO_3^- leaching, NO_3^- can be further

reduced to gaseous nitrogen oxide (NO_x) forms by denitrifying microbes mainly under anaerobic conditions (e.g. after heavy rains and slow drainage). The most prominent product is nitrous oxide (N_2O) which is a very potent greenhouse gas (GHG) that could contribute to a major extent to climate change with an estimated global warming potential of 265 times as CO_2 (IPCC 2014). However, N_2O emission could also occur directly after the first step of the nitrification process (1) denitrification contributes to a larger extent to N_2O production (Firestone & Davidson 1989).

Generally, natural grasslands or forest are characterized by low nitrification and denitrification rates and by a tight N cycle, however exceptions exist (Robertson & Groffman 2015). In this regard, N leaching losses are usually small and not severe for the N balance of most ecosystems. This is in stark contrast to agricultural systems, that are nowadays oversaturated with reactive N due to high amounts of N fertilization in different forms. Large amounts of N are applied to crops but N use efficiency (NUE) of the systems are poor and losses of N to the environment are high (Raun & Johnson 1999; Coskun et al. 2017a). In terms of N fertilizer losses, nitrification has an important role and predominant N losses occur in the form of NO_3^- or in the gaseous form of N_2O. Therefore, strategies to control nitrification might contribute to reduce the N footprint of agroecosystems.

1.2 Synthetic nitrification inhibitors

Several synthetic substances with the capacity to inhibit soil microbial nitrification in soils have been developed and tested decades ago (Fillery 2007). The substances block the enzymatic pathway of the ammonia monooxygenase (AMO) responsible for the first step in the oxidation of NH_4^+ to NO_3^- in soils (Ruser et al. 2015). The globally most important substances for synthetic nitrification inhibitors (SNIs) are nitrapyrin, dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) (Crawford & Chalk 1992; Zerulla et al. 2001; Upadhyay et al. 2011; Guo et al. 2013). Since nitrification and accordingly NO_3^- production is slowed-down, denitrification (under anaerobic conditions) rates have also been observed to be reduced by up to 35% under application of SNIs due to substrate (NO_3^-) shortage for denitrifiers (Ruser et al. 2015). Retardation of nitrification in agricultural systems is supposed to increase the time plants could take up N in the form of NH_4^+. Consequently, SNIs could thereby improve N

recovery and reduce (NO_3^-) leaching and diminish associated environmental impacts (Subbarao et al. 2006b; Gopalakrishnan et al. 2007).

Some SNIs such as DCD have been evaluated as highly specific to NH_4^+ oxidizers with no harmful effect on abundance of other microbes in the soil (Guo et al. 2013). However, others observed a significant reduction of non-target soil bacteria (Patra et al. 2006). SNIs appeared to differ in terms of inhibition effects on AOB and AOA (Wakelin et al. 2014; Ruser et al. 2015) or even between different AOB (O'Sullivan et al. 2017b). However, also side effects on N dynamics due to SNIs are reported as, for instance an increase of biological immobilization of NH_4^+. In this regard, mineral N is sustainably present in its cationic form, which is preferred over NO_3^- by N immobilizers and assimilated by them respectively (Sahrawat 1989; Hauck 1990; Crawford & Chalk 1992). However, it should be considered that N immobilization is strongly driven by available C and not mainly by inorganic N availability (Shen et al. 1984; Chalk et al. 1990).

Certain issues restrict the wide-spread use on SNIs primarily in smallholder farming systems of the tropics. High amounts of 15-30 kg ha^{-1} of DCD for proper nitrification inhibition are needed and the related high costs, the limited availability on the market, and its water solubility and consequently its susceptibility to leaching are some of the constraints for increasing application (Zerulla et al. 2001; Fillery 2007; Gopalakrishnan et al. 2009; Upadhyay et al. 2011). Various studies have been conducted to investigate the efficacy of many SNIs. Nitrapyrin can be easily volatilized and cannot be applied with solid N fertilizers and furthermore has shown reduced efficiency under higher temperatures (Chen et al. 2010), whereas this counts for all the here introduced SNIs (Ruser et al. 2015). With suggested application rates of 0.5 – 1.0 kg DMPP ha^{-1} this SNI seems to be very efficient in comparison to DCD (Zerulla et al. 2001) but case studies have shown that N_2O emissions were not more reduced by DMPP compared to DCD application (Di & Cameron 2012). The efficiency of DMPP seems to depend also on the applied form, which is either applied as liquid or sprayed on granulated fertilizers (Weiske et al. 2001; Di & Cameron 2012; Ruser et al. 2015). There is evidence that application of liquid DMPP might result in spatial separation of the SNI and NH_4^+ due to different diffusion or absorption to clay particles in the soil profile which makes NH_4^+ susceptible to nitrification again (Azam et al. 2001; Linzmeier et

al. 2001). Overall umpteen studies suggest that SNIs are playing a minor role in terms of nitrification control in tropical smallholder agro-ecosystems mainly due to the high costs and unpredictable efficiency. Further low-cost options to develop rather low nitrifying systems would therefore be an important contribution to reduce negative impacts of crop and livestock production (Subbarao et al. 2013b).

1.3 Biological nitrification inhibition (BNI)

Since N is indispensable for the synthetization of organic compounds containing amine ($-NH_2$) groups, this element often represents the rate-limiting step of growth for both, plant and microbe populations in ecosystems. Consequently, competition for N is always high and plants and microbes (and fungi) have evolutionary adapted to this situation and developed strategies to succeed in terms of N uptake and assimilation. Apparent from the already described environmental factors influencing nitrification, the influence on the nitrification process by plants themselves is a research field that re-gained major attention in the last decade (Fillery 2007; Skiba et al. 2011; Subbarao et al. 2013b; Coskun et al. 2017a). The ability of plants to control nitrifiers with secondary substances is one of these strategies to compete for scarce N resources in the soil (Skiba et al. 2011; Coskun et al. 2017b), and has been investigated some decades ago by Munro (1966), Meiklejohn (1968), particularly in forest based and grassland ecosystems. The release of tannins, phenolic acids or flavonoids from decaying leaves with an inhibiting effect on *Nitrosomonas* and *Nitrobacter* bacteria was studied for climax ecosystems (Rice & Pancholy 1973, 1974). In contrast to the work of Rice & Pancholy, Moleski (1976) concluded that the release of tannins inhibiting nitrification derived mainly by root exudation rather than from decaying leaves, stems or roots. However, Purchase et al. (1974) suggested that nitrification in *Hyparrhenia* grasslands was low due to NH_4^+ shortage caused by microbial immobilization of N, favored by turnover of grass roots that increased the amount of available carbon (C) and necessarily the N demand of microbes. Inhibition of *Nitrosomonas europaea* by monoterpenes derived from decaying needles from coastal redwood (*Sequoia sempervirens*) has been reported by Ward et al. (1997). Others, who applied the isolated substances directly to liquid cultures of *Nitrosomonas* (Bremner & McCarty 1988) investigated a possible inhibiting effect by terpenoids from ponderosa pine (*Pinus ponderosa* Dougl.). However, the effect observed under controlled liquid conditions, was absent even when higher amounts of the

substances were extracted from pine needles and applied to soils. Consequently, Bremner & McCarty (1988) came to the same conclusions as Purchase et al. (1974): low nitrification was rather due to low NH_4^+ availability caused by heterotrophic microbial immobilization stimulated by available organic C increase. In line with this, Stienstra et al. (1994) intended to separate the allelopathic effect from the N immobilization effect on the repression of nitrifiers in grasslands dominated by *Holcus lanatus*. The authors concluded that stimulation of NH_4^+ immobilization caused the effect. In contrast, the study of Paavolainen et al. (1998) concluded that nitrification inhibition in forest soil is a combined direct and indirect inhibitory effect by monoterpenes. Moore & Waid (1971) targeted the suppressive effect on nitrification of root washings applied to incubated soil with high NH_4^+ addition, to compensate for the N immobilization effect. It has been concluded that the substances had a direct inhibiting effect on nitrification, although the mechanism could not be explained in more detail.

Overall, the early work that targeted the link between plant derived substances and slowed-down NH_4^+ oxidation did not deliver clear evidence of two important points: firstly, if the plant derived substances, that slowed-down nitrification, are actively or passively released and secondly if the secondary metabolites that reached the soil from plant biomass either stimulated N immobilization or had a direct and specific toxic effect on nitrifiers (Erickson et al. 2000). Smits et al. (2010) further discovered that plant species affect the ecosystems as such that they may either repress or in contrast even increase the nitrification potential of a certain habitat. Furthermore, not yet understood environmental factors seem to play a key role in terms of nitrification inhibition. Field studies conducted in tropical grassland ecosystems have shown that even in close neighborhood, low- and high-nitrifying sites that contain the same plant species (*Hyparrhenia diplandra*) under the same soil type could co-exist (Lata et al. 1999). This finding provided evidence that plants, especially grasses in N poor savanna ecosystems, actively shape soil microbial nitrification (Lata et al. 2004; Smits et al. 2010; Coskun et al. 2017b).

One of the most prominent examples in terms of nitrification control is the tropical pasture grass *Brachiaria humidicola* (*Bh*) for which lower NO_3^- levels in soils have been reported already some decades ago (Sylvester-Bradley et al. 1988). *Bh* gained increasing intention in terms of its potential to control nitrification, since unique substances such as brachialactone in collected root exudates were

identified to be responsible for biological nitrification inhibition (BNI) (Subbarao et al. 2009). In the early work on altered nitrification by plants, the mechanisms for release of substances from plant roots could not be identified. Subsequently, BNI substance released by *Bh* in hydroponic studies could be triggered by supplying NH_4^+ (Subbarao et al. 2007b). However, BNI substance release could not be confirmed when roots were exposed to a NO_3^-. Furthermore, a low pH was identified as a factor fostering the release of root derived BNI substances. Apart for *Bh*, NH_4^+ and low pH have also been reported to be responsible for BNI substance release by sorghum (*Sorghum bicolor*) roots (Zakir et al. 2008). An additional factor that influences a significant BNI substance release of *Bh* seems to be plant age, since researchers working with young plants could not confirm a BNI effect by *Bh* (Miranda et al. 1994; Castoldi et al. 2013; O'Sullivan et al. 2017b). Concerning pot studies, substantial nitrification suppression by *Bh* has been confirmed one year after planting (Nuñez et al. 2018). However, there is lack of information concerning quantification of BNI substances released to the rhizosphere and therefore the interaction of plant age and effective BNI is poorly understood.

BNI by collected root exudates or extracts have, besides for *Bh* and sorghum, meanwhile also been documented for rice (Pariasca-Tanaka et al. 2010; Sun et al. 2016), a wild relative of wheat (*Leymus racemosus* [Lam.] Tzvelev) (Subbarao et al. 2007c), weeds such as wild radish, brome grass, wild oats, annual ryegrass (O'Sullivan et al. 2017a) and matgrass (*Nardo-Galion saxatilis*) (Smits et al. 2010). Whereas the exact active release mechanism of BNI substances for most of these plants, and also for *Bh*, is still unknown (Skiba et al. 2011), it is claimed for sorghum that BNI release is functionally linked to plasma membrane H^+-ATPase activity in roots (Zhu et al. 2012; Zeng et al. 2016). Concerning the mode of action, BNI exudates from *Bh* block both the ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) of *Nitrosomonas europaea* in contrast to SNIs that solely block the AMO pathway (McCarty 1999; Subbarao et al. 2007a; Gopalakrishnan et al. 2009).

1.4 Methods to characterize BNI

The methodological work that has been used to target BNI effects by plants are based on consequences of plant presence with BNI ability or BNI root exudates and extracts. In this regard, quantification of mineral N or gene abundance of nitrifiers are used to indicate a possible BNI effect. A common method

is the incubation of soil from field plots or pot experiments (rhizosphere soil), where plants with BNI potential were cultivated (Sylvester-Bradley et al. 1988; Gopalakrishnan et al. 2009; Castoldi et al. 2013; Karwat et al. 2017). Modified versions of this incubation studies were introduced to solely investigate the root exudate or extract effect on N dynamics: a standard soil was treated with prior collected root extracts or exudates and NO_3^- formation after NH_4^+ application monitored over time (Gopalakrishnan et al. 2009; Nardi et al. 2013; Meena et al. 2014). Furthermore, Ishikawa et al. (2003) simply monitored mineral N in frequently taken sub-samples from pots where plants with potential BNI capacity have been harvested previously. Short-term incubation methods as the shaken slurry method by Hart et al. (1994) were used to reduce the experimental time. Emphasizing on the determination of the potential nitrification activity (PNA) in soil samples with excess NH_4^+, the formation of nitrate (NO_3^-) is monitored in sub-samples taken over time from the permanent shaken slurry (Smits et al. 2010; Nardi et al. 2013). Another technique targets directly the nitrifying enzyme activity in soil from pot or field studies (adapted from Lensi et al. 1986): NO_3^- production after NH_4^+ addition in an aerobic incubation is deduced by N_2O measurements under addition of C_2H_2 (inhibits reduction of N_2O) in a subsequent anaerobic incubation (Lata et al. 2004). All of these methods alter nitrification due to changes of O_2, soil water content and destruction of soil aggregates. Consequently, soil incubations have the potential to uncover potential BNI effects, but straightforward conclusions to in-field BNI effects are difficult due to disturbance of the soil-plant system.

Soil-free incubation methods or so called bioassays, are also described in the literature, as the incubation of target bacteria (*Nitrosomonas europaea*, *Nitrosospira multiformis* or *Nitrobacter* spp.) with either root extracts or root exudates in liquid NH_4^+ cultures. For one method, nitrifying bacteria have been genetically modified, carrying genes for bioluminescence activity from the marine bacteria *Vibrio harveyi* coupled to the first NH_4^+ oxidation step (Iizumi et al. 1998; Subbarao et al. 2006a, b; Gopalakrishnan et al. 2007, 2009; Zeng et al. 2016). Other assays measure nitrification activity of non-modified nitrifiers simply by determining produced NO_2^- by a Griess reagent (O'Sullivan et al. 2016, 2017; Souri & Neumann 2018). However, the bioassay methods only take into account certain bacterial strains and exclude the role of other nitrifying bacteria and in particular ignore nitrifying archaea. Furthermore, the genetically modified *Nitrosomonas europaea* is not available on the market and,

besides of a luminometer, a laboratory with the status to work on genetic modified organisms is needed, which are certain constraints in a practical way. Using the Griess-method with simply non modified nitrifiers avoids these complications and even allows high throughput of samples via microplate reading (O'Sullivan et al. 2017b). However, the crucial point of reported discrepancy of plants expressing their BNI potential in hydroponic cultures and in field studies (Subbarao et al. 2007a) remains.

To investigate the BNI effect on total nitrifier populations in soils, the abundances of ammonia oxidizing bacteria (AOB) and archaea (AOA) have been determined by quantification of the respective ammonia monooxygenase subunit A gene (*amoA*). Data on gene copy numbers of nitrifiers determined by using real-time quantitative polymerase chain reaction (qPCR) have been used to indicate the suppressive effect by BNI on ammonia oxidizers (Ishikawa et al. 2003; Subbarao et al. 2009). This approach from the field of microbial ecology has certain constrains as huge discrepancies of DNA extraction protocols among laboratories, or alteration of target genes by the amplification signal (Smith & Osborn 2009). Furthermore, microbial abundance fluctuates and *amoA* gene numbers deliver only a snapshot of nitrifier community abundance. Furthermore, BNI release has been shown to underlie fluctuations over time due to stimuli in the soil (supporting information in Subbarao et al. 2009). It is therefore suggested that a single study, either soil or hydroponic based, delivers only an incomplete picture and to understand further the complex nature of BNI methods need to be combined and further developed.

Simple methods as the measurements of NO_3^- and NH_4^+ contents in soils where plants are grown in the field or in pots (Sylvester-Bradley et al. 1988; Karwat et al. 2017) give valuable reference information on BNI fluctuations. The advantage of this method is, that it is simple since soil sampling and mineral N determination requires cheap equipment such as an auger, KCl solution, funnels, flasks, paper filters and color agents and a device to read mineral N in the soil extracts. Constraints to be mentioned are biased mineral N concentrations by leaching, plant uptake and the microbial biomass, and the fact that mineral N could change quickly in the field.

N_2O measurements with the chamber method has been used to investigate a possible impact of BNI on both, N_2O emissions by nitrification and denitrification (Ishikawa et al. 2003; Subbarao et al. 2009;

Byrnes et al. 2017; Karwat et al. 2017). However, fluctuations of N_2O are high and, in terms of denitrification, are altered by the water saturation level, temperature, and soil organic matter contents.

1.5 The tropical pasture grass *Brachiaria humidicola*

Brachiaria humidicola (Rendle) Schweick (koroniviagrass, creeping signal grass, false creeping paspalum) is a C_4 pasture grass belonging to the family *Poaceae* and is originally from Eastern Africa and meanwhile used widely in South-American pasture systems established on previous natural savannas (Schultze-Kraft & Teitzel 1992; Miles et al. 2004). As most tropical African grasses it has an evolutionary developed tolerance to grazing pressure over Asian, European or American grasses due to its adaption to larger grazers that only survived in African savannas (Parsons 1972). Its aggressive, strong stoloniferous growth makes it very competitive and enabled it to enforce its distribution also from developed pastures into natural savanna systems of Latin America (Williams & Baruch 2000). *Brachiaria humidicola* (*Bh*) is mainly used in grazed pastures, but finds also use as ground cover in tree plantations or as erosion control on hillsides (Schultze-Kraft & Teitzel 1992). It is adapted to low fertility acidic soils with high Al-saturation, and is tolerant to flooding due to the ability to form aerenchyma as known for rice (Baruch et al. 1995; Cardoso et al. 2014). Its deep rooting ability (Rao et al. 1996; Fisher et al. 1994; Amézquita et al. 2004) might also enhance the organic matter content of deeper soil layers and store severe amounts of C there.

In terms of N uptake and assimilation *Bh* appeared to be less aggressive in N uptake after N dressing in the form of NH_4^+ compared to two other related grass species as *Brachiaria decumbens* and *Brachiaria brizantha* (Miranda et al. 1994; Nakamura et al. 2005). Another difference of *Bh* compared to the latter species is, that it (e.g., *Bh* cv. CIAT 679) can take up both N forms, whereas *Brachiaria brizantha* (e.g., cv. Marandú) has shown to take up only small amounts of NH_4^+, but rather feeds on NO_3^- (Castilla & Jackson 1991). The ability of *Bh* to bear even high quantities of available NH_4^+ might be an adaption simultaneously developed with BNI and could facilitate its reported higher tolerance to acidic soils compared to other *Brachiaria* species (Rao et al. 1996).

Due to its BNI ability it is expected to reduce the losses of N and tighten the nutrient cycling, which is of great importance to persist in low nutrient savanna soils (Rao 1998). Besides of *Sorghum bicolor*,

most studies on elucidating the BNI phenomenon in the recent years included *Bh*. The large collections of contrasting BNI genotypes derived by year-long breeding efforts at CIAT (Miles et al. 2004) together with a list of intensive investigations of the BNI process made it a prefect model plant for the research purposes of this dissertation.

1.6 Importance of BNI by *Brachiaria humidicola* in agroecosystems

During the 1970s, exotic grasses, mainly *Brachiaria* species, were introduced to the Brazilian savannas (*Cerrados*) and replaced the extensive cattle ranching systems with low livestock capacity of the native vegetation based pastures (Macedo 1995; Spain et al. 1996). To date, *Brachiaria* grasses are probably the most important grasses covering 50% of the total grassland area of Brazil (Jank et al. 2014). Also, the native savanna vegetation of the Colombian and Venezuelan plains (*Llanos*) have been replaced by mainly *Brachiaria* grasses (San José et al. 2003). Overall, out of the 250 million hectares of South America about 20 million hectares are planted with improved pastures, where *Brachiaria* grasses are the most important species used (Boddey et al. 1996). The demand for livestock products as meat and milk is expected to increase by 100% by the year 2050, and production systems need to be modified to decrease the negative environmental impacts that livestock production causes (Rao et al. 2015). The role of BNI by *Bh* in tropical savanna systems could be an important one, due to the wide acceptance of the grasses by farmers, and furthermore due to the increasing pressure to convert Latin American grassland systems into pastures (Lopes et al. 2004). Some points that need to be addressed to develop more environmental friendly pastures systems are the reduction of soil borne N_2O emissions, NO_3^- leaching and the linked pollution of water bodies. BNI by *Bh* could contribute to tighten the N cycle and reduce N leakage from pasture systems. Since *Bh* is widely used as a pasture grass not only in tropical Latin America, but also in Africa, the Pacific Islands and Australia (Miles et al. 2004), the observed BNI might already contribute to reduction of NO_3^- leaching and N_2O emissions on a large scale (Peters et al. 2013).

Another strategy to reduce N losses via nitrification and denitrification could be to include plants that govern the microbial oxidation of NH_4^+ via BNI into cropping-cycles, to create low-nitrifying agro-ecosystems where N losses are minimal (Subbarao et al. 2013b, 2015). This would not only have environmental benefits, but as well might reduce fertilizer expenditure due to higher NUE and

potentially increase crop yields. This is especially of crucial importance for smallholder farmers in so-called “developing countries”, where N fertilizer application is scarce due to economic reasons but low plant N availability often limits crop yields. Other visions include the detection of the genomic region responsible for BNI trait expression and transfer it through breeding strategies into modern crop varieties cultivated under high fertilizer N input (Subbarao et al. 2007c).

Whereas tropical natural grasslands used as pastures are not fertilized, and pastures with introduced grasses are only initially receive nutrient application during establishment (Fisher et al. 1997; Miles et al. 2004), improved pastures frequently receive P and N fertilization (Fonte et al. 2014). Effective BNI expression due to NH_4^+ presence (Subbarao et al. 2007b) could be a good argument for applying mineral N and contribute to avoid pasture degradation. Commonly, fertilization takes part when degraded pastures are converted into crop areas, since income of sold crop yields could cover the fertilizer N costs and justify the investment (Kluthcouski et al. 1991). In this case it is speculated that residual BNI by *Bh* reduces losses of applied N to the cropping system (Karwat et al. 2017). Further examination of a possible indirect efficacy on denitrification, and therefore on N_2O emissions from N fertilized crop systems on converted *Bh* pasture land are necessary (Ishikawa et al. 2003).

The rotation of crops and pastures (a.k.a. ley farming) as a tool of integrated management system to regenerate degraded pastures is used in Latin America (Lopes et al. 2004). For instance, the *barreirão* system of the Brazilian *Cerrados*, where pastures are sown simultaneously with crops (as e.g. rice, maize, sorghum or millet) to use the grass as N catch plant (sown deeper to delay establishment) after crop harvest where mineral fertilization has been applied (Kluthcouski et al. 2004; de Oliveira et al. 2004). *Brachiaria decumbens* and *Brachiaria brizantha* are widely distributed in the 200 million hectares of the Brazilian savannas. Enhanced yields of the subsequent crop (e.g. rice) under the *barreirão* system have been documented (Muzilli et al. 2004). If pastures are sown subsequently after the crop, the grass benefits from the residual fertilizer left-behind in the soil (or from mineralized N of crop residues) from the previous cropping system. Due to the improved nutrient availability by the previous N fertilized culture, crop-pasture rotations constitute a tool for grassland rehabilitation. BNI by *Brachiaria* grasses (Subbarao et al. 2006b) might therefore play a role in terms of N recycling in ley

farming systems. However, research that targets the contributing role of BNI on N recycling is still pending (Karwat et al. 2017). Furthermore, there is generally a growing interest in terms of using plants to manipulate the nitrification rate in soils with the target of reducing losses of N from the soil–plant system (Bowatte et al. 2015). N_2O emissions even from extensive pasture systems become important since bovine urine oversaturate certain spots of the pastures with N. Consequently, N losses of these spots could be reduced when pasture grasses as *Bh* with BNI are used and nitrification is slowed down after urea hydrolysis to NH_4^+ (Byrnes et al. 2017). Additionally, recycling of N excretion by animals in pasture systems is economical sound, since as already mentioned, fertilizer N addition is scarce due to economic reasons.

The meanwhile most widely distributed *Bh* accession in Latin America is cv. Tully (CIAT 679; CPI 16707; BRA 001627) and has been introduced to Ecuador already in 1983 and to Colombia in 1992 (Miles et al. 1996). Its BNI potential has been classified to be mid-high, but other less widespread used accessions with a significant higher BNI potential as, e.g. CIAT 26159 exist (Subbarao et al. 2007a, 2009; Nuñez et al., 2018). Consequently, this suggest that BNI contributes already to reduced N losses in large regions of tropical savanna regions of Latin America through the wide distribution of cv. Tully. However, not yet commercially available accessions identified in breeding populations at CIAT or EMPRAPA would have the potential to further increase the beneficial impact of BNI in terms of reduced N losses (Rao et al. 2014).

Other environmental or agroecological significant issues like C sequestration into soils (Gaitán et al. 2016) and reduction of soil erosion could be addressed by *Bh* due to its reported deep rooting and dense aboveground growth habit (Fisher et al. 1994; Rao et al. 1996; Amézquita et al. 2004). The grass could therefore, when managed in an adequate way, play an important role in terms of enhancing the usual low nutrient quality of savanna soils and ameliorant the C footprint of the agroecosystem (Rao 1998).

1.7 Project description

This dissertation was conducted in the framework of the project “Climate-smart crop-livestock systems for smallholders in the tropics: Integration of new forage hybrids to intensify agriculture and to mitigate climate change through regulation of nitrification in soil” (Rao et al. 2014). The project was financed

by BMZ-Beaf (GIZ Project Number: 11.7860.7-001.00) and was a joint project of the International Center for Tropical Agriculture (CIAT) in Colombia, University of Hohenheim (UHOH), Corporación Colombiana de Investigación Agropecuaria (CORPOICA), Universidad de los *Llanos*, Universidad Nacional Agraria (UNA), Consorcio para Manejo Integrado de Suelos (MIS), GIZ-Programa Manejo Sostenible de Recursos Naturales y Fomento de Competencia Empresariales (GIZ-MASRENACE). The project duration was from March 2012 – December 2015.

The main goal was to improve agricultural productivity and mitigate climate change through more efficient nutrient use and reduced greenhouse gas emissions from smallholder crop-livestock systems. The purpose was that small-scale farmers as well as research and development institutions apply the innovative approach of BNI by *Brachiaria humidicola* (*Bh*) forage grass hybrids to realize benefits in economic and environmental sustainability from integrated crop-livestock production systems.

The research (Chapter 2, 3 and 4) conducted for this dissertation contributed to the identification *B. humidicola* (*Bh*) hybrids with different levels of BNI. Therefore 118 apomictic hybrids of *B. humidicola* were evaluated for their growth and nutritive value and their potential ability to inhibit nitrification in soil under greenhouse conditions. A selection of *Bh* hybrids with contrasting levels of plant BNI were evaluate for their growth, nitrogen (N) uptake, N use efficiency and forage quality in the wet and dry season under field in the *Llanos* of Colombia two years. Indicators of BNI activity were developed for use under field conditions based on the role of BNI in improving the efficiency of utilization of N fertilizer. The methodological approaches included in Chapter 2, 3 and 4 of this dissertation were applied to evaluate contrasting hybrids with different BNI capacity to recover native and applied N and minimize leaching and gaseous losses of N_2O in soil column experiments in the greenhouse at UHOH and at CIAT Colombia. Furthermore, the research of this dissertation contributed to the determination of the residual value of the BNI function in long-term pastures on N use efficiency and grain yields of subsequent crops.

1.8 Research Justification

Nitrification and denitrification has been identified as the two main microbial processes contributing to N leakiness of agroecosystems. Retarded NH_4^+ oxidation could extent the time for plants to take up N and consequently might improve N uptake and NUE of the system (Subbarao et al. 2013b). Furthermore,

this would reduce the leaching losses of NO_3^- and consequently also the denitrification losses (e.g. N_2O), which illustrates potential environmental benefits. Controlling nitrification by the application of SNIs is not a viable option in the humid tropics since climatic factors have shown to constrain their efficacy. Furthermore, the high cost of SNIs restrict smallholder farmers from its adoption. The pasture grass Bh has shown to have a high BNI potential under controlled and field studies (Ishikawa et al. 2003; Subbarao et al. 2009; Byrnes et al. 2017). *Bh* is one of the most widely used forage grasses in the tropical Americas and beneficial effects in terms of low N losses and high N recycling might be linked to BNI (Miles et al. 2004; Peters et al. 2013). Although its BNI potential has been demonstrated, predictions of the *in vivo* BNI performance in the field are weak, due to the lack of feasible methods. Discrepancies have appeared between the measured BNI potential under controlled conditions and BNI performances of certain *Bh* genotypes (Subbarao et al. 2007a; Nuñez et al. 2018). Furthermore, reduced NO_3^- losses from soil under BNI influence have been hypothesized by many researchers, but the proof is still pending. New *Bh* genotypes are under development at CIAT Colombia whereby BNI is already on the list of considered traits for developing improved *Bh* genotypes (Rao et al. 2014). It is known that BNI is differently expressed among contrasting *Bh* CIAT accessions, and even higher BNI potentials as identified for the standard cv. CIAT 679 (Tully) have been documented (Subbarao et al. 2007a, b, 2009). New developed *Bh* hybrids from CIAT should be screened for BNI to identify high BNI candidates for further breeding purposes (Arango et al. 2014). Although, incubation methods, either hydropic or soil based, are widely accepted among the research community, additional methods for a rapid screening of *in vivo* BNI performance are needed. Approaches with minimal disturbance of the soil-plant system would further allow to characterize the BNI phenomenon over a longer period and clarify certain fluctuations on the ability of *Bh* to depress nitrifiers.

The pressure on native savannas in the Colombian *Llanos* increases and large areas have been replaced by *Brachiaria* grasses (San José et al. 2003). Furthermore, conversion of these *Brachiaria* pastures into maize or soybean systems are widely practiced (Lopes et al. 2004). However, the role of the residual BNI effect on subsequent N fertilized crops is still unknown e.g., the persistence of the BNI effect after pasture conversion and consequently a hypothesized higher N uptake by the subsequent crop. This is of main importance since N losses are severe from highly N fertilized crop systems, such as soybean or

maize systems. Although pasture systems generally have tight N recycling, certain spots of punctual N losses might be significant due to bovine urine disposal and justifies to further investigate the role of BNI by *Bh* (Byrnes et al. 2017). The potential of BNI to contribute to reduced N losses in agroecosystems is not yet exhausted on a large-scale. There is evidence that new developed *Bh* hybrids exceed the BNI potential known from the widely used standard cultivar CIAT 679 cv. Tully (Arango et al. 2014). Consequently, methods for screening the BNI potential and the *in vivo* performance in the field need to be developed to be able to identify the next generation of *Bh* germplasm with a significant beneficial BNI effect.

1.9 Hypotheses and Objectives

The following hypotheses were addressed in the framework of this dissertation:

1) The residual BNI effect caused by long-term cultivation of *Brachiaria humidicola* will be expressed after pasture conversion into a maize crop system in terms of higher N uptake, lower soil NO_3^- levels, lower N_2O emissions and lower N fertilizer losses by the subsequent crop compared to a non-BNI control site. Furthermore, reduced nitrification rates in incubated soil are expected in the previous *Bh* rangeland. The residual BNI effect will reduce over time, since BNI substances will be decomposed or leached, and consequently nitrification is expected to increase again. *Bh* biomass turnover will, apparent from BNI substance release, affect N nutrition of the maize crop positively.
2) Strong *in vivo* BNI performance by *Bh* in soil reduces formation of NO_3^- and as a consequence NO_3^- uptake by *Bh*. Therefore, low plant NO_3^- uptake is reflected in low activity of the enzyme nitrate reductase, responsible for NO_3^- assimilation, in plant tissues of *Bh*.
3) Effective BNI will reduce nitrification and consequently less ^{15}N discrimination among the NH_4^+ and the NO_3^- pool occurs in soil. High BNI will be expressed in a respective lower $\delta^{15}N$ shoot signature of *Bh* under conditions where NO_3^- is leached from the system, since *Bh* would feed on a respective less ^{15}N enriched NH_4^+pool, as in contrast to low BNI and high nitrification.

The following objectives of this dissertation were:

1) To detect a possible residual BNI effect by *Bh* on a subsequent (non BNI) maize cropping system, with a main focus on N fertilizer recovery, and to characterize the persistence of the residual BNI effect and additional factors influencing nitrification. (Chapter 2)
2) To proof that the activity of the enzyme nitrate reductase in *Bh* tissues is inversely related to nitrification inhibition and therefore a suitable method to detect differences in terms of *in vivo* BNI (Chapter 3)
3) To investigate if the fractionation of stable N isotopes due to the nitrification process as well as N losses (e.g. NO_3^- leaching) will influence the natural ^{15}N abundance in *Bh* tissues, and hence if plant $\delta^{15}N$ signals could serve as indicator for BNI and long-term N losses (Chapter 4)

1.10 Outline of the study

This dissertation has been submitted as a cumulative thesis and is composed by three scientific articles, of which one has been published (Chapter 2) and two have been submitted (Chapter 3 and Chapter 4). The general introduction (Chapter 1) approaches the relevance of the microbial processes nitrification and denitrification from an agroecological and enviro-agronomical point of view, explains the possibility to inhibit or retard these microbial processes with synthetic derived substances to inhibit primarily nitrification and secondly denitrification. The process of biological nitrification inhibition (BNI) by the tropical pasture grass *Bh* is the central focus of this dissertation. Knowledge gaps of the BNI topic are addressed in the following parts of the thesis. Chapter 2 examines the residual BNI effect with a focus on its persistence in terms of N uptake by maize, N losses, nitrification rates, nitrous oxide emissions and its contribution to yield formation of the two subsequent maize crops in the following two rainy seasons in the *Llanos* region of Colombia. It further discusses a combined effect of residual BNI substances and the effect of *Brachiaria* residues on the following maize cropping system. Chapter 3 focuses on the link between BNI impact on soil parameters and the enzymatic activity of nitrate reductase in leaves of *Bh*, influenced by NO_3^- uptake and consequently by nitrification. The aim was to test the method as a proxy for *in vivo* BNI by *Bh* with minimum disturbance of the soil-plant system. Chapter 4 aims to link BNI to isotopic N fractionation between different soil mineral N pools caused by

nitrification The aim was to use the ^{15}N natural abundance as indicator for BNI and N losses under field and greenhouse conditions. This dissertation closes with the general discussion (Chapter 4) including also an outlook concerning the future research needs of the topic.

2. Residual effect of BNI by *Brachiaria humidicola* pasture on nitrogen recovery and grain yield of subsequent maize

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https://link.springer.com/article/10.1007/s11104-017-3381-z

2.1 Abstract

Background and Aims

The forage grass *Brachiaria humidicola* (*Bh*) has been shown to reduce soil microbial nitrification. However, it is not known if biological nitrification inhibition (BNI) also has an effect on nitrogen (N) cycling during cultivation of subsequent crops. Therefore, the objective of this study was to investigate the residual BNI effect of a converted long-term *Bh* pasture on subsequent maize (*Zea mays* L.) cropping, where a long-term maize monocrop field (M) served as control.

Methods

Four levels of N fertilizer rates (0, 60, 120 and 240 kg N ha^{-1}) and synthetic nitrification inhibitor (dicyandiamide) treatments allowed for comparison of BNI effects, while ^{15}N labelled micro-plots were used to trace the fate of applied fertilizer N. Soil was incubated to investigate N dynamics.

Results

A significant maize yield increase after *Bh* was evident in the first year compared to the M treatment. The second cropping season showed an eased residual effect of the *Bh* pasture. Soil incubation studies suggested that nitrification was significantly lower in *Bh* soil but this BNI declined one year after pasture conversion. Plant N uptake was markedly greater under previous *Bh* compared with M. The N balance of the ^{15}N micro-plots revealed that N was derived mainly (68-86%) from the mineralized soil organic N pool in Bh while plant fertilizer N recovery (18-24%) was not enhanced.

Conclusions

Applied N was strongly immobilized due to long-term root turnover effects, while a significant residual BNI effect from *Bh* prevented re-mineralized N from nitrification resulting in improved maize performance. However, a significant residual *Bh* BNI effect was evident for less than one year only.

Keywords: Biological nitrification inhibition; N use efficiency; N recovery; Soil incubation; Nitrate leaching; ^{15}N.

2.2 Introduction

Lost fertilizer nitrogen (N) from agricultural systems harms the environment via increased nitrate (NO_3^-) amounts in water-bodies and nitrous oxide (N_2O) emissions to the atmosphere (Baligar et al. 2001). Nitrification is the microbial oxidation of ammonium (NH_4^+) to NO_3^-, whereas the latter mineral N form, due to its anionic charge, is very mobile in the mainly negative charged soil matrix compared to cationic NH_4^+. Denitrifying microbes use NO_3^- for their metabolism mainly under anaerobic conditions and contribute to N losses in gaseous forms. Natural grassland or forest systems generally have lower nitrification potentials compared with human made agricultural systems supplied with high N inputs that spur soil nitrification and consequently favor high N losses (Robertson and Groffman 2015).

Strategies to suppress nitrification after N fertilization could result in higher uptake of N in the form of NH_4^+ by crops and might be additionally beneficial for plants preferring N-NH_4^+ (Boudsocq et al. 2012), while reducing N losses to the environment. Synthetic nitrification inhibitors (SNIs) like nitrapyrin, 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) were shown to inhibit the activity of soil nitrifiers, but persistence of their effectiveness depends strongly on environmental factors (Zerulla et al. 2001, Fillery 2007, Ruser & Schulz 2015). In addition, prices for SNIs are beyond the reach of smallholders that manage low input systems in tropical and subtropical regions.

Research evidence for inhibition of NO_3^- accumulation in a Colombian pasture soil under *Brachiaria humidicola* (Rendle) Schweick (*Bh*) was generated for the first time by Sylvester-Bradley et al. (1988). The development of a bioassay (Subbarao et al. 2006a) using the inhibitory action of *Bh* root exudates on recombinant *Nitrosomonas europaea* (Iizumi et al. 1998) subsequently allowed the detection of biological nitrification inhibition (BNI) in several tropical pasture grasses (Subbarao et al. 2007a). Using hydroponic systems, it was claimed that the exudation of BNI substances from roots of *Bh* is an active release of biological nitrification inhibitors that are triggered by the presence of NH_4^+ (Subbarao et al. 2007b). These *Bh* exudates contained BNI active substances like methyl-p-coumarate and methyl ferulate, while the shoot tissue contained other very effective BNI substances such as linoleic acid (LA) and linolenic (LN) acid (Gopalakrishnan et al. 2009). Additionally, the cyclic diterpene brachialactone

was suggested to be the main substance in root exudates of *Bh* that suppresses nitrification (Subbarao et al. 2009). Moreover, BNI exudation is favored by low pH (Subbarao et al. 2007b).

However, little is known about the turnover of such compounds in soils. First studies under controlled conditions by Subbarao et al. (2008) indicated that LA maintained 50% of its inhibitory effect in terms of NO_3^- produced per g dry soil after 120 days, whereas the BNI effect of LN was stable until the end of the incubation period (4 months). Since it was observed that a major BNI effect in *Bh* pastures can be established within 3 years (Subbarao et al. 2009), it is also likely that next to the active release of BNI substances root decomposition could contribute to the accumulation of BNI products in soil. Consequently, such a mechanism could result in a steady release of BNI substances inhibiting microbial nitrification for a prolonged time. This would be advantageous over SNIs that are exposed to rapid leaching under high rainfall and temperature conditions. Furthermore, *Brachiaria* grasses are known for their deep rooting systems (Fisher et al. 1994; Boddey et al. 1996; Rao 1998) that could release BNI substances and inhibit nitrification and denitrification also in deeper soil layers.

Not much is known about how long and to what extent the BNI effect in soil remains after removal of *Bh* pastures. Furthermore, most of these substances are apparently anionic (Subbarao et al. 2007b) and will not be fixed by predominantly negatively charged soil aggregates. Additionally, microbes may start decomposing BNI substances once released to the soil as observed for other organic root derived substances (Lynch & Whipps 1990). A BNI persistence is of major interest for arable systems that obtain substantial N inputs often associated with high N losses. Tropical natural grasslands used as pastures are not fertilized and pastures with introduced grasses are commonly only initially fertilized during establishment (Miles et al. 2004). However, fertilization takes part when degraded pastures are converted to crop areas (Kluthcouski et al. 2004). For instance, intensive cropping systems like mono-cropped maize often have low nitrogen use efficiency (NUE) (Raun & Johnson 1999). This is evidenced by high unaccounted N losses of 52 – 73% (Francis et al. 1993) in maize cropping systems via NO_3^- leaching and N_2O emission (Hilton et al. 1994) under high rainfall conditions. Specifically, when plants are still young and the root system is still small $N\text{-}NH_4^+$ fertilizer is rapidly nitrified accelerating environmental problems (Schröder et al. 2000).

To counteract these N losses, it could be speculated that pre-cropping of BNI plants contributes to inhibition of nitrification and consequently reduces N losses of applied fertilizer of subsequent cropping systems if the BNI active substances persist sufficiently long enough in soils. This is of great importance since *Bh* is one of the most exploited species planted in approximately 118 million hectares in Latin America (Miles et al. 2004). Potential benefits for subsequent crops in agro-pastoral rotation systems have been shown for upland rice after pasture (Sanz et al. 2004) although a possible contribution of BNI has not been established. The present study was conducted as part of a larger project on BNI (Rao et al. 2014). The first objective of this study was to test the residual BNI effect from a *Bh* pasture on subsequent maize crop performance in terms of grain yield, total N uptake and fertilizer N recovery. The second objective was to investigate the impact of residual BNI effect on soil N dynamics. Three hypotheses were tested: (I) A significant residual BNI effect is still present in the soil after removal of *Bh*, but is diminished in the second maize crop season compared to the first year after pasture conversion. (II) Nitrification inhibition is reflected by lower NO_3^- amounts, lower nitrous oxide emissions, lower N fertilizer losses and consequently by improved soil N recovery in converted soils. (III) The turnover of *Bh* pasture residues in the converted soils have a positive effect on N nutrition, NUE and improved maize grain yield.

2.3 Materials and Methods

2.3.1 Field site and experimental set-up

The study location was established at the Corpoica La Libertad Research Center in the Piedmont (Andean foothills) region of the *Llanos* of Colombia (4°03'46”N, 73°27'47”W). Mean annual rainfall is about 3,685 mm with an average temperature of 21.4°C at an elevation of 338 m above sea level.

Table 2.3.1 Topsoil chemical characteristics of the two field sites at Corpoica - La Libertad Research Center in the Andean foothills of Colombia. Soil samples were taken after lime application and before maize sowing in both years respectively.

Year	Field site	Depth (cm)	pH	OM (%)	N (%)	C:N	P Bray II (mg kg^{-1})	CEC	Al^{2+}	Ca^{2+}	Mg^{2+}	K^{+}
										(mmol kg^{-1})		
2013	Long-term *B. humidicola*	0-20	5.2	4.0	0.15	14.7	2.5	32.2	8.2	15.2	7.3	1.5
	Long-term *Zea mays*	0-20	5.4	3.0	0.11	12.4	16.3	28.9	8.2	13.1	6.0	1.2
2014	Long-term *B. humidicola*	0-20	5.5	3.8	0.17	10.3	3.0	72.2	0.9	16.9	7.8	2.2
	Long-term *Zea mays*	0-20	5.5	2.5	0.11	12.8	28.4	60	0.9	11.5	5.6	1.8

The experiment included two fields (each 0.5 ha) in close vicinity (~2.3 km away). The first one, a 15 years-old productive *Brachiaria humidicola* (Rendle) Schweick cultivar Tully (CIAT 679) pasture (Bh) with high BNI potential (Subbarao et al. 2007a, 2009) was used to establish a maize (*Zea mays* L.) cropping field to test the residual BNI effect. As non BNI control field, a nearby cropped area, where maize (M) was grown as monocrop for the last 15 years was chosen. Fields were chisel plowed 2 times on May 25, 2013 and sprayed with glyphosate afterwards to impede regrowth of *Bh* and weeds. Dolomitic lime was applied at a rate of 2 t ha^{-1} to the field of the previous pasture whereas the maize field received 0.5 t ha^{-1} of lime. The different amounts of chalk were applied to align the different pH values of the two field sites (Table 2.3.1).

Each field site was split into 3 blocks with each block containing 4 main plots (20 x 20 m) that received different N fertilizer rates (0, 60, 120 and 240 kg N ha^{-1}). A sub-plot was nested within the main plot with a size of 4.8 x 2.25 m (10.8 m^2) consisting of a synthetic nitrification inhibitor (DCD, dicyandiamide) treatment for the 3 levels of N (60, 120 and 240 kg N ha^{-1}). Basal fertilization included (per ha) 36 kg N applied in the form of diammonium phosphate (DAP), 75 kg potassium (K) and 40 (50 in 2014) kg phosphorus (P) combined with 30 kg Boronzinco® (4.5 kg Zn, 0.15 kg Cu, 0.75 kg B, 1.8 kg S) and 100 kg Delfoscamag® (3.3kg P_2O_5, 13 kg MgO, 30 kg CaO, 8 kg S). Control plots ("0 N") were not supplied with N but with all other mentioned elements. For the second and third N split dressing in 2013, N was supplied in the form of urea equally at 15 (17 in 2014) days after sowing (DAS) and 41 DAS, respectively. DCD was applied to respective sub-plots at the second and third N split application in both seasons with a total fertilizer N contribution rate of 20% DCD-N.

Maize was cropped in the last months (12 July 2013 and 27 June 2014) of the rainy seasons (Fig 2.3.1). In 2013, a maize hybrid (Pioneer 30K73) was sown in both fields using a planting density of 50,000 plants ha^{-1}. In the second season (2014) a maize hybrid (Monsanto Dekalb 1596) with improved tolerance to fungal infection was chosen for both sites and sown at the recommended 60,000 plants ha^{-1}. Due to different maturity of the two hybrids, maize cobs in the first season (2013) were harvested at 120 DAS, while in the second season (2014) they were harvested at 138 DAS. Grain yields were

extrapolated based on the number of rows ha^{-1}. Agronomic N use efficiency (ANUE) was calculated by dividing the applied fertilizer N amounts by N in maize grain yields.

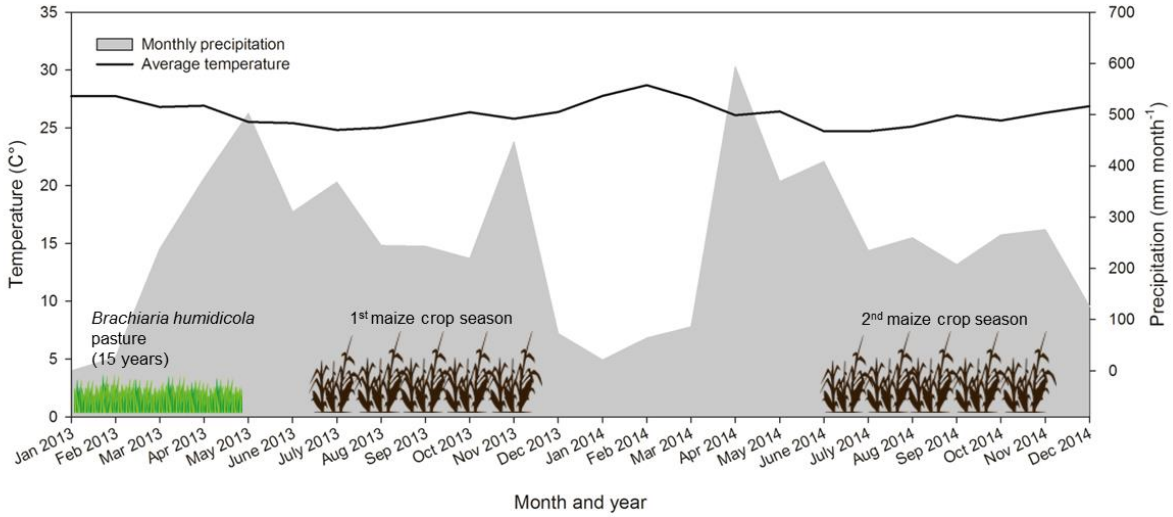

Fig 2.3.1 Distribution of monthly precipitation (mm $month^{-1}$) and average temperature (C°) during the years 2013 and 2014 at Corpoica La Libertad Research Center with indication of the *Brachiaria humidicola* CIAT 679 long-term pasture (Bh treatment) and the duration of the 2 maize crop experiments in the rainy seasons.

2.3.2 Mineral N determination

Mineral N (N_{min}) determination was done in 2013 at 41 DAS (before third N split application) and at 82 DAS. In 2014, mineral N was measured before second and after third N dressing. Soil N_{min} was extracted at the field site with 1 M KCl. At each sampling date, 8 samples were taken randomly with an auger from the topsoil layer (0-20 cm) from each main and DCD subplot from the Bh and M fields (in 2014 only in the most contrasting N treatment plots, 0 N and 240 N). Two representative sub-samples of 20 g fresh soil were taken. One sub-sample was used for determination of the soil dry matter by the gravimetric method, whereas another sub-sample was mixed in plastic bottles with 200 ml of KCl solution for N_{min} determination. The bottles were shaken intermittently by hand for about half an hour and filtered through Whatman Grade 2 filter paper. Filtered extracts kept at 4°C. NO_3^- were measured in yellow ionized form derived from alkalization with sodium salicylate and NH_4^+ as green ammonium salicylate complex with a Synergy Ht ultraviolet spectrophotometer and analyzed with Gen5™ Data Collection and Analysis Software (BioTek™).

2.3.3 Soil incubations

Soil was collected from the fields for incubation studies conducted under controlled conditions. This method was chosen to determine differences in nitrification activity among the field sites (I), and secondly to test the efficiency of DCD (II) that was used as an alternative for having a non-BNI control in the trial. Topsoil (0-20 cm) was collected from main plots before sowing in both seasons and air dried for 48 hours and sieved (2 mm mesh size) and visible root residues and small stones were removed. Afterwards, 5 g of soil were filled in small glass flasks followed by application of 1.5 ml ammonium sulfate ($(NH_4)_2SO_4$) solution as substrate for nitrifiers. The N concentration applied to the flask was 226.4 mg $N\text{-}NH_4^+$ kg dry $soil^{-1}$. DCD was diluted with the NH_4^+ solution at 20% of DCD-N contribution of the total N applied to the incubated soil (according to pre-tests, data not shown). Flasks were sealed with parafilm that contained 2 holes for aeration and incubated at 25°C in the dark for 11, 13, 15, 17 and 19 days when soil N_{min} was extracted with 50 ml 1 M KCl. Nitrification in soil was expressed as net increase (deviation from basal N) of µg $N\text{-}NO_3^-$ g organic N^{-1} (N_{org}^{-1} = total N – N_{min}), whereas apparent net mineralization was calculated as µg $N\text{-}NH_4^+$ g N_{org}^{-1} over time. Total N was measured with an isotope ratio mass spectrometer (IRMS) as for other soil samples explained below.

2.3.4 ^{15}N micro-plots

For the N treatments 60 N (only 2013) and 120 N (2013 and 2014), ^{15}N labeled N fertilizer was used to trace the fate of applied N and to determine ^{15}N recovery in different soil layers and in maize plant tissue. Micro-plots were set up within the respective main plots of the N treatments mentioned above. Aluminum sheets were riveted together to a frame that was penetrated into the soil to a depth of 50 cm below the soil surface. Micro-plots had a size of 1.2 m² (2 m x 0.6 m) in 2013 and 1.0 m² (2 m x 0.5 m) in 2014 (due to higher planting density) and included 6 maize plants. 10.3883 atom % ^{15}N enriched ammonium sulfate $(^{15}NH_4)_2SO_4$ (Sigma-Aldrich®) was used to prepare the applied nutrient solution that was sprayed with a hand pump on the soil surface. Two liters of the corresponding N solution were applied at sowing, 16 DAS and 41 DAS in 2013 according to the N rates of the respective main plots. Second and third N dressing date in 2014 were changed to 25 DAS and 46 DAS due to practical

workload. As control to the 120 N micro-plots, DCD was mixed with $(^{15}NH_4)_2SO_4$ and conventional $(NH_4)_2SO_4$ and applied to corresponding DCD micro-plots.

2.3.5 Maize and soil harvest in ^{15}N micro-plots

All plants from the micro-plots were harvested at the same time after sowing in the respective seasons (at 120 DAS in 2013 and at 138 DAS in 2014). Samples were oven dried at 60°C for 7 days, then separated into grains, spindle, shoot, leaves and roots and shredded separately. Plant materials were ball-milled and an amount of exactly measured 3-4 mg of the low N containing tissues (stems, roots, leaves) was filled into tin capsules (5 x 9 mm HEKAtech GmbH, Germany), while 2-3 mg of the ground maize grains were used for ^{15}N determination. For the calculation of the ^{15}N excess in the maize tissue, the background signal of non-enriched maize plant samples of the main and DCD subplots were measured.

Soil samples were taken right after harvest of the maize plants from the micro-plots. For estimation of soil ^{15}N recovery, firstly the topsoil layer 0-10 cm and 10-20 cm were removed from each of the micro-plots and placed separately on plastic sheets and homogenized and sub-samples were taken. Afterwards, 6 cores were taken with an auger for the respective soil layers 20-40 cm, 40-60 cm, 60-100 cm and sub-cores mixed to receive two samples per layer per micro-plot. To calculate the natural ^{15}N enrichment of the soil, corresponding samples were taken with an auger from the main plots at the same soil layers. All soil samples were oven dried (40°C), homogenized, ground with a porcelain mortar and ball-milled. Furthermore, a set of intact cores of the respective main plots were taken for determination of bulk density and texture analysis according to the method of Zamudio et al. (2006).

2.3.6 ^{15}N, total N analysis and calculation

Samples were analyzed for total N and ^{15}N by using a Euro Elemental analyzer coupled to a Finnigan Delta continuous-flow IRMS (Thermo Scientific, Bremen, Germany). The amount of the sampled material per capsule was calculated and adjusted to reach the optimal N target amount of about 50 µg N per sample. Yield and N recovery calculation were carried out following IAEA (2001) guidelines.

Maize yield for respective tissues was calculated according to the equation:

$$DM\ yield\ (kg/ha) = FW(kg) \times \frac{10000\ (m^2/\ ha)}{area\ harvested\ (m^2)} \times \frac{SDW\ (kg)}{SFW\ (kg)} \quad (1)$$

Where DM refers to dry matter and FW to fresh weight per area harvested. SDW and SFW correspond to dry weight and fresh weight of a sub-sample, respectively.

N yield for maize plants was estimated as:

$$N\ yield\ (g/m^2) = \frac{DM\ yield\ (g/m^2) \times N(\%)}{100} \quad (2)$$

$$N\ fertilizer\ yield\ (g/m^2) = N\ yield\ (g/m^2) \times \frac{\%Ndff}{100} \quad (3)$$

Where %Ndff corresponds to 'N derived from the fertilizer'

$$\%Ndff = \frac{^{15}N\ atom\ \%\ excess\ in\ sample}{^{15}N\ atom\ \%\ excess\ in\ fertilizer} \times 100 \quad (4)$$

Fertilizer N recovery in maize plants or soil samples was calculated according to

$$^{15}N\ recovery\ \% = \frac{^{15}\ Nexcess\ amount\ sample\ (g)}{^{15}N\ excess\ applied\ (g)} \times 100 \quad (5)$$

$$^{15}N\ excess\ amount\ sample\ (g) = \frac{N\ amount\ sample\ (g)\ \times\ ^{15}N\ atom\ excess\ sample}{100} \quad (6)$$

$$^{15}N\ excess\ applied\ (g) = \frac{N\ amount\ applied\ (g)\ \times\ ^{15}N\ atom\ excess\ fertilizer}{100} \quad (7)$$

$$^{15}N\ atom\ excess\ sample = \ ^{15}N\ atom\ \%\ sample\ -\ ^{15}N\ atom\ \%\ background \quad (8)$$

^{15}N atom% background was measured in maize plants or soil samples taken from the main plots where the respective micro-plots were installed.

2.3.7 Nitrous oxide emission measurements

Static rings with an inner diameter of 16 cm (2013) and 21 cm (2014) were installed in all main and sub-plots at the two fields M and Bh. For gas sampling, a chamber (volume 3901 cm³ in 2013, 6927 cm³ in 2014) was placed on the rings and sealed with a rubber band. A thermometer was installed through a

sealed hole inside the chamber for initial temperature determination. After a settling time of 15 minutes four gas samples (0, 10, 20 and 30 minutes after chamber placement on the ring) were taken per chamber. Samples were taken with a syringe (5 ml) through the septum on the top of the chamber. In 2013, the first gas sampling took place 4 days after the second N split application in the main and DCD sub-plots (18 DAS), while second gas sampling was at 45 DAS, four days after the last N split application. For the second maize crop season (2014), the methodology was slightly adjusted due to practical and financial constraints. Gas emission was measured after the last N split (41 DAS) at 43 DAS, 45 DAS and 48 DAS in the main and DCD sub-plots of the 0 N and 240 N treatments.

N_2O flux was calculated according to:

$$F_N = k_{N2O} (T0/T1) (V/A) (dc/dt)$$

where F_N is the flux of N-N_2O in µg $m^{-2}h^{-1}$, T_0 = 273 K, T_1 = initial temperature in the chamber in K, V = volume in m^3 of the chamber, A = chamber area in m^2, dc the slope across the four N_2O measurements per chamber in ppm N_2O and dt = slope of measurement intervals in h. Factor k_{N20} (1.25 µg N μL^{-1}) was used to convert N_2O in ppm to µg N-N_2O μL^{-1}.

2.3.8 Statistical analysis

For statistical analysis, the SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used. For intra-field data analysis, a linear model with effects for blocks, N fertilizer rates, +/-DCD application and seasons (2013 or 2014) was fitted for within-trial data analysis using the GLIMMIX procedure. Studentized residuals were inspected graphically for normality and homogeneity. Factors or interactions among factors being not significant for α=0.05 were removed from the model. Means of factors found significant in the type-III F-test (when p-value <0.05) for the respective model were compared by using the LINES option in the LSMEANS statement. Precise p-values are presented to point out to differences in terms of significance among treatments below the cut-off level (p=0.05). In order to account for pre-treatment field differences, mineral N results were partially expressed in relation to soil organic N content. For inter-field data analysis of not replicated pre-culture effects (Bh or M) we abstained from use of an adjusted (fixed effect) model but used simple plot t-tests for selected parameters without

directly inferring a causal relationship. Due to this limitation, we included additional controls (e.g. +/- DCD) in the intra-field trials to assess BNI effects.

2.4 Results

2.4.1 Soil incubations

Laboratory incubation of soils collected before sowing maize in the first season (2013) after *Bh* revealed significant lower evolving NO_3^- values during the incubation period compared to soils from the non BNI field M (Fig 2.4.1). The incubation results demonstrated also an efficient nitrification inhibition by the applied SNI (DCD). Additionally, nitrification inhibition with DCD was not significantly different from the BNI effect. Net mineralization was significantly higher for M compared to M+DCD or Bh. During the incubation M showed minor changes in terms of NH_4^+ mineralization, but decreasing N-NH_4^+ values under BNI (Bh) and SNI (M+DCD) suggested a strong immobilization of applied mineral N in the soil in 2013. In contrast, soil incubations before maize sowing in 2014 showed similarly high nitrification activity in soils from Bh and M (Fig 2.4.1). As already observed in 2013, the application of DCD in 2014 to both Bh and M reduced nitrification activity severely.

In 2013, field NO_3^- values in topsoil at 42 DAS (Table 2.4.1) one day before the third N split fertilizer application and at the end of the vegetative phase (82 DAS) were low in both fields with no major significant residual effect to previous applications of N or DCD. Comparison of the 2 sampling dates within each field indicated higher NO_3^- values at 82 DAS compared to 42 DAS for Bh (p=0.0012) whereas this was not significant for the M field (p=0.1488). In 2014, both soil mineral N forms were measured but only in the two most contrasting N rate treatments. At 32 DAS no significant N fertilization effect on the N form or quantities in the topsoil were observed. However, three weeks after third and last N dressing (66 DAS) Bh topsoil was significant higher in N-NH_4^+ compared to N-NO_3^- (p=0.0027) whereas this could not be observed for M treatment (p=0.1866) (data not shown).

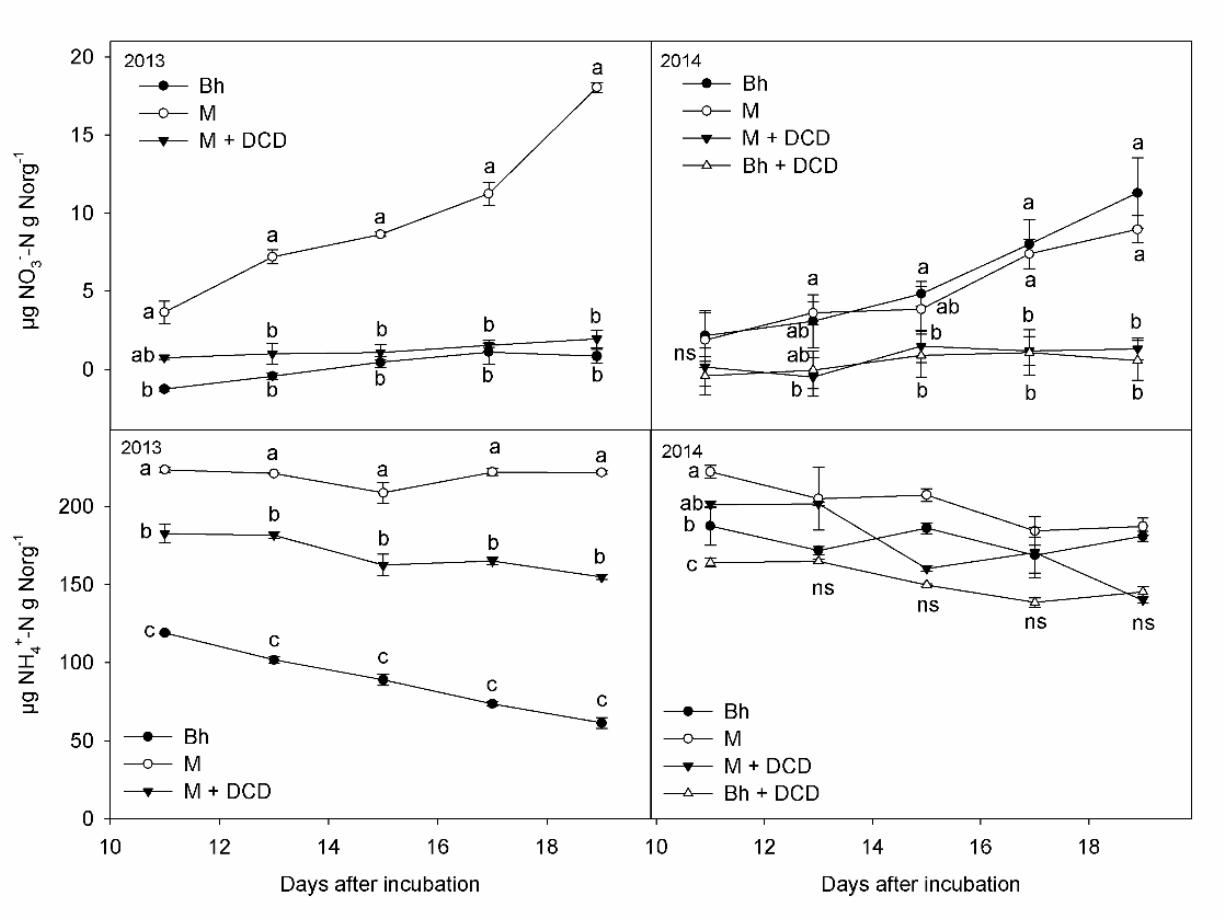

Fig 2.4.1 Mineral N dynamics in the incubated soils. Net nitrification activity expressed as µg N-NO_3^- g N_{org} (upper graphs) and net NH_4^+ mineralization activity in µg N-NH_4^+ g N_{org} (lower graphs) in incubated soils 11 to 19 days after initiation. Soil samples were taken before maize sowing in 2013 and 2014. Field sites differed in their preceding land use (Bh = long-term *Brachiaria humidicola* pasture, M = *Zea mays* mono-crop). DCD was added to M soil as synthetic nitrification inhibition control. Error bars indicate standard error (SE) of the mean. Values with same letters at equal sampling points are not statistically significantly different for least squares means (α = 0.05).

Table 2.4.1 Nitrate (NO_3^-) in topsoil (0-20 cm) at 42 DAS and 82 DAS in 2013 and at 32 DAS and 66 DAS in 2014. Furthermore, nitrous oxide emissions 3 days after second (18 DAS = days after sowing) and 3 days after third (45 DAS) N split application in the first maize cropping season (2013), and after 2 (43 DAS), 4 (45 DAS) and 7 (48 DAS) days after third N split application in the second maize planting season (2014). Field trials (Bh = long-term *Brachiaria humidicola* pasture, M = maize mono crop) differed in their previous land use. Main plots differed in fertilizer N amounts with +/- DCD (synthetic nitrification inhibitor). Values sharing the same letters in the same column within each field are not significantly different ($\alpha = 0.05$). SE = Standard error of the mean among 3 replicated field plots. nd= not determined.

		Nitrate in topsoil (0-20 cm) µg NO_3^--N g $Norg^{-1}$								Nitrous oxide emission µg N_2O-N m^2 h^{-1}									
		2013				2014				2013				2014					
Field site	N fertilizer treatment	42 DAS	SE	82 DAS	SE	32 DAS	SE	66 DAS	SE	18 DAS	SE	45 DAS	SE	43 DAS	SE	45 DAS	SE	48 DAS	SE
	kg N ha^{-1}																		
Bh	0	3.6^{a}	0.6	3.5^{b}	0.1	4.0^{a}	0.6	1.9^{a}	0.2	14.1^{b}	1.8	-2.1^{c}	3.0	-0.6^{a}	1.3	3.0^{a}	5.5	3.3^{a}	2.9
	60	3.2^{a}	0.1	4.7^{b}	0.8	nd	nd	nd	nd	19.2^{b}	2.7	26.7^{ab}	7.1	nd	nd	nd	nd	nd	nd
	60 + DCD	4.4^{a}	0.6	4.0^{b}	0.4	nd	nd	nd	nd	36.6^{ab}	13.2	42.0^{ab}	21.6	nd	nd	nd	nd	nd	nd
	120	3.4^{a}	0.1	9.4^{a}	2.9	nd	nd	nd	nd	21.6^{b}	9.8	31.1^{abc}	22.3	nd	nd	nd	nd	nd	nd
	120 + DCD	3.8^{a}	0.4	6.2^{ab}	1.3	nd	nd	nd	nd	58.8^{a}	38.7	15.5^{abc}	11.4	nd	nd	nd	nd	nd	nd
	240	4.1^{a}	0.7	7.0^{ab}	2.7	3.7^{a}	0.4	3.5^{a}	0.5	32.8^{ab}	5.5	13.3^{abc}	8.4	26.5^{a}	18.9	27.8^{a}	32.8	50.0^{a}	47.0
	240 + DCD	3.8^{a}	0.2	6.0^{ab}	0.5	nd	nd	nd	nd	39.3^{ab}	17.0	44.6^{a}	43.2	6.5^{a}	6.6	11.1^{a}	4.8	3.0^{a}	4.7
M	0	4.7^{a}	0.4	4.3^{a}	0.2	4.9^{a}	1.4	1.6^{a}	0.1	3.0^{a}	2.0	3.3^{a}	3.1	-1.1^{a}	3.8	-0.6^{a}	1.8	-1.0^{b}	1.3
	60	4.2^{a}	0.1	5.5^{a}	1	nd	nd	nd	nd	7.7^{a}	3.9	-1.8^{a}	5.5	nd	nd	nd	nd	nd	nd
	60 + DCD	5.1^{a}	0.8	4.3^{a}	0.2	nd	nd	nd	nd	6.9^{a}	3.5	0.9^{a}	1.2	nd	nd	nd	nd	nd	nd
	120	6.7^{a}	2.1	3.9^{a}	0.1	nd	nd	nd	nd	3.3^{a}	0.5	0.1^{a}	2.2	nd	nd	nd	nd	nd	nd
	120 + DCD	6.2^{a}	1.7	5.8^{a}	1.2	nd	nd	nd	nd	7.7^{a}	3.5	0.9^{a}	3.8	nd	nd	nd	nd	nd	nd
	240	5.6^{a}	1.3	4.1^{a}	0.2	4.7^{a}	0.7	2.1^{a}	0.9	1.6^{a}	1.0	2.7^{a}	2.2	7.1^{a}	7.6	6.2^{a}	4.9	21.8^{a}	4.0
	240 + DCD	4.7^{a}	0.5	4.0^{a}	0.2	nd	nd	nd	nd	8.3^{a}	1.3	1.8^{a}	2.0	6.8^{a}	4.9	6.5^{a}	4.2	-0.5^{b}	3.4

2.4.2 Maize yields in first cropping season, 2013

Maize grain yields from plots established after the previous *Bh* pasture did not differ among 0 N and 60 N (Fig 2.4.2 A). In contrast, a yield increase of more than 10 times (p=0.0012) was observed in the M field between yields of the 0 N treatment with 215 kg maize grain and 2226 kg maize grains at 60 N (Fig 2.4.2 B). Further N application above 60 kg N ha^{-1} did not result in higher yields in both fields, but yields after *Bh* were higher than the control. Plants in DCD sub-plots showed lower grain yields in Bh at 60 N and 120 N compared to DCD free Bh. However, this was not observed for the higher N levels in both fields. Maize yields were always higher for Bh than in the M field for all respective N and DCD treatments.

The agronomic N use efficiency (ANUE) in plots after the previous pasture (Fig 2.4.2 c and d) outperformed those in the continuous maize field with ANUE of 85 kg grain/kg N fertilizer and 37 kg grain/kg N fertilizer, respectively when 60 kg N/ha was applied. ANUE decreased with rising N fertilizer amounts in Bh (p<0.0001) and M (p=0.0023) from 60 N to 120 N and was significantly lower for the DCD treatment at 60 N but not for 120 N and 240 N.

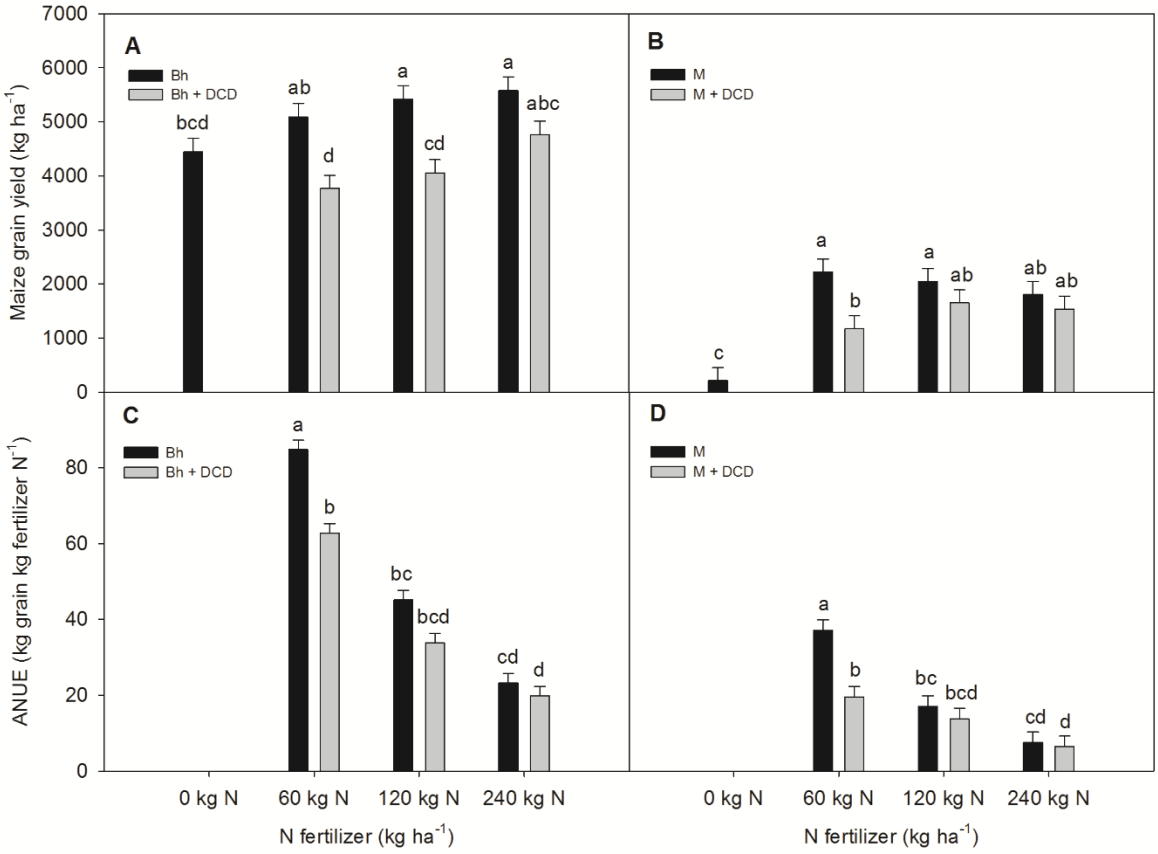

Fig 2.4.2 Maize grain yields (kg ha^{-1}) of the first season (2013) for Bh (a) and M (b). Agronomic nitrogen use efficiency (ANUE) (grain yield in kg ha^{-1} per kg of applied N) of the first cropping season (2013) for Bh (c) and M (d). Fields differed in their preceding land use (Bh = long-term *Brachiaria humidicola* pasture, M = *Zea mays* mono-crop). Each field site included four different N fertilization treatments from 0 – 240 kg N ha^{-1}. Data are mean values calculated from three randomized field plots. Additionally, a synthetic nitrification inhibitor (+ DCD) was used as control. Error bars indicate standard error calculated by the means of the whole sample size within the same field. Values with same letters within each graph are not statistically significantly different for least squares means (α =0.05).

2.4.3 Maize yields in second cropping season, 2014

In the second cropping season, a clear yield response (Fig 2.4.3 a and b) to rising N supply rates was observed in the main plots at both field sites up to a threshold of 120 kg N ha^{-1}, but N fertilization was of less importance to yield formation in Bh (p=0.0082) compared to M control (p<0.0001). Under both pre-crop treatments, DCD resulted in lower yields compared to respective N fertilizer levels. A *t*-test on same N fertilizer levels and DCD treatments suggested only for 0 N significant higher yields for Bh compared to M.

The ANUE (Fig 2.4.3 c and d) showed smaller differences among Bh and M than in 2013. Increasing N rates reduced ANUE stronger in M (p=0.003) than in Bh (p=0.0112). DCD application resulted in lower ANUE in Bh at 60 N and 120 N compared to non DCD plots whereas in M this was confirmed for low N comparison (60 N) only.

When ANUE in the first and second season were compared, higher ANUE was found in Bh in 2013 at 60 N and all + DCD treatments, but not for fertilization at 120 N and 240 N. In the control field M the trend was the opposite compared to Bh: generally higher ANUE in 2014 compared to the first season, except at 60 N and 120 N + DCD.

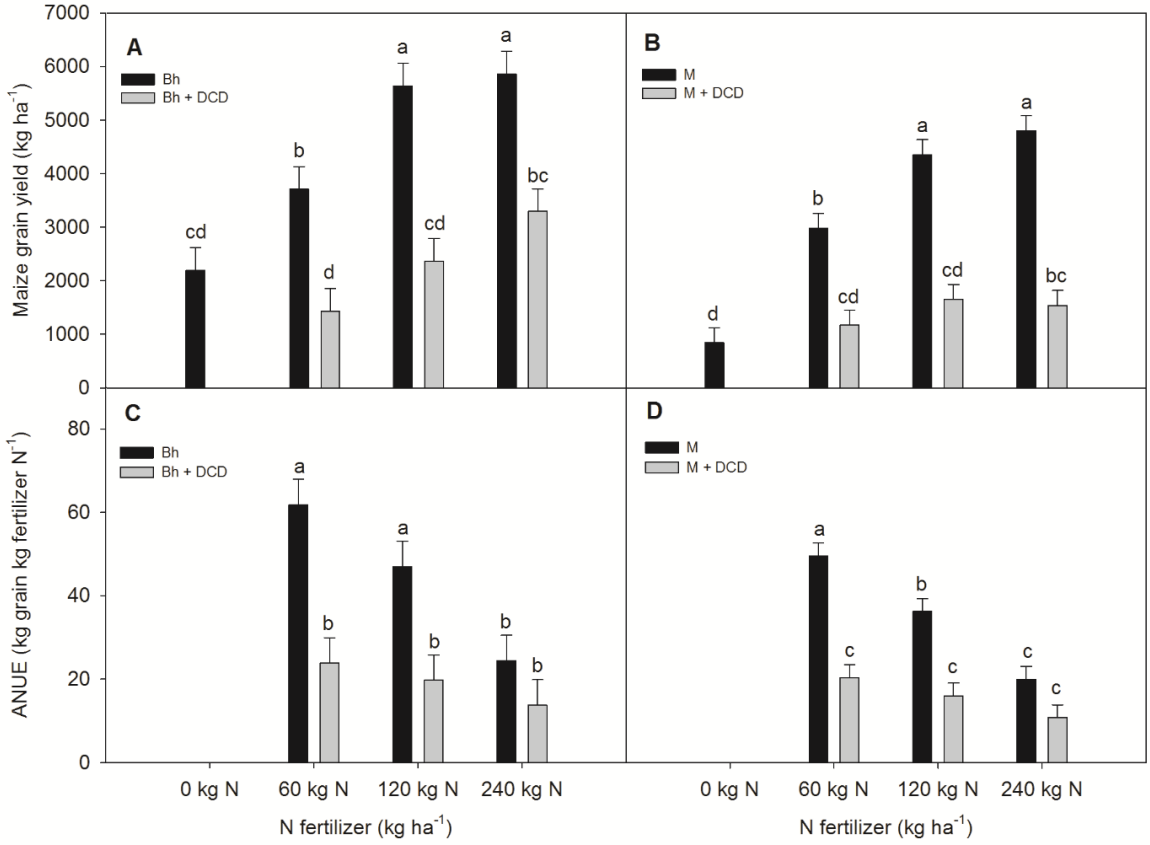

Fig 2.4.3 Maize grain yields (kg ha^{-1}) of the second season (2014) for Bh (a) and M (b). Agronomic nitrogen use efficiency (ANUE) (grain yield in kg ha^{-1} per kg of applied N) of the second cropping season (2014) for Bh (c) and M (d). Fields differed in their preceding land use (Bh = long-term *Brachiaria humidicola* pasture, M = *Zea mays* mono-crop). Each field site included four different N fertilization treatments from 0 – 240 kg N ha^{-1}. Data are mean values calculated from three randomized field plots. Additionally, a synthetic nitrification inhibitor (+ DCD) was used as control. Error bars indicate standard error calculated by the means of the whole sample size within the same field. Values with same letters within each graph are not statistically significantly different for least squares means (α =0.05).

2.4.4 ^{15}N recovery in maize plants and soil profile

In 2013 under low N fertilizer rates (60 kg N) an average of 12.0% of applied ^{15}N was recovered in plant parts in M plots compared to a not significant higher plant N recovery of 18.2% in Bh (Fig 2.4.4.1). Application of 120 kg N ha^{-1} showed as well no difference in plant ^{15}N recovery between M (26.2%) and Bh (23.6%). In M, plant ^{15}N recovery was increased at 120 N compared to 60 N, but DCD had no significant effect on plant N recovery at the same N fertilizer level.

In 2014 plant ^{15}N recovery values at 120 N were above 30% in both fields, however, they were not significantly higher than in the respective treatments in 2013. As in 2013, DCD did not significantly increase plant N recovery either. Overall, soil ^{15}N recovery was not affected significantly by N rate, DCD or the interaction of both (N rate*DCD) within the respective fields and seasons. Total soil ^{15}N recovery in 2014 for Bh ranged between 42.0% (120 N) and 48.6% (120 N + DCD) being statistically similar to M values of 46.9% at 120 N and 37.8% at 120 N + DCD.

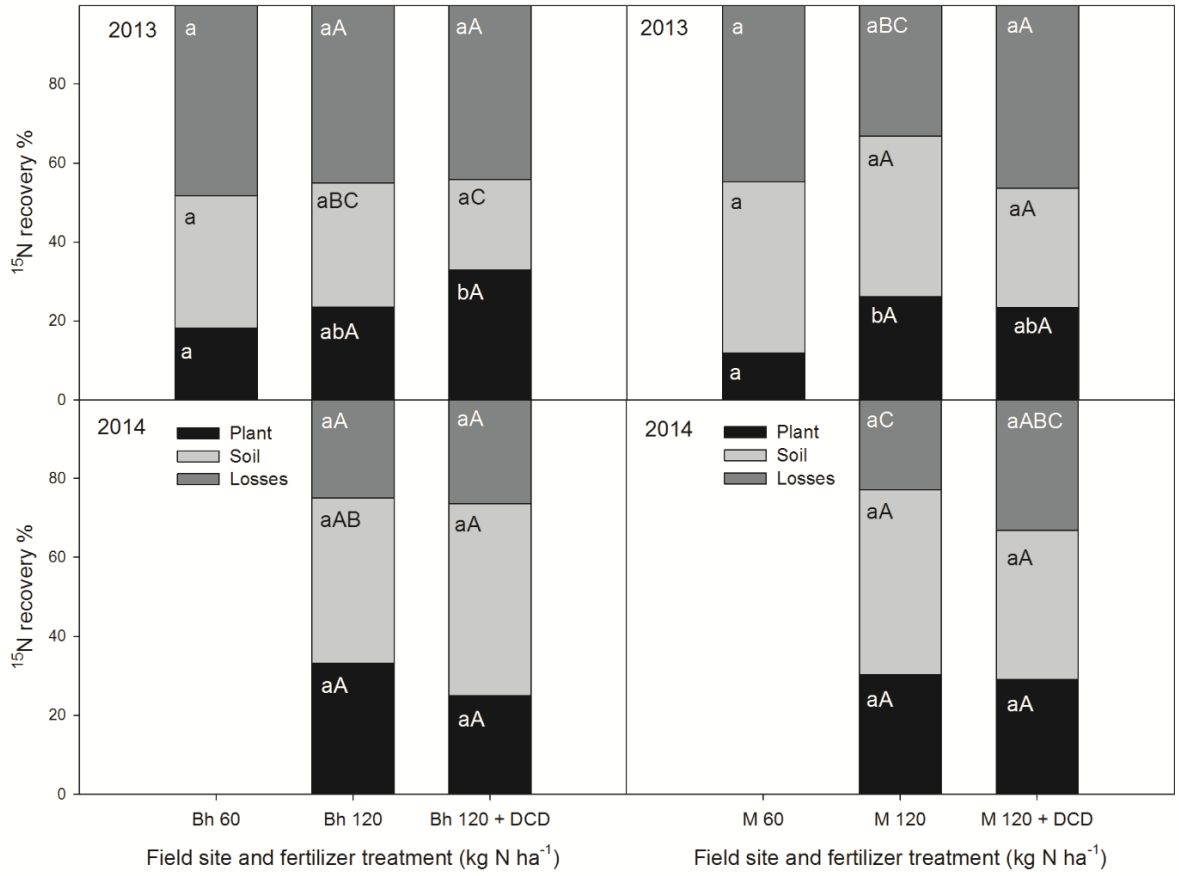

Fig 2.4.4.1 Percentage of recovered ^{15}N labeled N fertilizer in maize plants, soil profile (0-60 cm) and unaccounted N losses in ^{15}N micro-plots during two maize cropping seasons (2013 and 2014). Micro-plots have been installed in two field sites differing in terms of the previous land use (Bh = long-term *Brachiaria humidicola* pasture, M = *Zea mays* mono-crop system). Plant and soil samples were taken at 120 days after sowing (DAS). Values with same lowercase letters within the same site and year, and within similar colored bars (plant/soil/losses) are not statistical significant different ($\alpha = 0.05$). Same uppercase letters indicate no statistically significantly difference within the same site and bars of same color among the two field seasons (2013 and 2014).

In both cropping seasons, most of the detected ^{15}N within the soil profile was still found in the topsoil layer 0-10 cm (Fig 2.4.4.2). No consistent significant treatments effects on depth movement were found. However, apparently DCD application decreased the relative ^{15}N recovery in the subsoil (0-60 cm) in both fields in 2013, indicated by a ^{15}N topsoil:subsoil recovery ratio for Bh (p=0.054) of 2.9 (Bh 120 N) and 5.0 (Bh 120 N + DCD) and 2.1 (M 120 N) and 4.6 (M 120 N + DCD) (p=0.674). However, this pattern was not confirmed in the second season.

Unaccounted N losses (Fig 2.4.4.1) in 2013 for Bh varied between 45.1% (120 N) and 48.3% (120 N + DCD) compared to 44.8% and 46.7% for M in the respective N treatments (Fig 2.4.4.1). In Bh micro-

plots N losses were not affected by the fertilizer rate or DCD application or its interaction (N rate*DCD). Furthermore, the cropping season effect was significant (p=0.0224) for Bh. N loss comparison among the fields on the same N levels showed no significant (t-test) difference in terms of lost N amounts in both seasons.

In both years, Bh showed higher total plant N yields compared to M (Table 2.4.4) being expressed more strongly in the first season after *Bh* pasture conversion. Within Bh plant N uptake was not significantly affected by N rate, DCD, year or its interaction. Maize cultivated on the previous pasture field (Bh) took up higher amounts of N from the soil in 2013 than from applied fertilizer N (p<0.0001), however not in 2014 (p=0.6298). The opposite was observed for maize in the control field (M) in 2013, i.e. N uptake from the fertilizer was higher (p=0.0093) compared to soil N uptake when N rate was 120 N (+/- DCD) in the first season, whereas in 2014 it was vice versa.

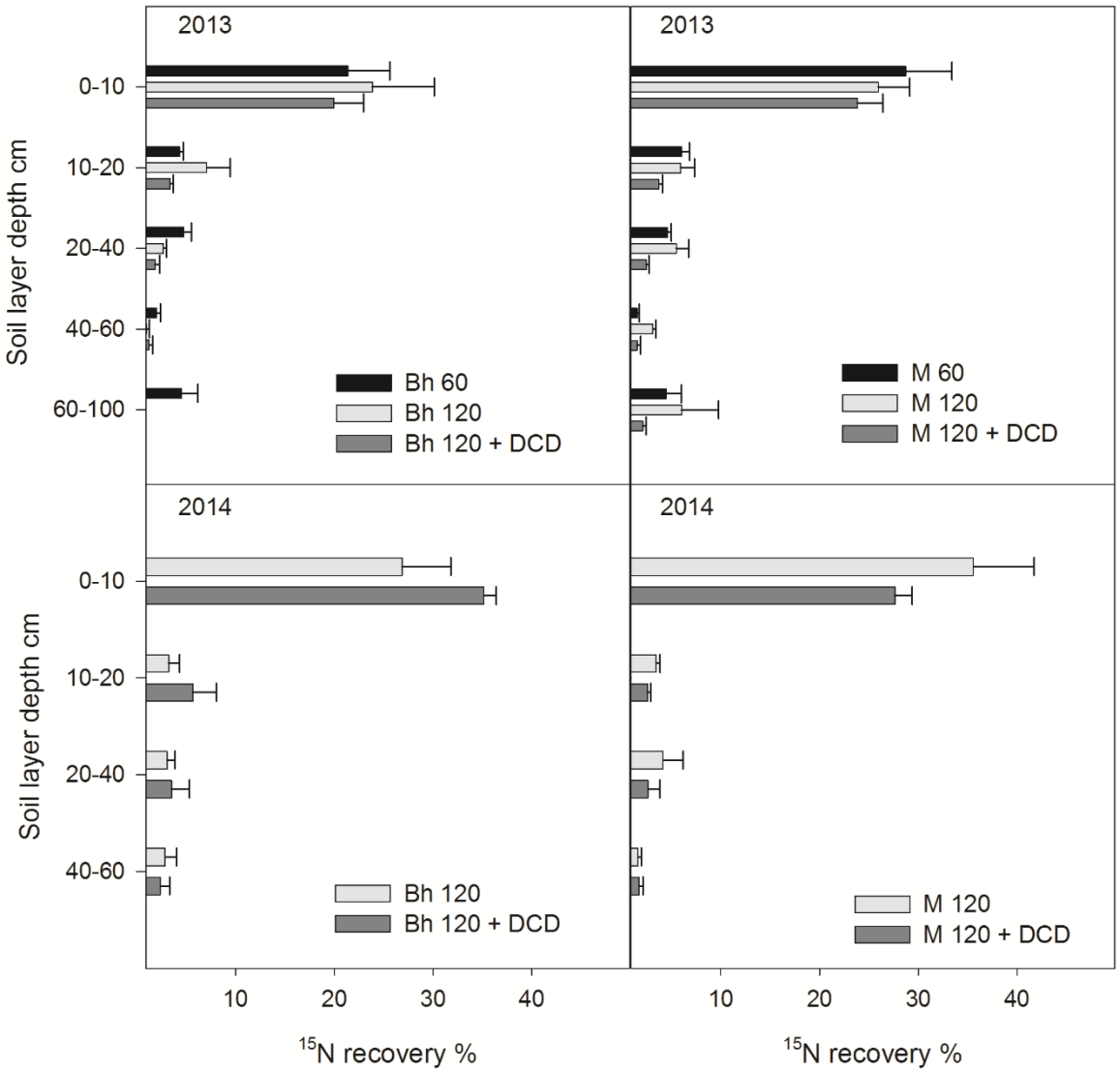

Fig 2.4.4.2 Percentage of recovered ^{15}N labeled N fertilizer in 5 different soil layers of the soil profile in the first (2013) and second (2014) season at 120 days after sowing (DAS), respectively. Due to stones samples from 60-100 cm could not always be taken. Soil samples were collected from ^{15}N micro-plots installed in main plots of different fertilizer N rates: 60 kg N ha^{-1}, 120 kg N ha^{-1} and 120 kg N ha^{-1} + DCD. Micro-plots have been installed in two field sites differing in terms of the previous land use (Bh = long-term *Brachiaria humidicola* pasture, M = *Zea mays* mono-crop system). Error bars indicate standard error of means of three samples taken from three randomized micro-plots.

Table 2.4.4 Summary of maize plant N recovery (%), plant N yield kg N ha^{-1}, plant N derived from applied N fertilizer (kg N ha^{-1}) and from soil in ^{15}N micro-plots for the two field sites M and Bh (M = maize mono crop, Bh = long-term *Brachiaria humidicola* pasture) for both maize cropping seasons (2013 and 2014). Values with same lowercase letters are not statistical significant different for least squares means separately for each year and field ($\alpha = 0.05$). Values with same uppercase letters are not statistical different for least squares means separately for each field ($\alpha = 0.05$). SE = standard error of least squares means for N rate effect. SE of the year and year*N rate effect are not shown in the table.

		kg N ha^{-1}	%	SE	kg N ha^{-1}	SE	kg N ha^{-1}	SE	kg N ha^{-1}	SE
Bh	2013	60 N	18.2^{bA}	2.7	80.2^{aA}	11.0	10.9^{cA}	2.7	69.2^{aA}	7.9
		120 N	23.6^{abA}	2.7	88.8^{aA}	11.0	28.3^{bA}	2.7	60.5^{aA}	7.9
		120 N + DCD	32.9^{aA}	2.7	95.2^{aA}	11.0	39.5^{aA}	2.7	55.8^{bA}	7.9
	2014	120 N	33.1^{aA}	6.6	89.1^{aA}	18.2	34.1^{aA}	10.6	55.0^{aA}	8.2
		120 N + DCD	24.9^{aA}	6.6	86.2^{aA}	18.2	48.6^{aA}	10.6	37.6^{aA}	8.2
M	2013	60 N	12.0^{bB}	3.5	23.0^{aC}	6.9	7.18^{bB}	4.1	15.8^{aB}	3.9
		120 N	26.2^{aA}	3.5	45.2^{aB}	6.9	31.4^{aA}	4.1	13.9^{aB}	3.9
		120 N + DCD	23.0^{abA}	3.5	35.5^{aBC}	6.9	28.0^{bA}	4.1	7.5^{aB}	3.9
	2014	120 N	30.3^{aA}	1.8	68.9^{aA}	4.2	22.0^{aA}	4.5	47.6^{aA}	4.2
		120 N + DCD	29.1^{aA}	1.8	65.0^{aA}	4.1	26.1^{aA}	4.3	38.8^{aA}	4.0

2.4.5 Nitrous oxide emission

Nitrous oxide emissions (N_2O) in the first maize season (2013) showed no significant N rate, DCD or N rate*DCD effect for both fields but large differences among the three field plot replications (Table 2.4.1). Inter-field analysis via *t*-test indicated significantly lower N_2O emissions in M than in Bh for some N rates or +/-DCD treatments, but a clear trend was absent. During the second cropping season (2014) after pasture conversion N_2O flux values were not significantly different than in 2013, due to the observed large variability. N fertilizer application increased N_2O emissions particularly at the third sampling date in 2014 (7 days after N fertilization, 48 DAS) for M (p=0.0432). However, at the same time N_2O emissions were decreased when DCD was applied together with 240 N being significant for M. The pattern looked similar for Bh although effects on emissions were not significant. The control (0 N) showed low N_2O emissions in both fields at all 3 sampling dates indicating that the observed increase in N_2O emissions under 240 N was due to N fertilization. The comparison of the trials Bh and M via *t*-test indicated no significant differences in N_2O emissions among the fields in 2014.

2.5 Discussion

2.5.1 Residual effect of *Bh* on maize crop performance

The hypothesis that the previous *Bh* CIAT 679 pasture has a positive effect on maize growth could be confirmed. Even when no N fertilizer was applied grain yields kept up with the commonly found range of 3.0 - 4.5 t ha^{-1} for the Colombian Eastern plains (Thomas et al. 2004). This is in line with observations by Moreta et al. (2014), who showed higher maize grain yields on a previous *Bh* pasture field compared to those on a previous maize-soybean rotation or on a converted native savanna field. Long-term pasture use (Bh) was likely to have enhanced the soil organic matter content compared to the control (M) field. Furthermore, the shift from pasture to crop could have had positive effects in terms of reduced pests and diseases as known from field research in the Brazilian *Cerrados* (Lopes et al. 2004). As shown by Fisher et al. (1994) and Amézquita et al. (2004), improved pastures of *Bh* can have positive effects on soil physical, chemical and biological properties. An estimated amount of root N of 18 kg ha^{-1} in *Brachiaria* pastures has been reported (Rao, 1998), and is likely to even increase under well managed long-term pasture use as found by De Oliveira et al. (2004). Decomposition studies with litter bags have shown

that even 140 days after incorporation 40% of nutrients can remain in the soil in the form of *Bh* litter (Thomas & Asakawa 1993), whereas the half-life (in terms of N release) for maize residues has been documented between only 43 – 35 days (Thomas et al. 2004). Therefore, it is suggested that mineralized N from enhanced organic matter contributed to the observed improved crop nutrition of the subsequent maize crop in the first season of the present study. Furthermore, the importance of additional fertilizer N on maize yield formation was higher for M than for Bh, which relied to a larger extent on N derived from soil. ANUE results further supported the beneficial effect of the long-term *Bh* pasture. However, Basamba et al. (2006) reported lower yields under no- and minimum tilled Colombian savanna Oxisols in the following maize cropping seasons compared to a maize monoculture, suggesting that converting (no BNI) native grassland into arable land does not necessarily result in a positive yield increase of the subsequent crop. Furthermore, an experiment in 1989 showed that upland rice yields were significantly higher after a 10-year-old *Brachiaria decumbens* (*Bd*) pasture compared to rice after the conversion of a savanna, even when no N was applied (Sanz et al. 2004). This is analog to the observation of the present study. However, BNI as additional benefit from *Bd* has not been mentioned in the article, although BNI activity has not only been detected in *Bh* but also in *Bd* (Subbarao et al. 2007a). Thus, the focus on the combined effect of organic matter dynamics as well as a BNI effect in our field trial on crop performance after *Bh* pasture use is therefore new and has probably not been studied since the residual BNI of *Brachiaria* grasses was not yet known or simply ignored.

2.5.2 Evidence of the residual BNI effect

We hypothesized that the BNI effect induced by *Brachiaria humidicola* will be present in the soil even when the pasture has been removed and that this has a positive effect on the succeeding maize crop. Residual BNI activity was evident in reduced nitrification in soil from the previous *Bh* pasture in 2013 in the incubation study. This is in line with similar early observations by Sylvester-Bradley et al. (1988), Ishikawa et al. (2003) and Subbarao et al. (2006a & 2007a) who also found lower nitrification in terms of low NO_3^- values over time and stable NH_4^+ values under existing *Bh* pastures. In terms of soil incubation effects, however, two processes might operate simultaneously, i.e. a net substance effect (exudates or extracts from plant tissues applied to soil) as well as a residual fine root effect influencing

N dynamics through N mineralization and immobilization (Purchase et al. 1974). According to Robertson and Groffman (2015), microbial nitrifiers are rather weak competitors for available NH_4^+, and nitrification accelerates when NH_4^+ supply exceeds the demand of the plants. Organic matter with a high C:N ratio causes microbial immobilization (Urquiaga et al. 1998; Sakala et al. 2000), and Thomas and Asakawa (1993) documented C:N ratios of *Bh* litter within a range of 89 – 160. However, immobilization and consequently substrate shortage was also observed in the control soil (M) since maize roots and maize stover as well have high C:N ratios (Paré et al. 2000), although net nitrification prevailed. Application of DCD to M soil resulted in low nitrification in both incubation studies, and on the other hand confirmed the presence of active BNI effects after *Bh*. However, the effect might be partly reduced by the accompanied decreasing NH_4^+, which was probably the consequence of immobilization favored by DCD (Juma & Paul, 1983; Clay et al. 1990), since soil microorganisms seem to have a preference for NH_4^+ rather than for NO_3^- (Recous et al. 1992). Under field conditions, the observed varying NO_3^- levels in topsoil indicated that nitrification was not consistently or completely inhibited by the residual BNI effect, not even throughout the first cropping season.

Subbarao et al. stated in their review (2006b) that the suppression of nitrification is a potential key strategy to improve N recovery and ANUE. The present study confirmed that in the first season the residual BNI effect contributed to enhanced N yields and improved ANUE. Uptake of soil derived N by the maize crop was higher for Bh compared to M, a potential consequence of enhanced retention of mineralized N and consequently N nutrition of maize. However, the residual BNI effect was not reflected in higher fertilizer ^{15}N uptake nor were fertilizer ^{15}N losses reduced compared to the control site. Our data suggested that applied ^{15}N was very rapidly immobilized in the topsoil and that maize in the Bh field compensated the low N availability by taking up mineralized N from sub-soil layers, which explains the enhanced N nutrition of maize in the Bh site.

According to our study, the lack of the residual BNI effect on N_2O emissions suggests that residual BNI does not necessarily have a similar effect as the *in situ* pasture BNI effect described by Subbarao et al. (2009) where N_2O emissions were found to be reduced. It has been shown by Fisher et al. (1994) and Amézquita et al. (2004) that accumulation of *Bh* root biomass can vastly enhance soil C. Others reported

that denitrification is also spurred by available C substrates (Bergstrom et al. 1994). For the Bh site enhanced C availability is expected as indicated by the higher organic matter contents favoring denitrifiers (Firestone & Davidson, 1989), that might have masked a sound N_2O reduction due to residual BNI in the subsequent maize crop. Due to the high rainfall (i.e. 3,685 mm/year) in the area temporal anaerobic conditions that favor denitrification are expected during and shortly after heavy rainfall despite reasonably good drainage of these Oxisols.

2.5.3 Persistence of BNI

Generally, turnover of plant residues in humid tropical systems is fast (Urquiaga et al. 1998). Therefore, it was hypothesized that the BNI effect from previously released root exudates or contained within incorporated *Bh* material would diminish over time. Subbarao et al. (2007a) could show that in soil from 10-year-old *Bh* field plots, including the CIAT 679 accession, inhibited nitrification activity lasted up to 30 days after incubation initiation. After 15 years of pasture use with *Bh* CIAT 679 we expected a substantial amount of BNI substances in the soil as shown by Subbarao et al. (2008), however a technique to measure these active compounds in the soil has still not been developed. The present study confirmed that nitrification inhibition in soil could be observed until about 6 weeks after removal of *Bh*. However, a BNI effect before the second cropping season 2014 could not be confirmed in the incubation assay any more. Our results thus suggest that a substantial residual BNI effect on soil processes lasted for less than one year. Consequently, it could be speculated that BNI inhibitor compounds are exposed to decomposition limiting their persistence in soils. Lower BNI persistence over time was expected since *Bh* root exudates contain hydrophobic and hydrophilic substances (Ipinmoroti et al. 2008) where the latter are prone to leaching, and furthermore, that several released substances from incorporated *Bh* litter may attract heterotrophic microbial populations that metabolize C (Lynch & Whipps 1990).

2.5.4 Effect of DCD on maize crop performance

The synthetic control treatment (DCD) showed no efficient nitrification inhibition in the field in contrast to its efficiency under controlled conditions. Furthermore, no significant beneficial effects of DCD on plant N recovery, soil N recovery nor a reduction of ^{15}N losses in the micro-plots in the previous *Bh* pasture could be found in both years, while for M even significant increased ^{15}N losses in both years

were observed. Regardless of its ability to prevent N losses from soil DCD in the presented study did not favor crop growth and performance. Losses of the mobile DCD from system with high rainfall have been observed by Amberger (1989). Weiske et al. (2001) could also not confirm a positive DCD effect on maize, but observed lower emission of N_2O since DCD has been shown to retard nitrification (Prasad et al. 1971) and consequently reduce NO_3^- as substrate for denitrifiers. Earlier, Clay et al. (1990) reported reduced maize grain yields combined with lower total N uptake by the crop. According to the reduced NH_4^+ mineralization results in the presented incubation study it is suggested that DCD favors N immobilization. Such an effect has been observed also by others (Guiraud et al. 1989; Clay et al. 1990; Ernfors et al. 2014) and could have had a negative impact on N nutrition of the maize plants. Its stability and effectiveness is also known to be negatively affected by high soil temperature (Ruser & Schulz 2015). We thus cannot confirm high effectiveness of DCD in the field, like others, e.g. Merino et al. (2001) who found no N_2O reduction in a DCD treated and mineral fertilized grassland soil in Northern Spain.

2.6 Conclusions

This is the first in depth study on the potential residual BNI effect of *Bh* on a following maize cropping system. The residual effect of the long-term *Bh* pasture increased maize grain yields in the first season under all evaluated N fertilizer rates and in the second season after pasture conversion for the treatment without N fertilizer compared to arable land. This was associated with a higher total N uptake under the previous *Bh* pasture compared to M as well as significant reduced nitrification in incubated soils under Bh confirming a residual BNI effect. The study further suggested that applied N was partially immobilized due to large amounts of incorporated *Bh* roots during conversion with a high C:N ratio; and N was subsequently slowly re-mineralized and consequently contributed to maize N nutrition. Hence, maize performance in Bh depended less on fertilizer N compared to the control field M. The contribution of applied N to yield formation increased in the second season due to a diminished influence of the *Bh* residues. Mineral N in topsoil was not significantly influenced by the amount of N fertilizer a few days after final N dressing in 2013 that supports the strong immobilization observed in vitro. At a later stage increased amounts of available N-NO_3^- were visible being a good indication for the superior

plant growth and grain production in the BNI field however also a possible reduced residual BNI effect in the topsoil at 120 days after *Bh* incorporation.

It was challenging to identify the net residual BNI effect of the previous *Bh* pasture due to interactions with decomposing *Bh* plant material. Thus, parallel processes interact where previously released BNI substances from *Bh* inhibit or retard nitrification while decomposition is accompanied by a mineralization and green manure effect, that provides NH_4^+ as substrate for nitrifiers and the immobilization effect due to a high C:N ratio that can indirectly decrease nitrification. Furthermore, the link between BNI and N_2O emission seems not to be clear yet and more frequent sampling might capture the peak of N_2O emission and identify a possible positive effect due to residual BNI. As control to BNI in the field DCD turned out to lose its nitrification inhibition effect too fast under these humid tropical conditions and therefore alternative substances (DMPP) should be considered for further testing.

Generally N losses from extensively managed pasture systems are low even when N is supplied to fight degradation of the grassland over time. BNI could therefore play a more important role in terms of its residual effect for subsequent crops under high N fertilization compared to possible N loss reduction under present *Bh* pastures. Therefore, it is suggested to investigate further mechanism and persistence of a residual BNI effect and its influence on soil N dynamics in field studies.

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Table 2.8 Bulk soil density (g cm^{-3}) and texture analysis from intact soil cores taken from the field sites Bh (long-term *Brachiaria humidicola* pasture) and M (long-term mono-crop system of *Zea mays*). Soil cores were taken after liming and plowing in 2013. n.d = not determined

	Bh				M			
		Texture				Texture		
Soil depth	Bulk soil density	Sand	Silt	Clay	Bulk soil density	Sand	Silt	Clay
cm	g cm^{-3}		%		g cm^{-3}		%	
0-5	1.33	42	26	32	1.40	36	26	38
5-10	1.42	39	21	41	1.39	40	19	41
10-20	1.27	32	22	46	1.37	37	17	46
20-30	1.45	27	23	50	1.47	37	16	47
30-40	1.50	27	21	52	1.57	36	17	47
40-60	1.48	27	22	51	1.61	35	17	48
60-80	1.55	30	21	50	1.63	35	14	51
80-100	n.d.[1]	n.d.	n.d.	n.d.	1.50	35	14	51

3. Nitrate reductase activity in leaves as a proxy for *in vivo* performance of biological nitrification inhibition by *Brachiaria humidicola*

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3.1 Abstract

Brachiaria humidicola (*Bh*) controls soil microbial nitrification via biological nitrification inhibition (BNI). The aim of our study was to verify if nitrate reductase activity (NRA) in *Bh* roots or leaves are a proxy of *in vivo* performance of BNI in soils.

NRA was measured using roots and leaves of contrasting accessions and apomictic hybrids of *Bh* grown under controlled greenhouse and natural field conditions. Nitrate (NO_3^-) contents were measured in soil solution and in *Bh* stem sap to validate NRA data.

NRA was detected in *Bh* leaves rather than roots, regardless of NO_3^- availability. NRA correlated with NO_3^- contents in soils and stem sap of contrasting *Bh* genotypes substantiating its use as a proxy of *in vivo* performance of BNI. The leaf NRA assay facilitated a rapid screening of contrasting *Bh* genotypes for their differences in *in vivo* performance of BNI under field and greenhouse conditions, but inconsistency of the BNI potential by *Bh* germplasm were observed.

In conclusion, it is suggested that the NRA in leaves of *Bh* serves as a proxy to assess potential *in vivo* BNI activity under greenhouse and field conditions.

Key words: biological nitrification inhibition (BNI), nitrate reductase activity (NRA), nitrate assimilation, plant nitrate status, enzymatic assay, tropical forages, 3,4-dimethylpyrazole phosphate (DMPP), *Brachiaria humidicola*

3.2 Introduction

Plants control soil nitrification via root exudation, a process termed biological nitrification inhibition (BNI) (Coskun et al. 2017b). BNI is induced by a wide range of forage species including *Brachiaria humidicola* (*Bh*) (Sylvester-Bradley et al. 1988; Ishikawa et al. 2003; Subbarao et al. 2007a). So far, detection of BNI in *Bh* relied solely on the application of a bioluminescence assay using a recombinant *Nitrosomonas europaea* (NE) strain (Subbarao et al. 2006; Subbarao et al. 2007a). A modified assay using a non-modified NE strain along with a *Nitrosospira multiformis* (NM) strain has been published recently (O'Sullivan et al. 2016, 2017). Both methods rely on hydroponics in which plants have to be cultivated for root exudate collection. It could be assumed that this procedure may lead to an overestimation of the BNI potential and may also be obscured by either active or passive (e.g., root damage) release of BNI substances. Hence, determined nitrification inhibition by NE and NM *in vivo* might not reflect the actual BNI performance in the intact plant-soil system. There is thus an urgent need to establish alternative proxies for both pot and field studies to monitor the *in vivo* performance of BNI and to link this with identified BNI potentials from experiments under laboratory conditions without disturbance of the soil-plant system.

If ammonium (NH_4^+) availability in soils is high and BNI is low, microbial nitrification produces substantial amounts to nitrate (NO_3^-). If not leached or denitrified, NO_3^- is taken up by *Bh* followed by two reduction steps to NH_4^+ for further assimilation, while excess NO_3^- is stored in vacuoles (Tegeder & Masclaux-Daubresse 2017). The responsible enzyme catalyzing NAD(P)H reduction of NO_3^- to nitrite (NO_2^-) is nitrate reductase (Evans & Nason 1953). Nitrate reductase activity (NRA) can be measured *in vivo* in intact plant tissue based on this first reduction step prior to NO_3^- assimilation (Jaworski 1971). The relationship between soil nitrification and leaf NRA has been earlier demonstrated for the savanna grass *Hyparrhenia diplandra* (Lata et al. 1999), but the actual link between leaf NRA in *Bh* and its BNI under environmental conditions is yet to be verified. Cazetta & Villela (2004) reported that NRA, measured *in vivo* in *Brachiaria radicans*, was higher in leaves than in stems. Macedo et al. (2013) found higher NRA rates in leaves compared to roots of *Brachiaria brizantha*. However, it is not known so far if *Bh* reduces NO_3^- in leaves and roots so that NRA could be used as a proxy for *in vivo* performance of BNI, specifically for *Bh* genotypes with acknowledged contrasting BNI potential (Subbarao et al. 2009).

In this study, it was our primary goal to verify the potential of NRA as a proxy for the detection of *in vivo* performance of BNI by selected *Bh* accessions and genotypes grown under contrasting fertilization regimes. Accordingly, we tested the following hypotheses: (1) NRA in roots and leaves of *Bh* is controlled by different N forms and concentrations (Experiment 1). (2) There is a strong correlation between leaf NRA, soil NO_3^- and plant NO_3^- (Experiment 2). (3) NRA of contrasting *Bh* genotypes under field conditions can be linked to their BNI potential, whereby low NO_3^- availability due to strong *in vivo* BNI performance by *Bh* is reflected in low NRA in plant tissues of *Bh*.

3.3 Material and Methods

3.3.1 Nitrate reductase activity measured in intact plant tissues of *Bh*

In vivo nitrate reductase activity (NRA) was measured according to Jaworski (1971). Segments of freshly cut roots, stems or recently expanded leaves (~1 cm^2) were used as incubation tissue. The enzyme nitrate reductase (NR) and the reduction equivalent nicotinamide adenine dinucleotide phosphate (NADP) were provided internally by the fresh plant material. The *in vivo* assay solution comprised 100 mM phosphate buffer with a pH of 7.5, 30 mM KNO_3 and 5% propanol, as well as simulated cytosolic plant cell conditions to ensure adequate enzyme activation. Potassium nitrate (KNO_3) ensured unlimited substrate availability for NR. Propanol was added to strengthen the reduction of NO_3^- to NO_2^- and to hinder further reduction of NO_2^- to NH_4^+. Anaerobic conditions during the incubation avoided the interference of molecular oxygen (O_2) and NO_3^- as oxidation substrate for NR. All operations were conducted under dim light to reduce photosynthetic activity. A polystyrene isolating box filled with ice pads was used to diminish the metabolic activity and enzyme degradation in plant tissue. Afterwards, 600 mg plant material was homogenized and split into 2 equal sub-samples (T_0 and T_{30}). Sub-samples were transferred into 50 ml falcon tubes and 10 ml of the *in vivo* solution was added. The T_0 (control) tubes were placed in a water bath at 100°C for 5 minutes and stirred at 80 rpm to degrade and inactivate NR. Thereafter, T_0 and T_{30} tubes were incubated at 35°C in a water bath for 30 minutes at 80 rpm. T_0 tubes were treated equally since a complete NR inactivation cannot be assured despite boiling since small amounts of NO_2^- were still present. Therefore, evolved NO_2^- in the T_0 samples was set as point

zero. Moreover, T_{30} tubes were put into a boiling water bath to minimize further reduction of NO_3^- through NR after the 30 min incubation period. Afterwards, all tubes were cooled to room temperature and 10 ml of color reagent were added to determine NO_2^- via a staining procedure consisting of 1% sulfanilamide in HCl and 0.02% Griess reagent (*N*-(1-naphthyl)-ethylenediamine hydrochloride). A NO_2^- stock solution with potassium nitrite (KNO_2) of 25 µM was prepared to calibrate the color reaction. The absorbance was determined at 540 nm with a stationary multi-mode microplate reader SIAFR model (BioTek Instruments, Vermont, USA). For measurements under field conditions, a portable DR 1900 spectrophotometer (Hach Company, Loveland, USA) was used.

3.3.2 Experiment 1: NRA initiation in roots and leaves of *Bh* under different N forms

At the University of Hohenheim (UHOH), young *Bh* CIAT 679 cv. Tully stolons were transferred into 2 L plastic pots filled with a sand-perlite (70:30) substrate. There, twenty pots containing one stolon per pot were installed in a greenhouse under light bulbs with a photosynthetically active radiation (PAR) of 800 µmol m^{-2} during a photoperiod of 12 h day^{-1}. Plants were irrigated daily with 100 ml of a nutrient solution according to Yoshida *et al.* (1976) for 30 days. N was provided by either as NH_4^+ (BNI & nitrification stimulation) or NH_4^++DMPP (3,4-dimethylpyrazole phosphate, synthetic nitrification inhibition as control) or NO_3^- (to detect maximum NRA as control). The latter N treatment contained 3 different N-NO_3^- concentrations (low [0.1 mM], mid [1 mM] and high [10 mM]) to detect NRA sensitivity to substrate availability. Each treatment was replicated 4 times and pots were arranged as complete randomized block design. After this pre-establishment phase, the grass was cut back to 10 cm and irrigated with 500 ml tap water to leach remaining N of the substrate. Ten days later, N depletion was apparent by N deficiency symptoms (i.e., light green leaves of plants). Then, irrigation was repeated using the respective nutrient solutions to induce a *de novo* synthesis of the NR. The NRA baseline sample was collected one day before N fertilization. Sampling of leaves was conducted 12 hours after plants were re-supplied with the respective N form and amount. Final harvest leaf and root tissue was performed 72 hours after N supply and NRA determination was conducted as described above.

3.3.3 Experiment 2: Relationship among NRA activity and soil nitrification under different N forms in a *Bh* hybrid population under controlled conditions

A two factorial (genotype × N fertilizer form) experiment under the same conditions as *Experiment 1* (in terms of light & photoperiod) was performed with 4 replications arranged in two blocks (α design) to perform a genotypic evaluation on BNI potential using the novel NRA assay. The experimental pots were manufactured from common PVC-drainpipes (Ø 11cm × 100 cm) to enable deep rooting and monitoring of NO_3^- dislocation within the soil profile. A ferralitic substrate was used, resembling similar soil characteristics of a tropical Oxisol. The soil derived from a site named "Eiserne Hose" (50°31'2.0' latitude and 8°50'55.9' longitude, Lich, Germany) and was characterized as a fossil tertiary clay loam (laterite) with a pH of 5.7, 0.25% carbon (C) and 0.029% N. This substrate was amended with sand (25 vol%) to improve drainage properties. PVC drainpipes were equipped with rhizons (Eijkelkamp Agrisearch Equipment, Rhizon Soil Moisture Sampler, Ø 2.3 × 50 mm, hydrophilic polymer, porosity 0.1 µm) installed horizontally at 7.5 cm and 50 cm depth within the soil column. This enabled non-destructive sampling of soil solution (e.g., monitoring of real-time NO_3^- levels as nitrification indicator in the topsoil) by applying a suction pressure through a common medical syringe. The experiment included 5 *Bh* apomictic hybrids (i.e., Bh08-population) with unknown BNI capacities provided by CIAT Colombia (Rao et al. 2014). Two CIAT standard accessions (CIAT 679 cv. Tully, CIAT 16888), which were reported with mid-high and high BNI activity, respectively (Subbarao et al. 2006, 2009), were included as controls. *Bh* stolons were planted in August 2014 and frequently cut and fertilized with macro- and micro-nutrients. N was applied according to the three N form treatments of *Experiment 1*. The sampling period started in December 2015 when plants had been cultivated for 16 months. Prior to sampling, the grass was fertilized with Yoshida solution containing NPK (in kg ha^{-1}) analog to 50 N, 50 P and 20 K. Fertilizer N was applied according to the three N treatments to trigger NRA differently, as described in *Experiment 1*. After 2 weeks, plants were cut back to 10 cm above soil surface and fertilized with 150 ml of nutrient solution containing 50 kg N ha^{-1} of the respective N treatment to re-induce synthesis of NR. To determine the dynamics of NRA among genotypes, detailed sampling of newly developed and fully expanded leaves was conducted for 2 contrasting (selected based on soil NO_3^- monitoring, see below) genotypes (CIAT 679 versus Bh08-675) before, 2 and 5 days after N fertilization

(DAF) for all 3 N treatments. As baseline, leaves were collected before N supply to all pots. At final harvest (9 DAF), leaves were obtained from all 7 *Bh* genotypes to determine the intraspecific leaf NRA and soil nitrification activity (described below) linkage. NRA was measured as described above.

Simultaneously to each NRA sampling, soil solution samples through the installed rhizons were taken from the topsoil to measure real-time soil NO_3^- levels as an indicator for soil nitrification. Soil solution sampling was conducted 3 hours after irrigation with 100 ml of tap water to ensure sufficient soil moisture and time for equilibrium establishment regarding NO_3^- concentration in the soil solution. Ten ml of soil solution was collected by syringes, filtered through Whatman No 2 and frozen immediately until NO_3^- was quantified photometrically (AutoAnalyzer 3 / QuAAtro AQ2, SEAL Analytical, Southampton, UK). A first soil NO_3^- measurement was conducted before N fertilization to ensure that further measured NO_3^- at 2, 5 and 9 DAF were mainly due the effect of the applied N fertilizer.

3.3.4 Experiment 3: Leaf NRA as BNI indicator under field conditions

The field site was situated at La Libertad Research Station of Corpoica (Corporación Colombiana de Investigación Agropecuaria) in the Piedmont region of Colombia at an altitude of 336 meters above sea level with a mean annual temperature of 26°C and annual rainfall of 2,933 mm. The soil was classified as an Oxisol (USDA soil taxonomy) with a pH of 5.5. The trial was established by CIAT Colombia in August 2013 and arranged as randomized complete block design. Intraspecific *Bh* hybrids were planted with each 3 replicates to evaluate their BNI activity compared to CIAT *Bh* accessions. Each plot had a size of 4×4 m (16 m^2). Before planting in 2012, the plots received a basal fertilization (in kg ha^{-1}): 100 N, 40 P, 75 K, 110 Ca, 65 Mg, 19 S and 35 Borozinco®.

At the end of the rainy season in October 2015, all *Bh* genotypes selected for this study were depleted in N fertilization for 27 months. All test plots were then separated into N fertilized and N unfertilized (control) split-plots. Subplots of 1 m^2 were installed randomly with strings within each split-plot. The N dosage for the N fertilized split-plot was 100 kg N ha^{-1} (as di-ammonium-phosphate (DAP) and urea). Additionally, each plot including the N free plots received fertilization (in kg ha^{-1}) of 25 P, 50 K, 50 Ca, 15 Mg, 11 S, 0.5 B, 0.0875 Cu, 1.5 Si and 2.5 Zn in solid form. For NRA determination, the hybrids CIAT 16888 (high BNI control) and CIAT 679 (mid-high BNI control), CIAT 26146 (mid-low BNI

control according to Rao et al. 2014 and Arango et al. 2014) were selected for leaf sampling. Additionally, 3 Bh08 apomictic hybrids (Bh08-1149, Bh08-700, Bh08-675) were included in this experiment.

Leaf samples were collected from the subplots from all 6 selected *Bh* genotypes before, 3, 8 and 11 DAF. The samples taken from the N unfertilized part were used to determine the baseline NRA, whereas NRA determined in leaves from the N fertilized plots served to assess the effect of N fertilization on NRA. As further indicator for contrasting NO_3^- uptake patterns among *Bh* hybrids and accessions, NO_3^- was measured in stem sap collected at 3, 8 and 11 DAF. For this step, finely cut stems of the respective plants for NRA measurement were squeezed into a plastic syringe. The effluent sap was collected in a petri dish and homogenized with a pipette tip and transferred onto NO_3^- test strips and analyzed using Nitracheck 404 (both Merck Millipore, Billerica, USA).

Soil samples from the topsoil (0-10 cm depth) of each subplot were taken with an auger (Ø 2.5 cm) at 8 DAF. From each plot, 2 representative subsamples of 20 g fresh soil were taken. Determination of the soil dry matter by gravimetry was conducted with one subsample, whereas another subsample was mixed in plastic bottles with 200 ml of 1 M KCl solution for NO_3^- extraction. The bottles were shaken for 30 min and filtered through Whatman Grade 2 filters. Extracts were kept at 4°C until NO_3^- was measured with a microplate reader (BioTek Instruments), in yellow ionized form derived from alkalization with sodium salicylate.

To monitor nitrification dynamics, topsoil samples were collected before N application from the plots, air dried for 48 hours and sieved (2 mm mesh size). Small stones and visible root residues were removed. A representative sample of 5 g of soil from each plot were filled in small glass tubes followed by application of 1.5 ml ammonium sulfate ($(NH_4)_2SO_4$) solution as substrate for nitrifiers. Tubes were sealed with parafilm that contained 2 holes for aeration and placed to a dark incubation chamber with constant 25°C and 60% air humidity. Soil NO_3^- was extracted before incubation start (basal), and after 5, 11, 14, 20 and 25 days (based on pre-tests) with 50 ml 1 M KCl. Soil NO_3^- was corrected by basal NO_3^- levels for each sampling time. NO_3^- concentration in incubated soil was expressed as mg $N\text{-}NO_3^-$ kg dry $soil^{-1}$.

3.3.5 Statistical analysis

SAS version 9.4 was used for statistical analysis (SAS Institute Inc., Cary, NC, USA). For *Experiment 1, proc glimmix* procedure was chosen to fit a mixed model with fixed effects and respective interactions for supplied N form (either NH_4^+, NH_4^+ + DMPP, NO_3^-), tissue (roots or stems), N concentration supplied (low, mid, high NO_3^-), and sampling time (0, 12 or 72 hours after N fertilization). Interactions of factors were removed from the model when interactions were not significant (p>0.05). Replication (REP) × sampling time was set as random effect. The mixed model for *Experiment 2* was developed with *proc mixed* procedure using genotype (GT), days after fertilization (DAF), and N form and respective interactions as fixed and REP × block (BLK) and REP × BLK × DAF as random effects. The *proc mixed* and *glimmix* approaches were also used for analyzing the data of *Experiment 3*. The mixed models included the fixed factors of N fertilization (N applied, no N applied), GT and DAF. REP × DAF, REP × GT and DAF × REP × GT were set as random and DAF was set as *repeated* statement. For all mixed model approaches, the following statistical procedure was similar: studentized residuals were inspected graphically for normality and homogeneity. Factors or interactions among factors being not significant at α=0.05 were removed from the model. Means of factors found significant for the respective model were compared by using the *lines* option in the *lsmeans* statement. Linear regressions presented from *Experiments 2* and *3* were conducted with SigmaPlot version 12. When data passed the normality test (Shapiro-Wilk) and the constant variance test, the R squared (R^2) and p-values were taken from the estimate of the procedure.

3.4 Results

3.4.1 NRA in roots and leaves and its induction by different N forms and NO_3^- concentrations (Experiment 1)

NRA was strongly expressed in leaf tissue but not in roots (p<0.0001) in all 3 N treatments (NH_4^+, NH_4^++DMPP, NO_3^-) when sampled 72 hours after N supply (Fig 3.4.1.1). The N form influenced NRA in leaves (p<0.0001), but not in roots (p=0.364). Highest NRA was detected in leaves of plants fertilized with N-NO_3^- and the lowest NRA was measured in plants fertilized with N-NH_4^+ + DMPP. NRA rates

increased over time (Fig 3.4.1.2a) being higher in leaves sampled at 72 hours after N fertilization compared to sampling at 12 hours after N supply (p<0.0005). The difference among the two sampling time points was strongly expressed under NO_3^- followed by NH_4^+ nutrition and the NH_4^+ + DMPP treatment. Low NO_3^- supply resulted in lowest NRA induction (p<0.0001), whereas intermediate and high NO_3^- availability showed no difference in NRA rates (p=0.0639) (Fig 3.4.1.2b).

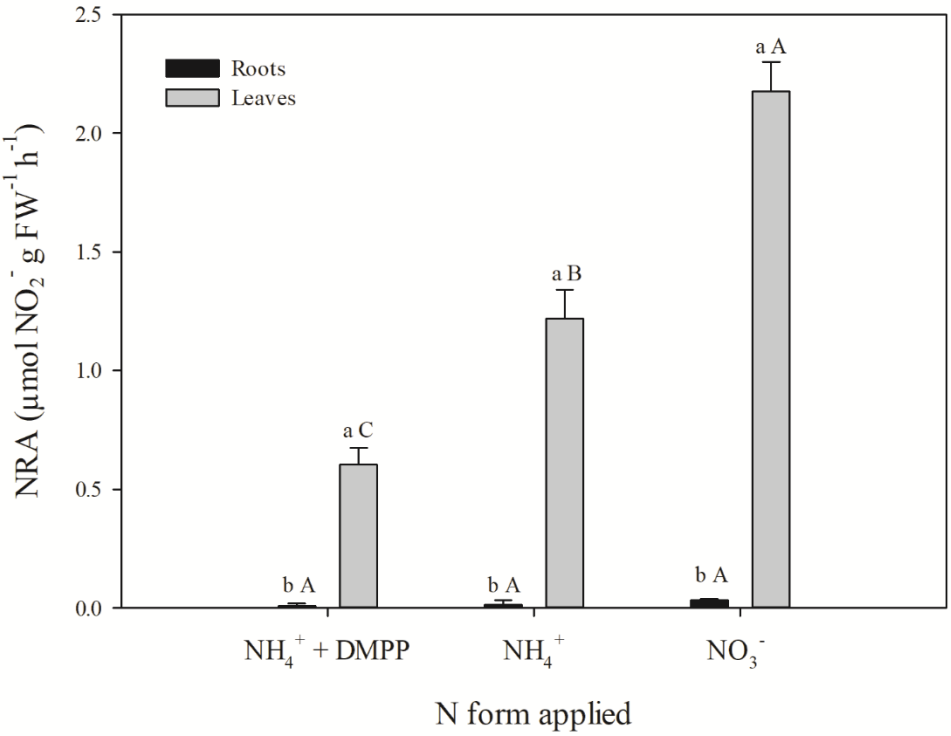

Fig 3.4.1.1 Nitrate reductase activity (NRA) in root and leaf tissue of *Brachiaria humidicola* (accession CIAT 679) under 3 different nutritional N forms (*Experiment 1)*. Bars are means of 4 replications. Standard errors are given in error bars. Least square means that share a common letter are not significantly different for α = 0.05 within the respective fertilizer form treatment. Same upper case letters indicate no significant difference for α = 0.05 between the N form treatments tested for each tissue separately.

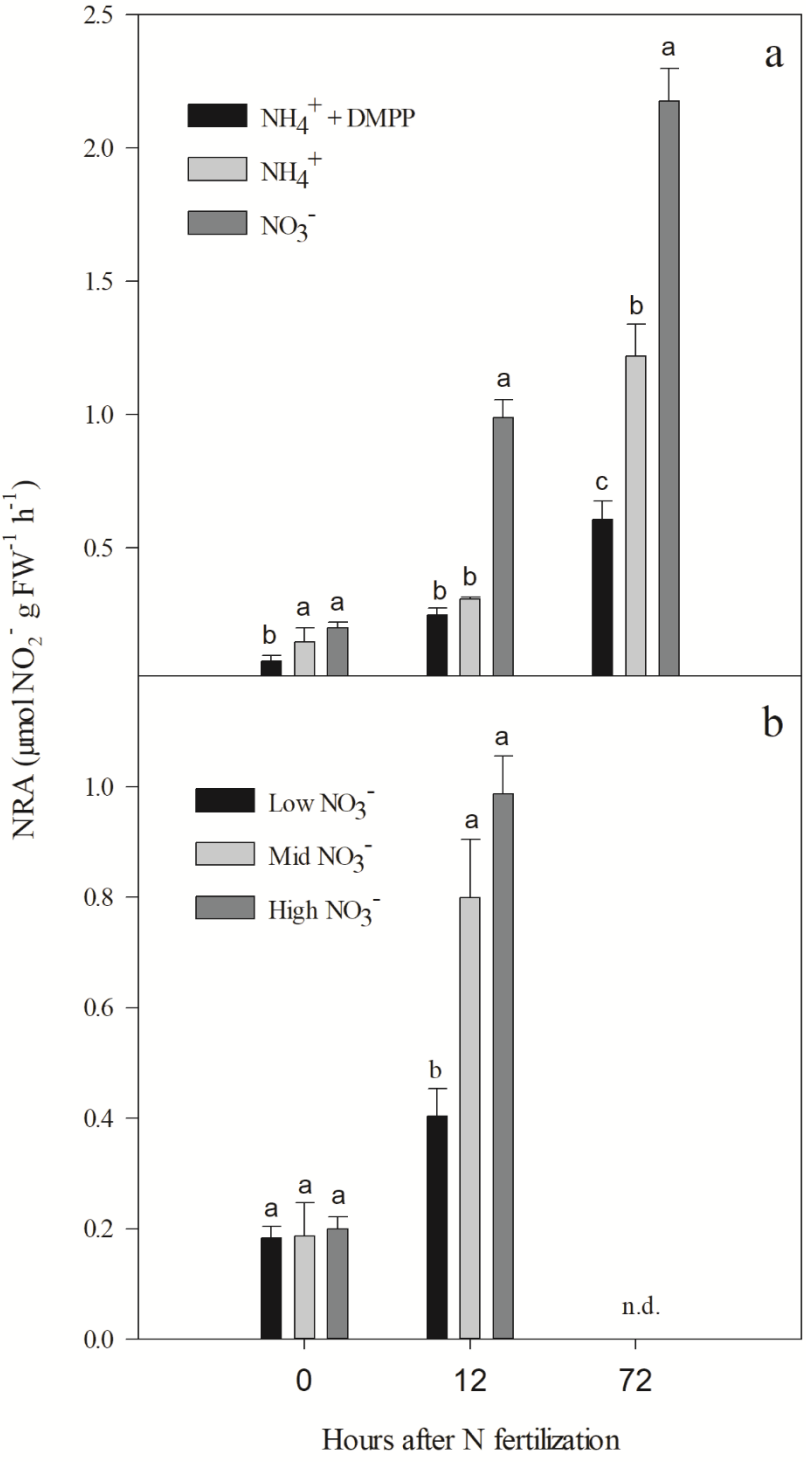

Fig 3.4.1.2 Nitrate reductase activity (NRA) in leaf tissue of *Brachiaria humidicola* (accession CIAT 679) before N supply, at 12 h and 72 h after N fertilization (*Experiment 1)*. N was applied in 3 different nutritional N forms shown in Fig 2a. NO_3^- treatment included 3 different nutrient solutions (high N, intermediate N and low N supply), shown in Fig 2b. Bars represent means of 4 replications. Standard errors are given in error bars. Least square means that share a common letter are not significantly different for $\alpha = 0.05$ within the respective sampling time.

3.4.2 Relationship between leaf NRA, soil NO_3^- and plant NO_3^- concentration under controlled (Experiment 2) and field conditions (Experiment 3)

A positive linear regression (p=0.012) between NO_3^- in the topsoil at 2 DAF and NRA at 9 DAF was observed (Fig 3.4.2.1a) for the 7 *Bh* genotypes of the pot trial (*Experiment 2*). The positive relationship (p=0.0032) between soil NO_3^- at 5 DAF and leaf NRA at 9 DAF was even stronger (R^2=0.85) (Fig 3.4.2.1b). However, no significant correlation was detected for leaf NRA and soil NO_3^- measured at the same date (p=0.3429, regression not shown).

NO_3^- measured in stems of the 6 *Bh* genotypes in the field (*Experiment 3*) correlated with NRA (Fig 3.4.2.1c) measured at the same sampling date (11 DAF) (p=0.0394), but not when sampled at 3 (p=0.2526) or 8 (p=0.2437) DAF. However, a nonlinear regressions analysis including all sampling dates (3, 8 and 11 DAF) and all the 6 *Bh* genotypes tested in *Experiment 3* showed that NRA in leaves increased contiguously with NO_3^- in stems (p<0.0001, regression not shown). Analog to the observed relationship between soil NO_3^- and leaf NRA in the greenhouse, the trend of the 6 *Bh* genotypes tested in the field was similar: increased NO_3^- in topsoil (0-10 cm, 8 DAF) was correlated positively with NRA (p=0.0083) measured in leaves 3 days later (11 DAF) (Fig 3.4.2.1d).

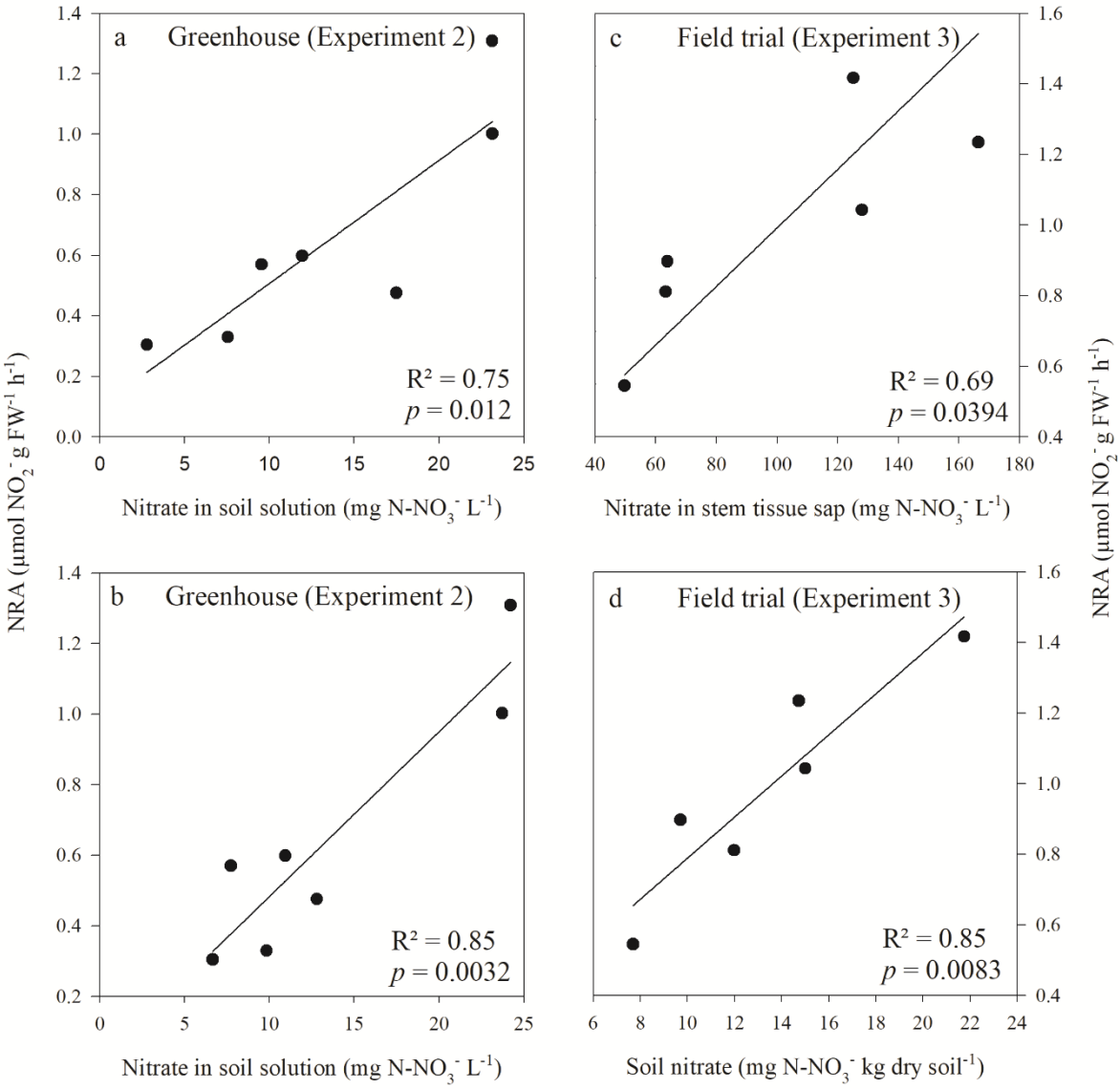

Fig 3.4.2.1 Linear regression between nitrate reductase activity (NRA) in leaf tissue at 9 days after $N\text{-}NH_4^+$ fertilization (DAF) and NO_3^- measured in soil solution from the topsoil at 2 (a), 5 DAF (b) of 7 *Brachiaria humidicola* (*Bh*) genotypes. Sampling was conducted at 16 months after establishment of the greenhouse trial described in *Experiment 2*. Results of *Experiment 3* (field trial) are shown in graphs c and d: linear regression between NRA in leaf tissue and NO_3^- concentration in stem sap (c) that was simultaneously sampled at 3 DAF of 6 Bh genotypes. Sampling was conducted at 27 months after establishment of the field trial. Linear regression among NRA measured *in vivo* in leaf tissue at 11 DAF and NO_3^- (d) in topsoil at 8 DAF.

3.4.3 Leaf NRA development for 2 contrasting *Bh* genotypes under different N fertilization forms (Experiment 2)

The influence of the fertilizer N form on leaf NRA was highly significant ($p<0.0001$) for the *Bh* grasses of the greenhouse pot trial. Among the 2 selected *Bh* genotypes (i.e., CIAT 679, hybrid Bh08-675), the dynamics of NRA was similar in terms of the 2 different N fertilizer control treatments (Fig 3.4.3.1, a & e). NH_4^+ + DMPP nutrition resulted in lowest NRA, whereas NO_3^- nutrition showed greatest NRA rates. NH_4^+ nutrition (BNI trigger, Subbarao et al. 2007b) was reflected in higher NRA values as the treatment NH_4^+ + DMPP, indicating nitrification activity when DMPP was not added. On the other hand, NRA was lower under NH_4^+ nutrition than under pure NO_3^- supply, indicating BNI activity for both genotypes. NRA differences among the genotypes appeared only under NH_4^+ supply between 2 and 5 days after N supply (Fig 3.4.3.1c). In this case, NRA was higher in CIAT 679 than in the hybrid Bh08-675 at 5 and 9 days after fertilization for the NH_4^+ treatment. NO_3^- in the topsoil showed a similar trend as the NRA for the respective genotypes (Fig 3.4.3.1, b, d, f). The nitrification inhibiting effect of DMPP was clearly reflected in constant low value of NO_3^- in solution. Collected soil solution samples from 50 cm depth depicted NO_3^- concentrations in the soil solution that were below the detection limit of 5 mg $N\text{-}NO_3^-$ L^{-1} (data not shown). This indicated that NO_3^- losses via leaching through the soil column were very small and did not affect NO_3^- uptake by the grass. NO_3^- measured in stems simultaneously to the leaf NRA assessment and the soil NO_3^-sampling at day 5 showed higher NO_3^- in the stem sap of CIAT 679 than Bh08-675 (data not shown).

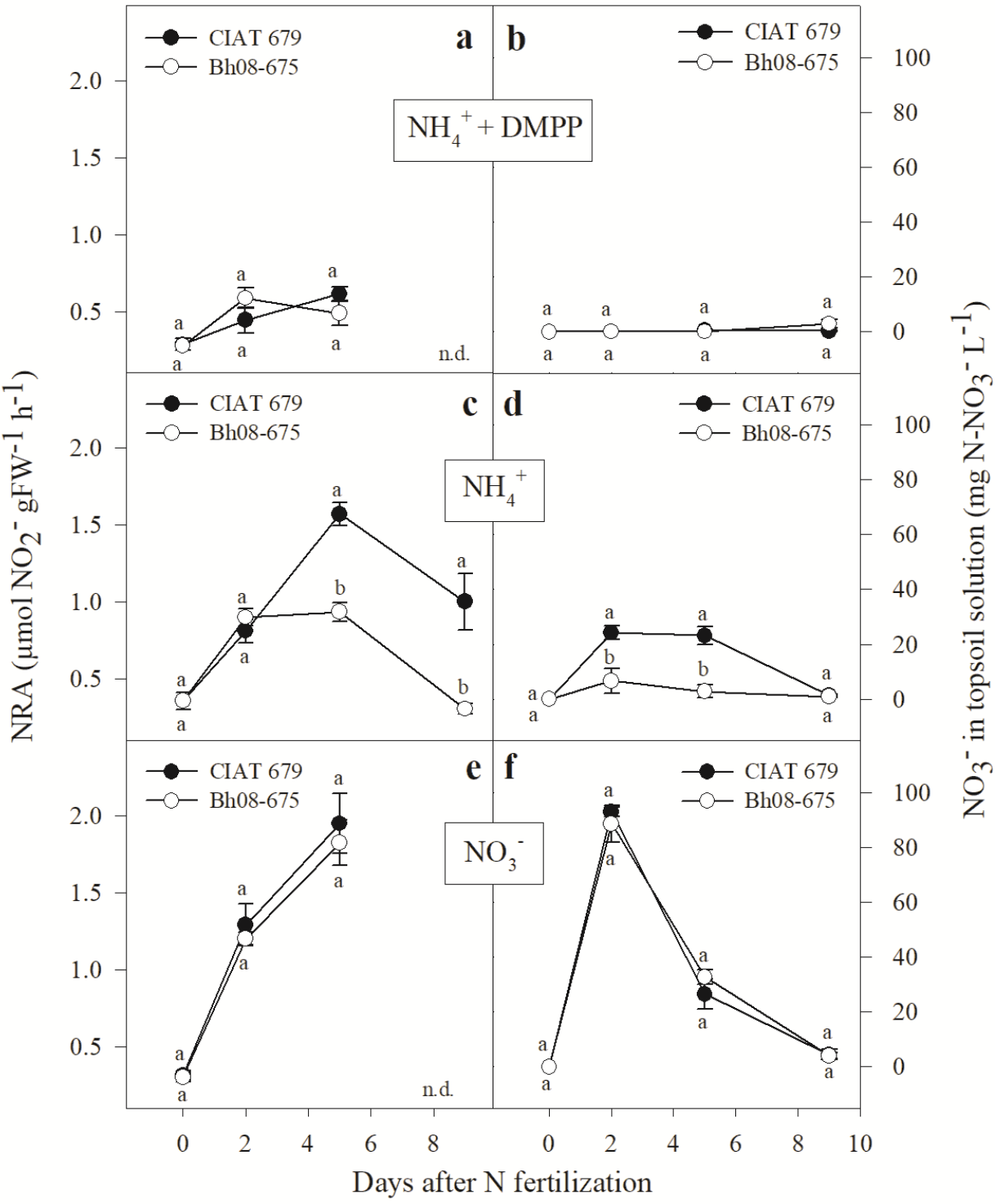

Fig 3.4.3.1 Nitrate reductase activity (NRA) measured *in vivo* in leaf tissue (left) and NO_3^- (mg N-NO_3^- L^{-1}) in soil solution (right). The contrasting *Brachiaria humidicola* genotypes CIAT 679 (mid-high BNI control) and Bh08-675 hybrid (unknown BNI potential) of 4 replications (plants from randomized soil columns). N fertilizer was supplied in 3 different N forms. Leaf samples were collected before N supply, and at 2 days and 5 days after N fertilization (DAF). NH_4^+ fertilized plants were additionally sampled at 9 DAF. Sampling was conducted at 16 months after establishment of the greenhouse trial (*Experiment 2*).

3.4.4 Leaf NRA of contrasting *Bh* genotypes under field conditions (Experiment 3)

NRA in leaves sampled in the field (Fig 3.4.4.1) before N fertilization from 6 contrasting *Bh* genotypes (*Experiment 3*) detected already significant differences among the hybrids (p<0.0001). The high BNI accessions (CIAT 16888) and mid-high BNI cultivar (CIAT 679) showed the lowest NRA rates and were different from NRA of the hybrids Bh08-1149 and Bh08-675. A general trend of NRA over time until 11 DAF was determined with relatively clear patterns for all accessions and genotypes tested. At the final sampling, NRA for CIAT 16888 was the lowest followed by CIAT 679. CIAT 26146 as low BNI control showed higher NRA in comparison to CIAT 16888 (p=0.001).

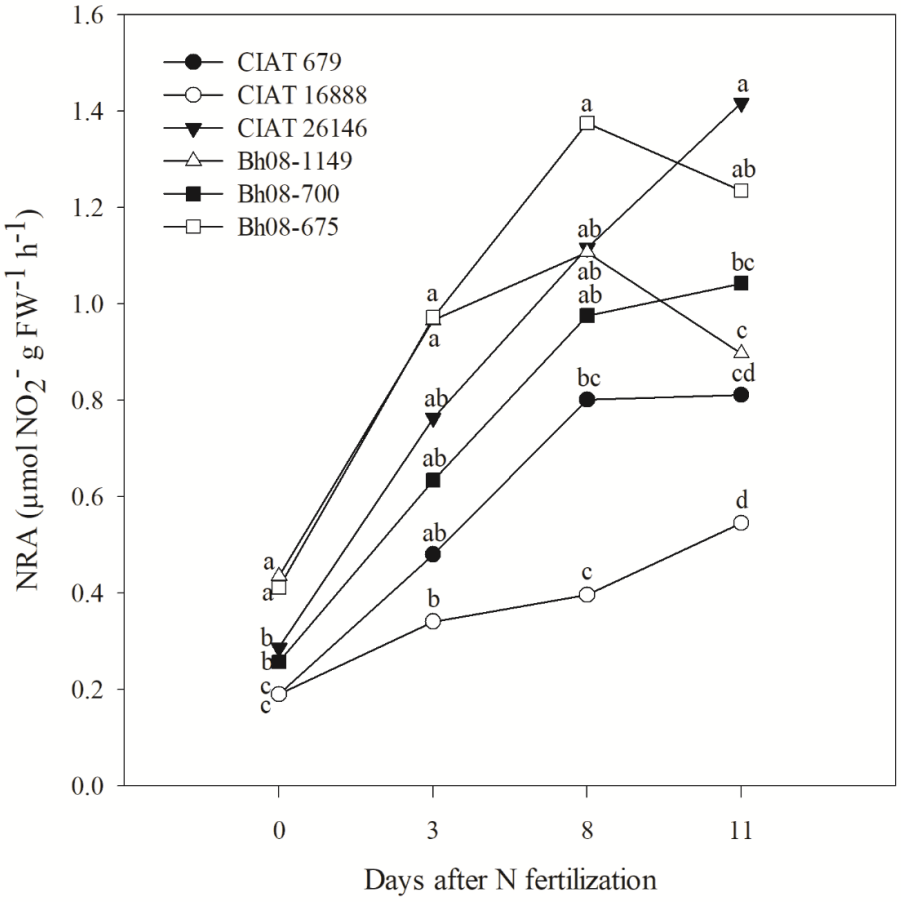

Fig 3.4.4.1 Nitrate reductase activity (NRA) measured *in vivo* in leaf tissue over time of *Brachiaria humidicola* genotypes including CIAT 679 (mid-high BNI control), CIAT 16888 (high BNI control), CIAT 26146 (low BNI control) and 3 Bh08 hybrids (unknown BNI potentials). Samples were taken at 0, 3, 8 and 11 days after NH_4^+ fertilization (DAF) from plants of 3 fully randomized field plots. Least square means with the same letter indicate no significantly different NRA least square means at α = 0.05 at equal DAF. Sampling was conducted at 27 months after establishment of the field trial (*Experiment 3*).

3.4.5 Incubation of soil from contrasting *Bh* genotypes from the field site (Experiment 3)

Differences of nitrification were analyzed in terms of significance of the interaction of NO_3^- concentrations (mg N-NO_3^- kg dry soil^{-1}) × days after incubation initiation (Fig 3.4.5.1). Incubated soil from CIAT 26146 (low BNI control) showed the strongest NO_3^- increase over time (p<0.0001), whereas it was less pronounced for high BNI control CIAT 16888 (p=0.0292) and not significant for mid-high BNI control CIAT 679 (p=0.0897). The nitrification development of Bh08-1149 (p=0.1102) was comparable to CIAT 679, and Bh08-675 (p=0.0317) close to CIAT 16888. Bh08-700 revealed the second strongest NO_3^- increase over time (p=0.0016) after low BNI control CIAT 26146.

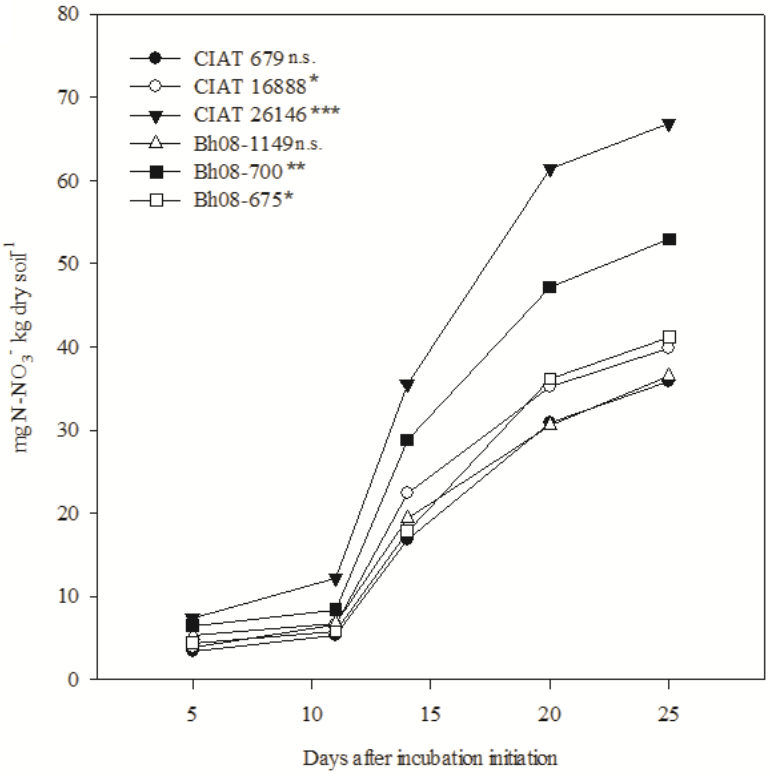

Fig 3.4.5.1 Nitrate (NO_3^-) formation over time in incubated soil sampled from replicated field plots (3 per genotype) where *Brachiaria humidicola* (*Bh*) genotypes including CIAT 679 (mid-high BNI control), CIAT 16888 (high BNI control), CIAT 26146 (low-mid BNI control) and 3 Bh08 hybrids (unknown BNI potentials) have been grown for about 27 months (*Experiment 3*). Soil was taken before N fertilization. One representative sample per field plot was incubated in the laboratory, dots indicate means of the 3 field plots of each respective genotype. Significance levels: n.s. = not significant "NO_3^- in soil concentration" × "days after incubation initiation" effect; significance of the tested interaction effect was indicated at *p-level < 0.05*, <0.01**, <0.001**.

3.5 Discussion

3.5.1 NRA measured in vivo in *Bh* leaves serves as a BNI indicator

To date, there is no suitable method available for sensitive BNI screening in *Brachiaria humidicola* (Bh) with minimal disturbance of the soil-plant system for both field and pot studies (Subbarao et al. 2006, 2017; Lata et al. 1999). To overcome this constraint, we demonstrate here that nitrate reductase activity (NRA) in grass leaves serves as a sound proxy of *in vivo* performance of BNI in *Bh*, an approach substantiated by combining earlier reports studying BNI potentials (Subbarao et al. 2009) or NRA in *Brachiaria* (Cazetta & Villela 2004; Macedo et al. 2013). Its potential for a reliable determination of BNI was verified by strong correlations of NO_3^- in solution, either through enhanced soil nitrification activity or direct NO_3^- supply via fertilization. However, no significant correlation was detected for leaf NRA and soil NO_3^- measured at the same date, indicating a delay between re-supplied NO_3^-, its uptake by roots and transfer into xylem for transportation to the cytoplasm, where it was finally reduced via NR (Li et al. 2013; Tegeder & Masclaux-Daubresse 2017). This retarded reflection of nitrification derived NO_3^- in NRA should be emphasized in prospective studies.

Specifically, under field conditions, CIAT 16888 with its reported high BNI potential (Subbarao et al. 2009) revealed the lowest NRA in leaves throughout the measurement period and lowest contents of soil NO_3^- than tested *Bh* lines with low BNI but high NRA (i.e., Bh08 hybrids, CIAT 26146) (Nuñez et al. 2018). On the other hand, we also observed different levels of *in vivo* BNI performances via leaf NRA along with correlated (nitrification derived) NO_3^- in soil solution between CIAT 679 and Bh08-675 in the greenhouse versus field study. This clearly exemplified the complex and yet poorly understood nature of BNI expression in *Bh* germplasm. Our observation was further in line with that of Subbarao et al. (2006) who firstly classified CIAT 679 as a medium BNI ecotype, while the same accession was later classified as high BNI capacity close to CIAT 16888 (Subbarao et al. 2009).

In vivo BNI in the *Bh* accessions measured via NRA in the field was not related to observed BNI potentials in soil incubation assays. Such discrepancy between BNI released in hydroponics and nitrification levels in a field study for different *Bh* genotypes have been previously reported (Subbarao et al. 2006). There is evidence that *Bh* root exudates comprise about ten, mostly anionic compounds with BNI potential (Subbarao et al. 2007b), although their biochemical nature is still not identified. It

could be thus assumed that different *Bh* genotypes release BNI substances of different composition and concentration (Subbarao et al. 2007a), whereby hydrophobic and hydrophilic phases, as well as their cationic and anionic nature (Ipinmoroti et al. 2008) is supposedly influencing their ability to be adsorbed to soil particles. However, it is still unknown if this mechanism as an effect on the persistence and efficacy of root derived substances, or if this rather leads to fixation and inactivation of the BNI relevant compounds. Consequently, genotypic BNI substance composition × edaphic interactions on BNI efficacy deserve more attention in research. Here, BNI exudate fingerprinting of contrasting *Bh* genotypes combined with *in vivo* BNI efficacy with the NRA assay could provide a concerted assessment of a specific *Bh* genotype under certain edaphic conditions. Moreover, it is known that the activation of NR or translation of an existing mRNA for the responsible enzyme depends on climatic factors such as the level of radiation and temperature during the day (Beevers & Hageman 1969). Therefore, it is suggested that absolute NRA values among different genotypes shall be performed in fields under similar environmental conditions to allow a reliable and comparable determination of *in vivo* BNI in *Bh*. To compensate for such natural fluctuations, NRA values obtained from the field experiment represented the net NRA.

3.5.2 Different forms and availability of N influence the in vivo NRA in *Bh* leaves

BNI activity reduces soil nitrification and hence alters the ratio of plant available NH_4^+:NO_3^- in soils resulting in plant uptake of predominantly NH_4^+ (under high BNI) or NO_3^- (under low/no BNI and high soil nitrification). According to this ecological concept, sole NH_4^+ nutrition, particularly when combined with high BNI or a nitrification inhibitor (+DMPP), revealed thus a lower NRA expression. This again confirmed that NRA of *Bh* is strongly coupled to NO_3^- nutrition of *Brachiaria* (Cazetta & Villela 2004; Macedo et al. 2013) and other plant species (Andrews 1986; Beevers & Hageman 1969). Moreover, Castilla & Jackson (1991) reported that hydroponics, where N was supplied as NH_4^+ in conjunction with NO_3^-, forced Bh to take up both N forms, without any observed preference for either one mineral N from. This is of main importance for linking *in vivo* NRA with BNI since privileging NH_4^+ and avoiding NO_3^- uptake might not reflect differences among candidates of medium and high BNI. This would be the case when substrate (NH_4^+) availability exceeds plant demand. The close relationship between NRA

of the incubated leaves and the soil NO_3^- combined with plant NO_3^- uptake indications revealed that *in vivo* NRA was assessed by the pre-sampled N status of the plant, and that a *de novo* synthesis of NR in post-sampled leaves could be excluded. Likewise, significant NRA differences of contrasting *Bh* CIAT accessions were measured before N addition in the field study. Accordingly, it is suggested to further investigate the applicability of the NRA assay on potential long-term BNI accumulation under low N availability besides from the presented *in vivo* BNI expression after NH_4^+ application.

3.6 Conclusions

Our NRA assay was verified as BNI proxy applicable for greenhouse and field studies. It was validated as a rapid and reliable method linked to the actual soil nitrification after NH_4^+ fertilizer supply. The possibility to perform several sampling intervals using the same plants allowed to detect fluctuations of BNI without major disturbance of the studied plant-soil environment. In comparison to commonly used hydroponic-root exudation studies, this methodological advancement represents a clear advantage for reliable real-time BNI performance monitoring for many food and feed crops under natural conditions (Zakir et al. 2008; Pariasca-Tanaka et al. 2010; Subbarao et al. 2013b; Sun et al. 2016; O'Sullivan et al. 2016).

It was demonstrated that NO_3^- is mainly reduced in leaves of *Bh* genotypes, regardless of NO_3^- availability in soil. The close relationship between increase of both, soil NO_3^- and NRA suggested that NRA might serve as a valuable indicator of *in vivo* performance of BNI in *Bh*. A delay effect between increasing NO_3^- availability in the soil and its reflection in the *in vivo* leaf NRA was observed and should be considered in determining the suitable time for sampling of leaf tissue. Our results confirmed the BNI potentials of included *Bh* CIAT accessions (Subbarao et al. 2006, 2009; Rao et al. 2014; Arango et al. 2014) in the field, however, this potential could not be expressed under greenhouse conditions. Accordingly, future studies need to elucidate synergistic effects of edaphic and biochemical origin that potentially alter *in vivo* BNI, apparent from detected triggers (NH_4^+ and low pH) of BNI.

Further testing of the possibility to use leaf NRA as an indicator of *in vivo* performance of BNI would allow the investigation of the role of BNI over a longer period. This would enhance the understanding of the relationship between *in vivo* BNI expression and N supply from soil to plant. It should be further investigated if other plants, where BNI has been detected (Zakir et al. 2008; Pariasca-Tanaka et al. 2010;

Subbarao et al. 2013; Sun et al. 2016; O'Sullivan et al. 2016), also reduce NO_3^- mainly in leaves and if the developed NRA assay could be adopted for *in vivo* monitoring of BNI performance of plants.

3.7 Funding

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4. Low ^{15}N natural abundance in shoot tissue of *Brachiaria humidicola* is as indicator of reduced N losses due to biological nitrification inhibition (BNI)

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4.1 Abstract

The tropical forage grass *Brachiaria humidicola* (Bh) suppresses the activity of soil nitrifiers through biological nitrification inhibition (BNI). As a result, nitrate (NO_3^-) formation and leaching are reduced which is also expected to tighten the soil nitrogen (N) cycle. However, the beneficial relationship between reduced NO_3^- losses and enhanced N uptake due to BNI has not been experimentally demonstrated yet. Nitrification discriminates against the ^{15}N isotope and leads to ^{15}N depleted NO_3^-, but ^{15}N enriched NH_4^+ in soils. Leaching of ^{15}N depleted NO_3^- enriches the residual N pool in the soil with ^{15}N. We hypothesized that altered nitrification and NO_3^- leaching due to diverging BNI magnitudes in contrasting Bh genotypes influence soil ^{15}N natural abundance ($\delta^{15}N$), which in turn is reflected in distinct $\delta^{15}N$ in Bh shoot biomass. Consequently, high BNI was expected to be reflected in low plant $\delta^{15}N$ of Bh. It was our objective to investigate under controlled conditions the link between shoot value of $\delta^{15}N$ in several Bh genotypes and leached NO_3^- amounts and shoot N uptake. Additionally, plant ^{15}N and N% was monitored among a wide range of Bh genotypes with contrasting BNI potentials in field plots for 3 years. We measured leaf $\delta^{15}N$ of young leaves (regrown after cutback) of Bh and combined it with nitrification rates (NRs) of incubated soil to test whether there is a direct relationship between plant $\delta^{15}N$ and BNI. Increased leached NO_3^- was positively correlated with higher $\delta^{15}N$ in Bh, whereas the correlation between shoot N uptake and shoot $\delta^{15}N$ was inverse. Field cultivation of a wide range of Bh genotypes over 3 years decreased NRs in incubated soil, while shoot $\delta^{15}N$ declined and shoot N% increased over time. Leaf $\delta^{15}N$ of Bh genotypes correlated positively with NRs of incubated soil. It was concluded that decreasing plant $\delta^{15}N$ of Bh genotypes over time reflects the long-term effect of BNI as linked to lower NO_3^- formation and reduced NO_3^- leaching. Accordingly, a low $\delta^{15}N$ in Bh shoot tissue verified its potential as indicator of high BNI activity of Bh genotypes.

Key words: isotopic discrimination, isotopic fractionation, N uptake, N assimilation, nitrate leaching, soil incubation

4.2 Introduction

Biological nitrification inhibition (BNI) by the tropical forage grass *Brachiaria humidicola* (Sylvester-Bradley et al. 1998; Subbarao et al. 2007) is an ecologically evolved trait to compete with nitrifying soil organisms for available ammonium (NH_4^+). Certain root derived exudates (Coskun et al. 2017b), e.g. brachialactone, have been verified to block the activity of ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) pathways in nitrifiers such as *Nitrosomonas europaea* (Subbarao et al. 2009). By preventing the microbial transformation of less soil mobile NH_4^+ to more soil mobile nitrate (NO_3^-), BNI is expected to reduce leaching of nitrogen (N) from ecosystems (Subbarao et al. 2009; 2013; Coskun et al. 2017a). Recent research has been undertaken to investigate the BNI effect by Bh on N dynamics, for example on the reduction of nitrous oxide (N_2O) emissions from soils (Byrnes et al. 2017), or on the influence of residual BNI effect on N uptake of subsequent crops (Karwat et al. 2017). However, indicators for reduced NO_3^- leaching losses by effective BNI do not exist yet. The reduction of NO_3^- leaching from agroecosystems due to BNI is one of the claimed central features in terms of BNI implementation (Subbarao et al. 2013; Coskun et al. 2017a).

Nitrification and N leaching are two main soil processes that lead to ^{15}N:^{14}N isotope fractionation (Mariotti et al. 1981). This results in a ^{15}N enriched NH_4^+ and a ^{15}N depleted NO_3^- pool in soils (Delwiche & Steyn 1970; Herman & Rundel 1986; Jones & Dalal 2017). Under high rainfall conditions, nitrification derived ^{15}N depleted NO_3^- is exposed to high leaching, whereby plants then would feed on remaining ^{15}N enriched NH_4^+. In contrast, plants with effective BNI are expected to suppress microbial NO_3^- formation. Consequently, less ^{15}N depleted NO_3^- would be lost from the system. Under both scenarios, Bh feeds mainly on NH_4^+, towards which it has a higher tolerance than other *Brachiaria* species (Castilla & Jackson 1991; Rao et al. 1996). The ^{15}N signature of soil mineral N can be reflected in a corresponding shoot ^{15}N signature (Takebayashi et al. 2010). Previous field studies demonstrated that foliar $\delta^{15}N$ increased in conjunction with increasing nitrification and N leaching by precipitation (Pardo et al. 2002; Stamatiadis et al. 2006; Huber et al. 2013; Yé et al. 2015). From these studies, the relationship between plant $\delta^{15}N$ and soil nitrification and N losses was apparent, which indicates a possible strong relationship between BNI and the $\delta^{15}N$ in plant tissue.

What remains unclear is if shoot $\delta^{15}N$ of Bh genotypes is linked to plant induced BNI, and if this relates to reduced nitrification, thus reduced NO_3^- leaching. We therefore hypothesized that (i) effective BNI is linked to reduced soil nitrification rates, enhanced N nutrition, reduced NO_3^- leaching; and that (ii) this link is expressed in respective low $\delta^{15}N$ in plant shoot tissue due to nutrition of Bh on a less naturally enriched N pool of ^{15}N. In contrast, it was expected that respectively higher nitrification rates (NRs) and higher NO_3^- leaching is expressed in higher $\delta^{15}N$ of shoot biomass due to the uptake of ^{15}N enriched soil N.

4.3 Material and Methods

4.3.1 Experiment 1: Relationship between $\delta^{15}N$ of Bh, N uptake and NO_3^- leaching under controlled conditions

A greenhouse study at the University of Hohenheim (UHOH), Stuttgart, Germany, was implemented as an α-design, i.e. a design with incomplete blocks, that are grouped into complete replicates. The trial contained four complete replicates with two blocks per replicate. The aim was to monitor the effect of N loss and N uptake after N fertilization on the $\delta^{15}N$ in shoot biomass of different Bh genotypes. Experimental soil columns were manufactured from PVC-drainpipes (Ø 11 cm × 100 cm) in order to enable deep rooting of Bh and monitoring of NO_3^- dislocation within the soil profile. A fossil tertiary clay loam (collected in Lich, Germany, 50°31'2.0"N, 8°50'55.9"E) with pH 5.7, 36% clay content, 0.25% total C and 0.025% total N was used. The original soil was amended with sand (25 vol%) in order to improve drainage, and the resulting substrate was filled into the experimental pipes.

Five apomictic Bh hybrids (Bh08 selection: Bh08-1149, Bh08-700, Bh08-675, Bh08-696, Bh08-1253) and two germplasm accessions of Bh (CIAT 16888, CIAT 679 cv. Tully) were used as test genotypes (Rao et al. 2014). Bh stolons were first propagated from a Bh stock collection at UHOH and transferred to a turf-based culture substrate for 3 weeks for root establishment. Then, the young Bh plants were transplanted in August 2014 to the experimental pipes and were exposed to supplementary artificial light (photosynthetically active radiation averaging 800 µmol m^{-2} s^{-1}) for 12 h photoperiod. Day and night temperature regimes were adjusted to 25°C and 20°C, respectively. Plants were raised with a basal fertilizer of N-P-K-S (analog to 30-60-150-35 kg ha^{-1}). After the establishment phase of 6 weeks, plants

were cut to 2-3 cm height to facilitate regrowth. The experimental phase commenced with application of 150 kg $N\text{-}NH_4^+$ ha^{-1} as $(NH_4)_2SO_4$ ($\delta^{15}N$ = -0.1) to stimulate the growth and activity of soil nitrifiers. Plants were watered every second day (with 100 ml H_2O), whereas the amount of water applied was doubled at 8, 19 and 28 days after N fertilization (DANF) in order to increase leaching of NO_3^- derived from nitrification.

Rhizons (Soil Moisture Sampler, Ø 2.3 × 50 mm, porosity 0.1 µm, Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands) were installed horizontally into the pipes at 7.5 cm and 50 cm depth. This procedure enabled sampling of soil solution by suction pressure using a common medical syringe. Soil solution sampling (0, 3, 6, 7, 10, 14 and 20 DANF from topsoil and at 0, 18 and 27 DANF from 50 cm depth) was conducted at 3 hours after irrigation with 100 ml of tap water to warrant sufficient soil moisture and time for establishing equilibrium for NO_3^- level in the topsoil. A sample of 10 ml of soil solution was collected and frozen immediately. Concentration of NO_3^- in soil solution samples was analyzed by complete reduction to NO_2^- through hydrazine (in alkaline solution, with copper as catalyst) and subsequent reaction with sulphanilamid and Griess reagent (N-(1-Naphthyl)ethylenediamine) to form a pink compound measured photometrically at 550 nm (method DIN38405, ISO/DIS 13395; photometer: AutoAnalyzer 3, QuAAtro AQ2, SEAL Analytical Ltd., Southampton, UK).

Resin bags (ion-exchange resin in a fine nylon mesh) were installed at the bottom of the experimental pipe to trap leached NO_3^-. This allowed the quantification of cumulative NO_3^--losses during the experiment. For this purpose, Resinex MX-11 (Jacobi Carbons GmbH, Frankfurt, Germany), a mixed anion/cation exchange resin with a maximum anion sorption capacity of 0.4 eq l^{-1} was used. Each bag contained 200 ml resin amended with 200 ml of washed sand in order to slow down percolate passage through the coarse resin matrix. The anion traps were removed at the end of the experiment (i.e. at 42 DANF). The resin was thoroughly homogenized and an aliquot of 40 ml resin of each bag was further processed. Extraction of NO_3^- was performed twice with 200 ml 2 M KCl and extracts were subsequently analyzed photometrically for NO_3^- content (see analysis of percolate samples). A pre-test on the extractability of Resinex MX-11 verified that a two-fold extraction was sufficient to achieve extraction rates of 98% of trapped NO_3^-.

At 42 DANF, soil solution sampling showed NO_3^- levels below the detection limit using a fast NO_3^- test (< 5 mg NO_3^- L^{-1}). This indicated that the main effect of N fertilization should be reflected in terms of the $\delta^{15}N$ and N uptake amount (mg N g plant dry matter^{-1}). The aboveground shoot biomass (cut 3 cm above soil surface) of all 7 Bh genotypes was sampled subsequently and oven-dried (3 days 60°C). Dry matter was determined and ground aliquots of plant material were filled into tin capsules (HEKAtech GmbH, Wegberg, Germany).

^{15}N and N% were measured for all plant samples from Experiments 1-3 at UHOH by using a Euro Elemental analyzer coupled to a Finnigan Delta continuous-flow isotope ratio mass spectrometer IRMS (Thermo Scientific, Bremen, Germany). The ^{15}N natural abundance of the sample relative to the standard (atmospheric N_2) was expressed as: $\delta^{15}N‰ = [(R_{sample} / R_{standard}) -1] \times 1000$ (‰) where R represents the isotope ratio ($^{15}N/^{14}N$) and $R_{standard}$ is $^{15}N/^{14}N$ for atmospheric N_2 that is 0.0036765 ($\delta^{15}N‰ = 0$) (Robinson 2001).

4.3.2 Experiment 2: Differences in $\delta^{15}N$ leaf and shoot of a wide range of Bh genotypes grown under field conditions

A field trial was established in August 2013 with a range of Bh genotypes with contrasting BNI activity at Corpoica-La Libertad Research Center in the Piedmont region of Colombia (4°03'46" N, 73°27'47" W). The experimental field site was located at an altitude of 338 meter above sea level with an annual mean temperature of 21.4°C and an average annual rainfall of 3,685 mm. The soil is classified as an Oxisol with a pH of 4.9, clay content of 42%, total N of 0.11% and C/N ratio of 12.4. The trial included 4 out of the 5 Bh apomictic hybrids used in Experiment 1 and 4 Bh germplasm accessions. Each main plot had a size of 4×4 (16 m^2) and received basal fertilization (kg ha^{-1}) in September 2013 of 69 N (urea, $\delta^{15}N$=0.05), 25 P, 50 K, 50 Ca, 15 Mg, 10 S, 0.5 B and 2.6 Zn. The second N (100 kg N ha^{-1} as urea) fertilization (26 October 2015) was conducted after leaf sampling for Experiment 3 to stimulate both, BNI and soil nitrification.

For forage productivity and forage quality evaluation, grasses were cut every six weeks from October 2013 to November 2015. Aboveground shoot biomass samples of each plot were oven dried at 60°C. A sub-sample was ground and sent to UHOH for IRMS analysis (see above in Experiment 1).

4.3.3 Experiment 3: $\delta^{15}N$ of young regrown Bh leaves linked to BNI indicated by soil incubation

Two years after initiation of the field trial described for Experiment 2, a substantial BNI effect was expected based on preliminary tests (soil incubation, data not shown). The grass was cut at 5 October 2015 (end of the rainy season) and all plots received maintenance fertilization (kg ha^{-1}) of 40 P, 75 K, 110 Ca, 65 Mg, 19 S, 0.9 B and 5.3 Zn. No N fertilizer was applied. It was intended that the plants take up soil mineral N and consequently reflect the $\delta^{15}N$ of the soil mineral N. To avoid border effects during sampling, one sub-plot (1 m^2) per main plot were was randomly defined and marked. At 11 days after grass cutting (16 October), recently fully expended (second youngest) leaves from the regrown plants within the sub-plots were collected from the plots of 3 CIAT accessions as well as from Bh08 hybrids. Oven-dried and ground shoot samples were sent to UHOH for ^{15}N and N% measurement (see above in Experiment 1).

Before cutting the grass in the field trial (Experiment 3) and adding the fertilizer in October 2015, topsoil samples (0-10 cm) were collected with an auger from 8 randomly chosen points within each sub-plot. About 500 g of soil per sub-plot was thoroughly mixed, air-dried for 48 h, sieved (2 mm mesh size) and small stones as well as visible root material were removed. Representative sub-samples of 5 g of soil from each plot were filled in small glass tubes followed by application of 1.5 ml ammonium sulfate ($(NH_4)_2SO_4$) solution as substrate for soil nitrifiers. Tubes were sealed with parafilm that contained 2 holes for aeration and placed in a dark incubation chamber with constant 25°C and 60% air humidity. Soil NO3- was extracted before starting the incubation (basal) and after 5 and 25 days (main active phase of nitrification) with 50 ml of 1 M KCl. NO_3^- concentrations were determined as described above (Experiment 1). Nitrification rates (NRs) were expressed as mg $N\text{-}NO_3^-$ kg dry $soil^{-1}$ day^{-1}.

4.3.4 Statistical analysis

Statistical analysis was performed using the SAS ® version 9.4 (SAS Institute Inc., Cary, NC, USA) with a mixed model approach. The assumptions of homogeneity of variance and normal distribution of

residual errors were tested through the plots of studentized residuals vs. predicted value and quantile-quantile-plots, respectively, for all data sets. For the analysis of data from Experiment 2, the following model was set up: Genotype + Year + Rep + Genotype × Year. Block was set as random factor. Model based least square means were used for data visualization with SigmaPlot for Windows version 12.0. (Copyright Systat Software, Inc.). The same software was also used for correlation analysis conducted for Experiments 1 and 3.

4.4 Results

4.4.1 Experiment 1 (Greenhouse at UHOH, Germany)

Plant $\delta^{15}N$ at harvest (42 DANF) was found to be negatively correlated with plant N uptake amounts ($p<0.001$) (Fig 1a). Furthermore, the relationship between plant $\delta^{15}N$ and the amount of cumulative leached NO_3^- ($p=0.018$; $R^2=0.16$) was moderately positive (Fig 4.4.1.1b). In case of low N uptake, plant $\delta^{15}N$ was high, whereas plants with high N uptake were found to have lower plant $\delta^{15}N$ (Fig 4.4.1.1a). Increased leaching of NO_3^--N lead to an increase of the $\delta^{15}N$ of remaining N accumulating in the grass shoot biomass (Table 4.4.1). Furthermore, plant $\delta^{15}N$ of all samples was relatively enriched compared to the $\delta^{15}N$ of the applied NH_4^+ fertilizer ($\delta^{15}N=-0.1$). A linear regression analysis showed an inverse relationship ($p<0.001$; $R^2=0.37$) between plant N uptake and leached N (supplementary material).

Table 4.4.1 Plant $\delta^{15}N$ (‰), Plant N uptake (mg N pot^{-1}) and leached N (mg N pot^{-1}) 42 days after N ($(NH_4^+)_2SO_4$; $\delta^{15}N$=-0.1‰) fertilization of 2 *Brachiaria humidicola* (Bh) CIAT accessions and 5 Bh08 hybrids. Standard error of mean (±) is given behind the mean values. Means that share a common letter within the same line do not differ significantly at the α=5% level (LSD-test). Plants (6 replications of CIAT 679 and CIAT 16888; 4 replications of Bh08-hybrids) were sampled 42 days after N fertilization from a pot trial installed in a greenhouse at the University of Hohenheim (Experiment 1).

	CIAT 679	CIAT 16888	Bh08-675	Bh08-1149	Bh08-700	Bh08-696	Bh08-1253
Plant $\delta^{15}N$ (‰)	4.08bc ±0.34	4.35bc ±0.34	4.72bc ±0.42	3.80c ±0.34	5.10b ±0.42	6.70a ±0.41	5.07b ±0.42
Plant N uptake (mg N pot^{-1})	87.08a ±1.62	53.52c ±5.81	59.42bc ±5.15	87.05a ±1.73	63.16bc ±3.16	30.94d ±6.30	68.49b ±6.54
Leached NO_3^- (mg N pot^{-1})	48.27b ±2.29	67.30a ±3.00	60.30ab ±6.83	57.07ab ±3.07	66.08a ±7.87	69.93a ±8.52	66.43a ±8.32
Dry matter (g)	7.13a ±0.31	3.66c ±0.39	4.19c ±0.63	6.06ab ±0.46	4.56bc ±0.13	2.18d ±0.24	4.23c ±0.13

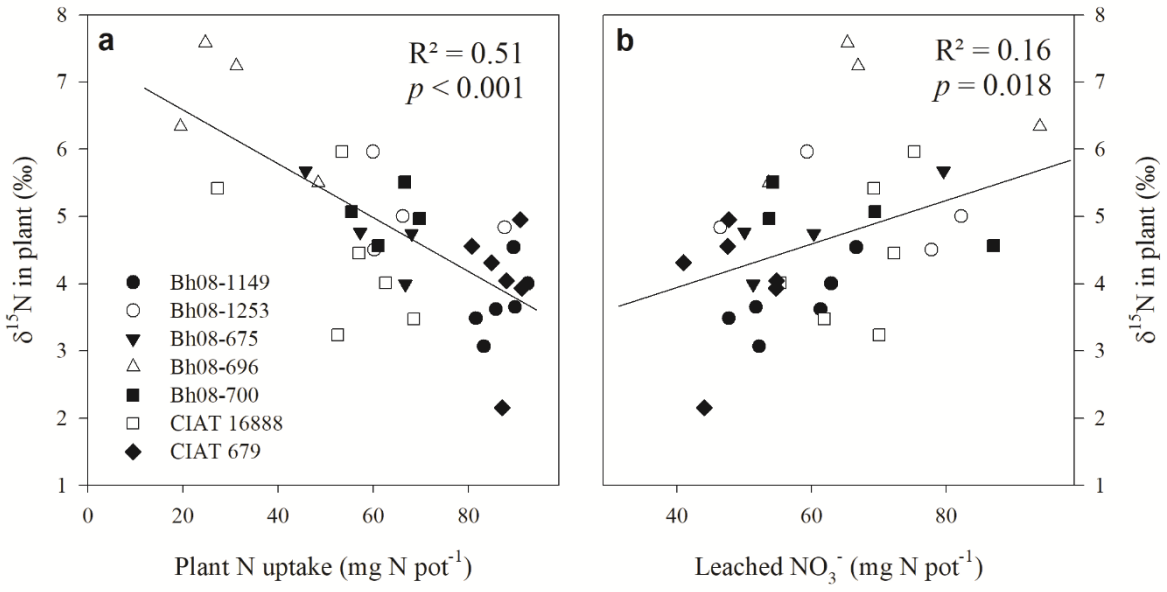


Fig 4.4.1.1 Linear regression of $\delta^{15}N$ (‰) values of *Brachiaria humidicola* (*Bh*) aboveground biomass and plant N uptake (mg N pot^{-1}) (**a**) and cumulative leached NO_3^- (mg N pot^{-1}) (**b**). The greenhouse experiment included 7 Bh genotypes and was established in August 2014. Plants were sampled at 42 days after NH_4^+ fertilization (6 weeks after transplanting to experimental pots). Plant N uptake and leached NO_3^- are cumulative amounts determined at the date of harvest.

4.4.2 Experiment 2: Yearly $\delta^{15}N$ monitoring of Bh genotypes in the field (Field trial La Libertad, Colombia)

The overall year effect showed an obvious trend of decreasing plant $\delta^{15}N$ over the experimental seasons (p<0.0001) (Fig 4.4.2.1). One month after experiment establishment and N fertilization (October 2013), most genotypes tended to a plant $\delta^{15}N$ of around 7‰, except for the Bh08-1149 hybrid with a $\delta^{15}N$ of almost 8‰. One year later (October 2014), $\delta^{15}N$ of all genotypes had dropped below 5‰, however, a genotypic effect on $\delta^{15}N$ abundance was absent (p=0.13). At the last sampling (November 2015), $\delta^{15}N$ of CIAT 26149 and CIAT 26146 had higher $\delta^{15}N$ than the other 3 CIAT accessions and the two Bh08-hybrids (p=0.02).

To investigate an expected relationship between $\delta^{15}N$ and N uptake by Bh, a regression analysis was conducted between the measured plant $\delta^{15}N$ and the N concentration (N%) in the respective sampled Bh

grass genotypes (Fig 4.4.2.2). A negative correlation (p<0.001) was observed between plant $\delta^{15}N$ and plant N%, indicating that the higher the N status of the plant is the lower the respective $\delta^{15}N$ becomes.

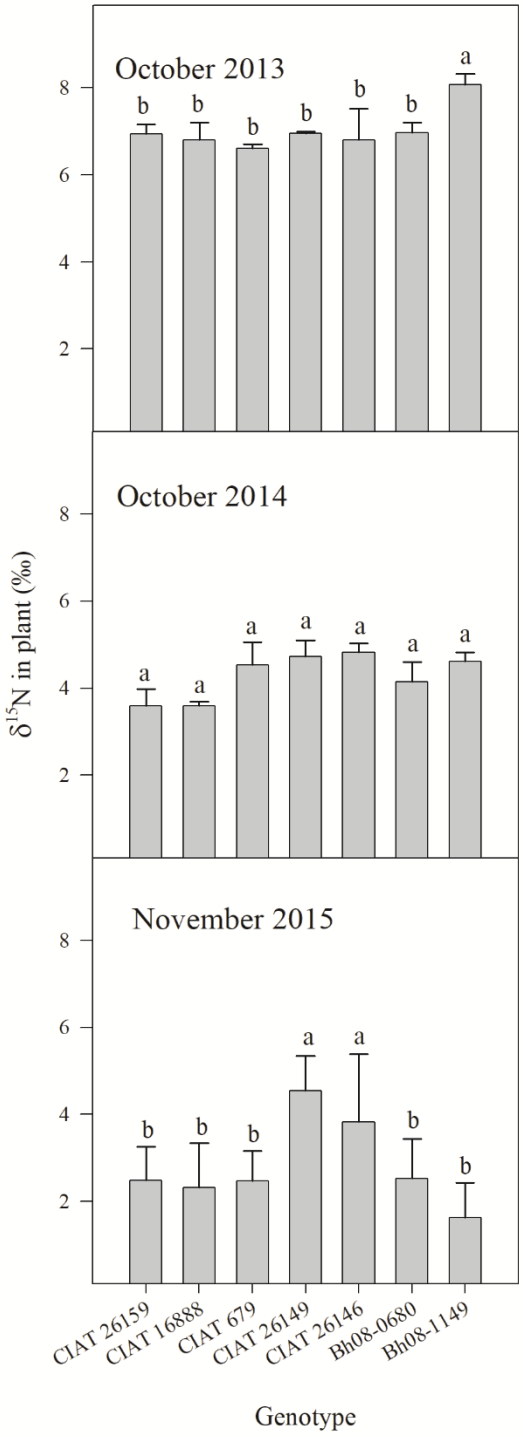


Fig 4.4.2.1 $\delta^{15}N$ (‰) plant signature of *Brachiaria humidicola* (*Bh*) aboveground biomass of 5 contrasting (in terms of BNI) CIAT accessions and 2 hybrids (Bh08-population) with unknown BNI capacity sampled after the rainy season in the Colombian *Llanos*. The field trail was established in August 2013 N fertilizer was applied as urea in September 2013 (69 kg N ha^{-1}) and in October 2015 (100 kg N ha^{-1}).

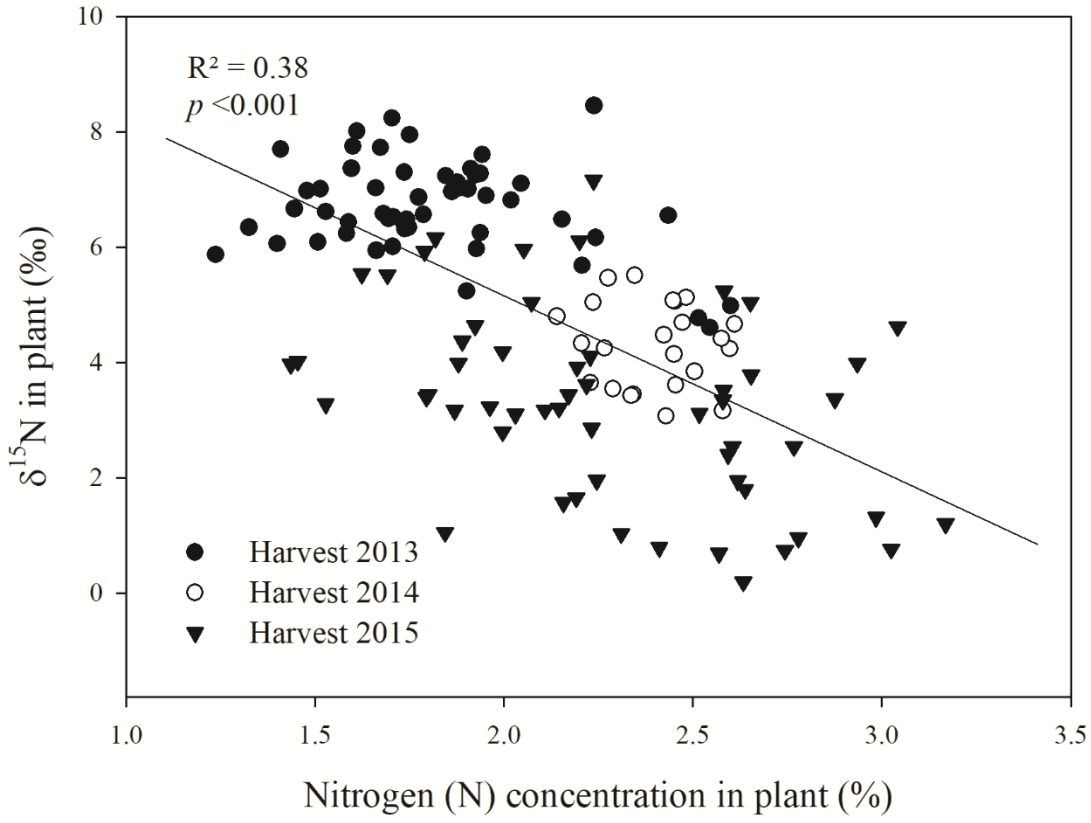


Fig. 4.4.2.2 Linear regression of $\delta^{15}N$ (‰) and N concentration (%)of *Brachiaria humidicola* (*Bh*) aboveground biomass of contrasting genotypes (in terms of BNI) sampled after the rainy season in the Colombian *Llanos*. The field trail was established in August 2013 N fertilizer was applied as urea in September 2013 (69 kg N ha^{-1}) and in October 2015 (100 kg N ha^{-1}).

4.4.3 Experiment 3: Genotypic leaf $\delta^{15}N$ and relation to soil nitrification (Field trial La Libertad, Colombia)

Means of $\delta^{15}N$ values of regrown leaves at 11 days after regrowth (October 2015) of the selected Bh genotypes were positively correlated with the observed nitrification rates of the respective Bh genotypes (p=0.007) (Fig 4.4.3.1). Leaf $\delta^{15}N$ means were found to be different among genotypes (p=0.001), whereas the genotype effect was not significant for NRs (p=0.74) (Table 4.4.3). In more detail, CIAT 26146 had highest $\delta^{15}N$ leaf signal and the corresponding incubated soil showed highest NR. CIAT 16888 and CIAT 679, with reported high BNI, had, compared to CIAT 26146, significantly lower ^{15}N leaf signals and were among the genotypes tested those with lower nitrification rates.

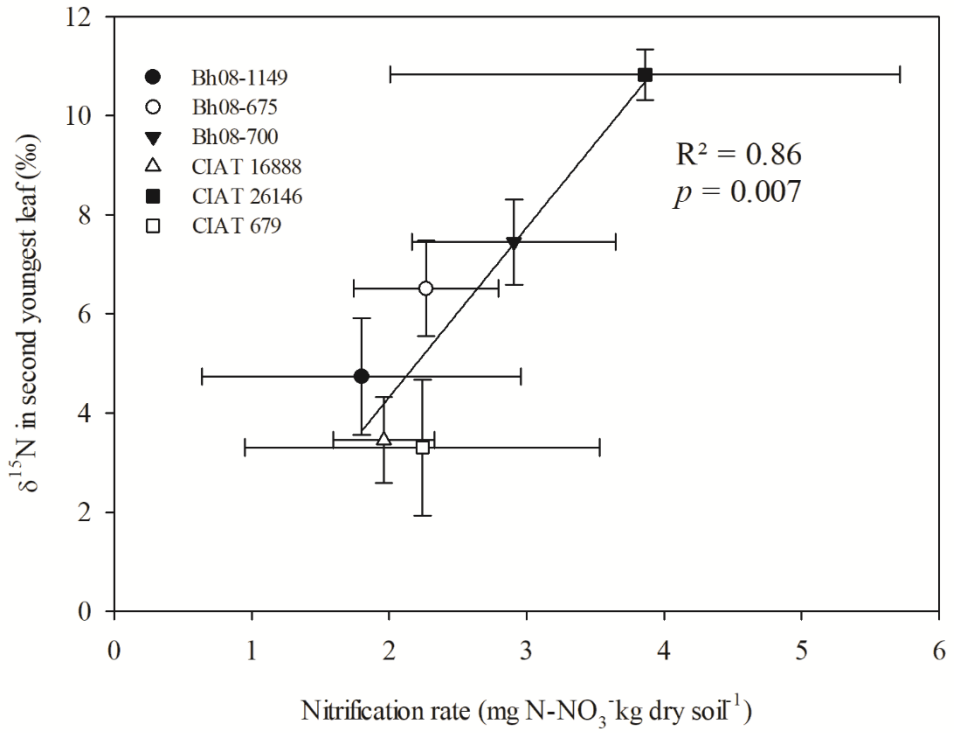


Fig 4.4.3.1 Linear regression of $\delta^{15}N$ (‰) in the second youngest leaf of *Brachiaria humidicola* (*Bh*) aboveground biomass and nitrification rates (mg $N\text{-}NO_3^-$ kg dry $soil^{-1}$) determined from incubated soil sampled from the respective field plots. The field trial included Bh CIAT accessions with potential of high BNI (CIAT 16888), mid-high BNI (CIAT 679) and low BNI (CIAT 26146). Additionally, 3 hybrids (Bh08-population) with unknown BNI capacity were sampled. The field trail was established in August 2013 in the Colombian *Llanos*. N fertilizer was applied as urea in September 2013 (69 kg N ha^{-1}). Plants were cut back at 5 October 2015 and topsoil (0-10 cm) samples were collected from incubation in the laboratory. Leaves of *Bh* were sampled 11 days after regrowth.

Table 4.4.3 Nitrification rates (NRs) determined from incubated soil sampled from field plots of 3 *Brachiaria humidicola* (*Bh*) CIAT accessions and 3 *Bh* hybrids. Plant $\delta^{15}N$ in leaves of the respective *Bh* plants. SE = standard error of the mean. Genotype effect of NRs was not significant (p=0.79). Genotype effect of plant $\delta^{15}N$ was p=0.02. Different letters indicate significant different means according to Multiple Comparison Procedures (Holm-Sidak method).

B.humidicola genotype	Nitrification rate mg $N\text{-}NO_3^-$ kg soil day^{-1}	SE	Plant $\delta^{15}N$	SE
CIAT 16888	2.24	1.3	3.3^{a}	1.4
CIAT 679	1.96	0.4	3.5^{a}	0.9
CIAT 26146	3.86	1.9	10.8^{b}	0.5
Bh08-1149	1.80	1.2	4.7^{a}	1.2
Bh08-700	2.91	0.7	7.5^{ab}	0.9
Bh08-675	2.27	0.5	6.5^{ab}	1.0

4.5 Discussion

4.5.1 Lower plant $\delta^{15}N$ is linked to reduced NO_3^- losses and high plant N uptake

Our results of the greenhouse study confirmed the hypothesis that low plant $\delta^{15}N$ is linked to enhanced N nutrition and reduced NO_3^- leaching. Hence, the altered plant isotopic signals were indirectly linked to isotopic fractionation of ^{15}N:^{14}N between product (NO_3^-) and substrate (NH_4^+) during bacterial and archaeal nitrification (Delwiche & Steyn 1970; Shearer et al. 1974). This led to subsequent leaching of the relatively ^{15}N depleted NO_3^-. Our observations (Experiment 1) indicated that with increasing amounts of leached NO_3^-, the $\delta^{15}N$ of the Bh grass increased (Fig. 1b). These results suggest that losses of ^{15}N depleted NO_3^- resulted in relative enrichment of the plant available N. Similar observations of leaching processes leading to an ^{15}N enrichment of the remaining soil NH_4^+ have been made by Pardo et al. (2007), Craine et al. (2009), and Stevenson et al. (2010). Furthermore, our observation of the positive relationship between increasing ^{15}N:^{14}N in vegetation due to increasing nitrification and N losses has also been described for leaves of forest trees (Pardo et al. 2002; Garten et al. 2008), cotton (Stamatiadis et al. 2006), mixed systems such as grass-heath-woodlands (Huber et al. 2013), as well as for comparative studies between perennial and annual grasses (Yé et al. 2015). Furthermore, evaluations at various sites demonstrated lower $\delta^{15}N$ of NO_3^- compared to $\delta^{15}N$ of NH_4^+ in soil due to nitrification (Takebayashi et al. 2010). The yearly field evaluation revealed that ^{15}N signals in Bh decreased with improved N status of the grass over time, indicating increased utilization of plant N as also observed in Central European grasslands by Kleinebecker et al. (2014). We suggest that in our field trial this was caused by decreasing NO_3^- losses from the system due to expanded plant (e.g. root system) growth and development over the years.

4.5.2 Long-term BNI effect in the field expressed in low plant $\delta^{15}N$

The general trend of decreasing plant $\delta^{15}N$ of Bh genotypes in the field study over the years indicated that N isotope fractionation and consequently the $\delta^{15}N$ of the mineral N in the soil changed over time. Thus, our results confirmed that during the early stage after establishment of the Bh genotypes, there was no significant influence of BNI on microbial nitrification. This observation is consistent with other studies on BNI expression with young Bh plants (Miranda et al. 1994; Castoldi et al. 2013; O'Sullivan

et al. 2016). Therefore, it is suggested that applied urea N (hydrolyzed to NH_4^+ within a few days) during transplanting had a strong stimulation effect on growth and activity of soil nitrifiers, and that the microbial formed NO_3^- was leached rapidly in the first rainy season when the grass was still small. BNI was unlikely to be strongly expressed, since plants were less than two months old and a strong BNI effect, due to an accumulation of BNI substances in the soil, needs about one year of Bh establishment (Nuñez et al. 2018). The high $\delta^{15}N$ of Bh plants observed in October 2013 could therefore reflect the strong ^{15}N enrichment of the soil mineral N pool caused by substantial nitrification and leaching loss of ^{15}N depleted NO_3^- during the establishment of the trial (Nadelhoffer & Fry 1994; Song et al. 2014). Plant $\delta^{15}N$ after the second rainy season (October 2014) were lower than the first sampling. BNI ability has been shown to be promoted with developing root biomass leading to less nitrification over time (Subbarao et al. 2009). Reduced N losses during the second rainy season explained the lower plant $\delta^{15}N$ caused by a lower ^{15}N enrichment of soil mineral N pool. The general tendency of decreasing ^{15}N natural abundance of Bh genotypes over the years was also visible in the third year. This indicated further reduction of NO_3^- formation and loss due to increase of BNI. This was verified by other soil incubation studies (Arango et al., unpublished) revealing low nitrification rates during the second (2014) rainy season. In contrast, incubation of soil sampled during the third rainy season (2015) evidenced significantly lower average nitrification rates (3.5 mg $N\text{-}NO_3^-$ kg dry $soil^{-1}$ day^{-1}). However, since root systems expand over the years a general higher uptake of NH_4^+ is expected. This could, additionally to BNI substance release, increase the competiveness of Bh for NH_4^+ and indirectly reduce nitrifier activity.

4.5.3 Link between high BNI and low leaf $\delta^{15}N$ of Bh genotypes

In our study, we linked leaf $\delta^{15}N$ to BNI by Bh. It was evident that low nitrification in incubated soil taken from plots, where Bh was cultivated for more than two years, correlated with lower leaf $\delta^{15}N$. Exudation of brachialactone by Bh and other known nitrification inhibiting substances (Subbarao et al. 2009; Gopalakrishnan et al. 2009) are supposed to increase the relative NH_4^+-to-NO_3^- uptake, thereby reflecting primarily the $\delta^{15}N$ signal of the plant available soil NH_4^+ pool (Kahmen et al. 2008). Furthermore, Bh root exudates have been shown to reduce *Nitrosmonas europaea* populations in soil (Gopalakrishnan et al. 2009). Our results thus support the hypothesis that high BNI (low nitrification

rates) results in lower leaf $\delta^{15}N$, as observed in our field study. Robinson (2001) suggested to measure whole plant $\delta^{15}N$ when studies intend to indicate source $\delta^{15}N$ in plant tissues to avoid uncertainties in isotopic discrimination during partitioning in the plant. In this respect, however, it has to be considered that intra-plant ^{15}N discrimination (e.g. root-to-shoot) is generally small when N availability is low or when NH_4^+ is the primary mineral N form taken up by the plant (Evans 2001).

4.5.4 Different plant $\delta^{15}N$ among contrasting Bh genotypes in terms of BNI

BNI differences among Bh accessions or hybrids have been revealed (Subbarao et al. 2007; Rao et al. 2014; Nuñez et al. 2018) and the effect of high BNI is expected to reduce NO_3^- formation and leaching (Subbarao et al. 2009, 2013). But experimental evidence for the latter is lacking. We observed a strong genotypic effect on leaf $\delta^{15}N$ in our field studies. For instance, Bh genotypes with low nitrification rates in the field (CIAT 16888, CIAT 679, CIAT 26159) showed a strong BNI effect from their root exudates on *Nitrosomonas europaea* (Subbarao et al. 2007). At the end of the field study, the same genotypes showed lower $\delta^{15}N$ than CIAT 26149 with known low BNI potential (Subbarao et al. 2007). Furthermore, the higher leaf $\delta^{15}N$ of CIAT 26146 compared to CIAT 16888 and CIAT 679 fit to our hypotheses, and earlier BNI evaluations (Subbarao et al. 2009; Nuñez et al. 2018). However, apart from BNI, other factors could have altered shoot $\delta^{15}N$ of Bh genotypes. Exemplary is the acknowledged symbiosis of mycorrhizae with plants (Evans 2001). However, under low N availability the cycling of N through the fungus to the plant is rather negligible for plant $\delta^{15}N$ (Högberg et al. 1999).

4.6 Conclusion

We studied the interlinkages of plant $\delta^{15}N$, BNI, microbial nitrification, N uptake and N leaching losses under controlled as well as under field conditions based on a selection of contrasting Bh genotypes. Our main conclusion is that high BNI activity decreases plant $\delta^{15}N$ of Bh. Thus, the ^{15}N natural abundance of grass tissue might be linked to BNI activity in soil, suppressing the growth and activity of bacterial and archaeal nitrifiers. As a result, this led to enhanced NH_4^+ uptake by Bh and reduced NO_3^- losses. This ecological concept is enhanced if: (i) BNI is expressed in soil due to long-term presence of Bh; (ii) there is a continuous substrate (NH_4^+) supply (mineralization, fertilization) so that the source N for plant uptake is never converted completely (into NO_3^-); and (iii) a significant amount of NO_3^- formed by

nitrification is leached of the rooting zone of the plants. Since Bh can take up both N forms (Castilla & Jackson 1991), the cumulative ^{15}N shoot signal would be confounded in case of NH_4^+ and NO_3^- uptake without nitrate loss. Furthermore, other microbial enzymatic reactions should not mask the discrimination process by nitrifiers, such as: (i) volatilization (driven by high soil pH, heat, not incorporated N fertilizer); (ii) denitrification (anaerobic conditions, high C availability, NO_3^- substrate present). However, nitrate substrate left would even be higher enriched in ^{15}N; and (iii) uptake of ^{15}N depleted N derived by free living N fixing bacteria.

Our observations suggest that high BNI along with reduced microbial nitrification (one of the main reactions causing ^{15}N:^{14}N fractionation) and N leaching (enriching the remaining soil mineral N with ^{15}N) are reflected in low $\delta^{15}N$ leaf or shoot biomass signals under environments with high NO_3^- leaching potential. We also suggest that the method described here can serve as an indicator of the extent of NO_3^- leakiness for BNI field evaluations over the years, if combined with other BNI indicators like the abundance and activity of soil nitrifiers under the given conditions set out above.

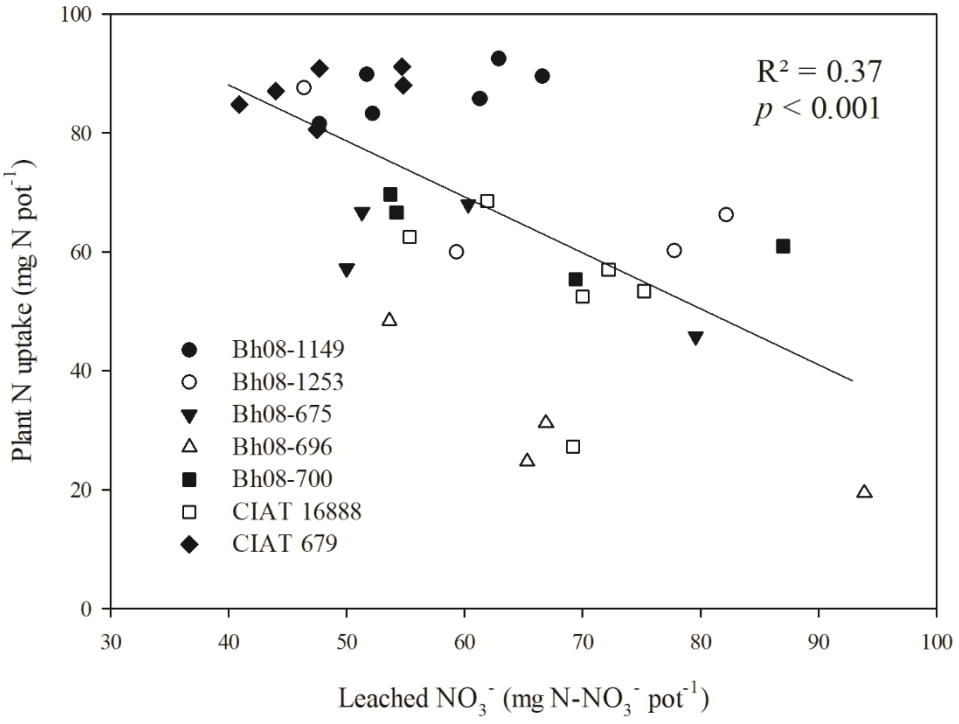
4.7 Acknowledgements

We would like to thank Marc-André Sparke for his cooperation during the sampling period in the greenhouse and in the field trials in Colombia. For the support with the IRMS we are grateful to Elke Dachtler, Carolin Röhl and Christian Brandt.

4.8 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.9 Supplementary material



Supplementary Figure 1. Linear regression of plant N uptake (mg N pot^{-1}) of *Brachiaria humidicola* (*Bh*) and cumulative leached NO_3^- (mg N pot^{-1}). The greenhouse study (Experiment 1) included 7 *Bh* genotypes and was established in August 2014. Plants were sampled at 42 days after fertilization (6 weeks after transplanting to experimental pots) with $(NH_4^+)_2SO_4$. Plant N uptake and leached NO_3^- are cumulative amounts determined at the date of harvest.

4.10 Author Contributions

HK wrote the manuscript and had the overall task to modify it according to suggestions and corrections of the co-authors. Furthermore, HK measured all samples with the IRMS that have been used for this study. KE installed the greenhouse trial (Experiment 1) and conducted the sampling. JN assisted in sampling of plant material (Experiment 3) and delivered further plant material from other experiments for pre-tests (data not shown). IR was the leading senior scientist of the BMZ project at CIAT Colombia. FR contributed to the scientific interpretation and the concept of the study. JA was the leading young professional of the BMZ project at CIAT Colombia and enabled HK the access to the field trial of Experiment 2 and 3 for sampling. DM had the responsibility of taking samples of Experiment 2. AA assisted in the incubation study of Experiment 3. GC was the leading senior scientist of this study. All authors contributed to manuscript revision, read and approved the submitted version.

4.11 Funding

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5. General Discussion

5.1 Does residual BNI improve nitrogen use efficiency of subsequent crops?

Bh pastures have been characterized by low soil NO_3^- levels and low nitrification rates (Sylvester-Bradley et al. 1988; Subbarao et al. 2009). However, the underlying reasons have been unknown for some time, until it could be demonstrated in studies under controlled environments that substances extracted from *Bh* (root and shoot) tissue or collected root exudates (Table 5.1) effectively slowed-down nitrification when applied to incubated soil or reduced the activity of nitrifying bacteria cultures in hydroponics (Subbarao et al. 2007b, 2008; Gopalakrishnan et al. 2009). BNI substances, that seemed to be actively released as brachialactone (Subbarao et al. 2009) might accumulate in soil due to continues release from *Bh* roots, indicated by increasing BNI over years in field and pot experiments (Nuñez et al. 2018). On the other hand, BNI effective free fatty acids and fatty acid esters detected in *Bh* shoots (Subbarao et al. 2008) as well as methyl-*p*-coumarate and methyl ferulate extracted from *Bh* roots (Gopalakrishnan et al. 2007) might be released during *Bh* biomass decomposition in the soil. It is likely that nitrification is inhibited for prolonged time due to an expected subsequent delivery of BNIs after *Bh* pasture conversion. Therefore, the residual BNI effect by *Bh* could play an important role in terms of N conservation of subsequent N fertilized cropping systems prone to N losses by nitrification (Karwat et al. 2017). Rotation of *Brachiaria* grass with crops, as e.g. maize, rice or soybean, is a known management tool in the Brazilian *Cerrados* to afford the N fertilizer costs for pasture regeneration (Kluthcouski et al. 2004; de Oliveira et al. 2004). The practice was established without the knowledge of a possible contribution of the residual BNI effect by *Brachiaria* grasses (Subbarao et al. 2006a, 2009) on enhanced N uptake (due to prolonged NH_4^+ presence by BNI) by subsequent crops (Muzilli et al. 2004; Lopes et al. 2004). The case study represented in this dissertation (Chapter 2) showed that the conversion of a 15-year-old *Bh* (cv. Tully) pasture into a maize crop system had a positive effect in terms of maize grain yields and plant total N uptake. However, reduced N fertilizer losses, or higher fertilizer N uptake by maize due to residual BNI could not be confirmed.

Table 5.1: BNI substances from *Brachiaria humidicola* (adapted and modified from: Coskun et al. 2017b)

Category	Compound	Source	Comments
Root exudate	Brachialactone	*Bh*	Blocks AMO and HAO
Tissue extract	Methyl ferulate	*Bh* roots	Released via root decomposition
	Methyl p-coumarate	*Bh* roots	Released via root decomposition
	Linoleic acid	*Bh* shoots	Blocks AMO and HAO; inhibits urease
	Linolenic acid	*Bh* shoots	Blocks AMO and HAO
	Methyl linoleate	*Bh* shoots	Most stable BNI in soil; inhibits urease

BNI substances released by *Bh* inhibit nitrification and consequently slow down microbial conversion of NH_4^+ to NO_3^-. Therefore, residual BNI due to substance delivery from decomposing *Bh* biomass in soil is expected to restrict nitrifiers' activity and consequently prolong N being available in the form of NH_4^+, which is less prone to be lost by leaching compared to highly soil mobile NO_3^-. Therefore, remaining BNI substances retaining nitrification might increase agronomic NUE of crop plants by a reduction of N losses from NH_4^+ derived by fertilization of from organic matter mineralization (Coskun et al. 2017a). In the case study presented in this dissertation (Chapter 2) higher maize grain yields were determined in the experimental field site (Bh) of a converted, previous *Bh* pasture compared to maize yields of the control field (M) where maize mono-cropping has been conducted for the previous 15 years. Furthermore, total N uptake was higher as well as agronomic N use efficiency (ANUE) in maize of the Bh field site. However, labeling of NH_4^+ fertilizer with ^{15}N allowed to distinguish of maize fertilizer N uptake and soil derived N uptake. It could be demonstrated that mineral N from organic matter turnover (e.g. in previous *Bh* plots, first cropping season) was of major importance compared to N from mineral fertilizer application in terms of maize N nutrition. ^{15}N recovery of ^{15}N labeled fertilizer N in the soil profile indicated in this respect that high amounts of applied N were accumulated in the soil profile after harvest, especially in the topsoil (0-10 cm), indicating a strong microbial NH_4^+ immobilization in the Bh field. Furthermore, information on ^{15}N recovery in maize plants and the soil profile allowed to calculate apparent losses of applied ^{15}N. Reduced losses of applied NH_4^+ fertilizer due

to BNI is one of the central promises of BNI (Subbarao et al. 2013b). However, in the presented case study this hypothesis could not be confirmed in terms of the residual BNI effect of *Bh* on a N fertilized maize-cropping system.

Other factors, besides from a potential residual BNI effect, need to be taken into account that likely contributed to boost maize yields and N uptake after *Bh* incorporation in the field: the higher organic matter of the Bh site compared to the M site. Furthermore, another factor that is expected to has interfered with nitrification was the observed N immobilization. Consequently, substrate (NH_4^+) shortage for nitrifiers caused by high NH_4^+ uptake by heterotrophic microbes was likely to have occurred due to long-term accumulation of *Bh* root biomass with a high C:N ratio (Urquiaga et al. 1998). Both mentioned factors might have an altered confounded effect with residual BNI on nitrification activity and maize N nutrition. Soil incubations indicated that a significant residual BNI effect was present at the beginning of the first maize crop season, but a corresponding low nitrification could not be detected in incubated soil taken before the second cropping season. Consequently, it was concluded that a residual BNI effect was present in the first maize-cropping season in the previous, converted *Bh* pasture, but that a significant residual BNI effect was absent in the second season. Since more direct methods to measure BNI substances, such as brachialactone, in the soil have so far not been developed and without the information of the quantity, effectivity and persistence of these substances, more general conclusion in terms of the residual BNI effect could not be drawn in the presented study. However, studies on crop-pasture systems in the Colombian *Llanos*, e.g. rice planted after conversion of a *Brachiaria decumbens* (*Bd*) pasture compared to rice cultivation after conversion of a native savanna site (NS) recorded rice grain yields exceeding 3 t ha^{-1} for production on the previous *Bd* pasture, whereas rice yields following NS were below 2 t ha^{-1} (Sanz et al. 1994). Years later, various grasses and cereal plants were screened for BNI (Subbarao et al. 2007a). Among the 18 plant species, *Bd* showed the second highest total BNI release after *Bh*, and *Bd* had even higher specific BNI (per g root dry weight) then *Bh*. Consequently, it could be assumed that in the field experiment of Sanz et al. (1994) higher rice yields after *Bd* compared to rice after NS was also influenced positively by a residual BNI effect, however this factor has not been accounted for in this early work on crop-pasture rotations.

5.2 How long does the residual *Bh* BNI effect in a cropping systems last?

Persistence of a potential residual BNI effect might depend on factors as turnover of BNI substances from *Bh* subsequent indirect delivery of BNI active compounds from decaying *Bh* biomass in soils. Different substances were extracted from both, shoots and roots of *Bh* and have shown to impede nitrification in incubated soil or inhibit directly the activity of nitrifying bacteria as, e.g. *Nitrosomonas* sp. (Subbarao et al. 2008, 2009; Gopalakrishnan et al. 2009). The BNI persistence by different substances (extracts from *Bh* shoots and roots or *Bh* root exudates) varied in studies from 30-120 days and depended on the soil type, water content, as well as on the applied amount of a respective BNI substance (Ipinmoroti et al. 2008; Gopalakrishnan et al. 2007, 2009). Although not all substances have been identified and isolated, there is strong evidence that compounds differ in biochemical terms as e.g. water solubility and ion charge patterns (Subbarao et al. 2007b; Ipinmoroti et al. 2008). Anionic or cationic charge of a BNI substance would decide over its ability to be adsorbed to soil particles. However, it is further unknown if BNI substance lose their effectiveness when attached to the soil matrix or if retention time would be positively affected. Furthermore, the hydrophobic and hydrophilic nature of a substance might determine the tendency of it to migrate to deeper soil layers, or even be leached from the soil matrix. The case study of this dissertation (Chapter 2) could determine that a residual BNI effect in incubated soil from the Bh site was still present 6 weeks after pasture conversion. However, a significant residual BNI efficacy could not be confirmed before the second maize crop season after *Bh* pasture conversion. Different results in terms of presence and persistence of the residual BNI effect appear from a comparable approach that investigated the residual BNI effect of a 10-year-old *Bh* pasture converted to maize cropping (Arango et al., unpublished). Soil Incubation results from this study suggested that the residual BNI effect was not present in the first year but rather in the second and third year after *Bh* pasture conversion. It is assumed that the residual BNI effect persisted longer in the study of Arango et al. (unpublished) compared to the study in this dissertation (Chapter 2) due to 3 major reasons: slower *Bh* plant residue turnover in the soil, and therefore delayed BNI substances release from *Bh* biomass (i); lower rainfall and consequently expected lower BNI substance losses due to leaching (ii); and higher BNI substance fixation in the previous *Bh* pasture due to high clay contents in the sub-soil. Summarizing the current findings on residual BNI persistence the operating point is the interaction

of the potential BNI substance amount (derived before pasture conversion and released during *Bh* composition) and the biochemical nature of the respective BNI substances with edaphic factors as soil texture, drainage and charge of soil particles are suggested to alter the persistence of a residual BNI effect.

5.3 Is BNI a pathway to a climate smart agriculture?

NH_4^+ based fertilizer is rapidly nitrified, specifically when plants are still young and the small NH_4^+ uptake is low due to small roots systems (Schröder et al. 2000). NO_3^- leaching and N_2O emissions represent major pathways of N losses from agricultural systems and contributing to the low nitrogen use efficiency (NUE) in N fertilized cropping systems under high rainfall conditions of the tropics (Francis et al. 1993; Hilton et al. 1994; Raun & Johnson 1999). Whereas NO_3^- itself is one of the nutrients that pollutes water bodies, N_2O emissions contributes negatively to climate change due to its high GHG potential (Baligar et al. 2001). Since BNI activity by plants potentially reduces NO_3^- formation and therefore indirectly N_2O emission it could be speculated that BNI could be used to make agroecosystems more environmental friendly (Subbarao et al. 2013b; Coskun et al. 2017a). Especially the indirect reducing effect of N_2O formation by induced NO_3^- shortage fostered by BNI is a promising point among BNI researchers (Rao et al. 2014). Punctual losses of N in tropical rangeland systems can occur by high amounts of urine by cattle (Ferreira et al. 1995). If *Brachiaria* grasses are used in pasture systems, it could result in reduced N_2O emissions as reported by Byrnes et al. (2017) and potentially also reduce N losses via NO_3^- leaching. The field study of this dissertation (Chapter 2) aimed to investigate for the first time the residual BNI effect on N_2O emissions. Significant reduced N_2O emission in the maize system established on the previous *Bh* pasture site could not be confirmed. Generally, the measured N_2O values were low for an intensive N fertilized maize systems. For instance, emissions for the M site were even lower than reported for a *Bh* CIAT 679 pasture (Subbarao et al. 2009). However, certain factors might have altered the N_2O emissions and masked a possible potential N_2O reduction effect by residual BNI. Denitrification depends on soil water saturation levels and linked O_2 levels, NO_3^- availability for denitrifiers, and availability of C for heterotrophic denitrifying microbes (Firestone & Davidson 1989). The expected large accumulated C in soil by *Bh* root biomass (Fisher et al. 1994; Amézquita et al. 2004), indicated by the soil organic matter levels in the Bh field site of the study (Chapter 2) might have spurred

denitrification. The lower organic matter of the soil from the control field M complicated a direct comparison of measured N_2O emissions between the fields. Furthermore, the synthetic nitrification inhibitor showed no depressing effect in terms of N_2O formation and could consequently not be used as a reliable control in the field study. According to the study of Chapter 2, it was suggested that the residual BNI effect on N_2O emissions does not necessarily reduce N_2O emissions as the *in situ* pasture BNI effect (Subbarao et al. 2009; Byrnes et al. 2017).

Both microbial reactions, nitrification and denitrification, contribute to N_2O emissions. Molecular methods could be used to detect contribution of either both groups. This is crucial since BNI primarily inhibits nitrification and only indirectly denitrification, by reducing the substrate (NO_3^-) for denitrifying microbes. For instance, DNA extraction from soil with subsequent quantification of nitrifiers genes (*amo*A) or denitrifiers genes (*nos*Z) combined with N_20 measurements (Henderson et al. 2010) would give a rather complete picture to clarify both, the effect of the actual and the effect of residual BNI on N_2O emissions.

5.4 $\delta^{15}N$ in plant tissue as proxy of BNI induced altered N losses

The negative economic and environmental factors due to NO_3^- leaching from agricultural land have been described by many authors (Coskun et al. 2017a). One of the main hypothesis related to BNI is that it could be used as tool to reduce NO_3^- leaching in agroecosystems (Di et al. 2016; Subbarao et al. 2017). Evidence of low soil NO_3^- levels linked to BNI activity by *Bh* appear in many studies (Sylvester-Bradley et al. 1988; Ishikawa et al. 2003; Subbarao et al. 2009). Furthermore, a reduction of nitrification itself by application of *Bh* root exudates could already be revealed (Gopalakrishnan et al. 2009). However, the theoretically linked reduced NO_3^- leaching due to actual or residual BNI has not yet been demonstrated (Chapter 3). In regard of this underlying idea, it is expected that BNI extends the retention time of N in the form of the less mobile NH_4^+. It is consequently speculated that plant N uptake is enhanced and N losses (in form of leached NO_3^- derived by nitrification and N_2O by reduction of NO_3^- due to denitrification) is reduced due to effective BNI (Subbarao et al. 2006a, b, c, 2013b).

Quantifying NO_3^- leaching losses in the field is labor intensive and prone to be biased my many factors. For instance, resin cores installed under an undisturbed soil profile to capture total leached NO_3^- over

certain periods and extraction of NO_3^- from the resin could give certain indices about cumulative NO_3^- leaching (Bischoff 2007). Whereas the use of NO_3^- traps with cationic resins is feasible in pot studies (Chapter 4) certain difficulties in the field complicate the use of this technique. For instance, biofilms could consume captured NO_3^- from the resin. Furthermore, water logging might cause denitrification and consequently provoke losses of NO_3^- from the trap in the form of N_2O, or lateral water flow biasing the trapped amount of NO_3^-. Another issue is the destruction of the soil profile during installation of the anion trap from a side tunnel in the field. The mentioned complications could have discouraged BNI research to examine if the hypothesis of reduced NO_3^- leaching due to BNI holds true. Consequently, development of simple indicators for NO_3^- leaching could contribute to check the mentioned hypothesis. Furthermore, enhanced plant N uptake linked to BNI is another promising point (Subbarao et al. 2012). However, this could only be shown for the residual BNI effect on maize (Karwat et al. 2017) and for BNI on NUE (NH_4^+ uptake by roots from nutrient solutions) in certain rice genotypes (Sun et al. 2016). Consequently, the expected positive impact of BNI on enhanced N uptake is still pending.

To illuminate the positive effects of both, reduced NO_3^- leaching and higher N uptake, a new method was tested, as described in Chapter 4. Briefly, it could be demonstrated in a greenhouse study, that the natural abundance of ^{15}N in *Bh* grass is certainly linked to N uptake and N leaching loss of NO_3^-. Furthermore, reduced NO_3^- formation in incubated soil from *Bh* field plots was linked to respective lower $\delta^{15}N$ values of *Bh*. Consequently, it was concluded, that plant $\delta^{15}N$ could serve to indicate differences in total NO_3^- leaching losses between *Bh* genotypes under the same experimental conditions. $\delta^{15}N$ of plants are influenced by physiological and biochemical processes of the N cycle (Högberg 1997; Robinson 2001) where nitrification is one of the main enzymatic reactions showing strong ^{15}N:^{14}N fractionation.

However, a major drawback of the ^{15}N natural abundance method is, that also other processes such as denitrification and NH_3 volatilization potentially alter the ^{15}N signature of soil mineral N pools. Possible isotopic effects due to denitrification (Mariotti et al. 1988) need to be considered under anaerobic conditions, e.g. in deeper soil layers were NO_3^- would consequently become enriched due to gaseous losses of ^{15}N depleted N_2O. If plant $\delta^{15}N$ is altered by denitrification depends on the spatial availability

of the remaining NO_3^- for the plant. The other important process, NH_3 volatilization, is driven by high temperatures and high soil pH, and furthermore is accelerated when NH_4^+ based fertilizers are not incorporated (Harrison & Webb 2001). Consequently, remaining mineral N would become ^{15}N enriched due to losses of ^{15}N depleted NH_3 (Ariz et al. 2011). Potential uptake of this relatively ^{15}N enriched N would increase plant $\delta^{15}N$. Eventually, the ^{15}N plant signature could not be related to nitrification if NH_3 volatilizations occurs. However, the presented experiments in the framework of this dissertation have been conducted with low pH soil. Consequently, NH_3 volatilization is supposed to be a negligible process in terms of ^{15}N: ^{14}N fractionation in our studies.

In terms of N isotopic fractionation by denitrification and NH_3 volatilization that potentially could mask the actual isotopic fractionation effect by nitrification, some theoretical considerations could be made to avoid complications. Consequently, nitrification is the main process responsible for ^{15}N:^{14}N fractionation in soils with pH < 7. On the other hand, if pH > 7, volatilization of NH_3 might also influence the ^{15}N signature of the remaining NH_4^+. To avoid complications with NH_3 volatilization, applied N should be quickly incorporated (e.g. applied as solution), especially under high temperatures. To minimize the influence of denitrification on ^{15}N abundance of NH_4^+, anaerobic conditions (e.g. over long periods) should be avoided. Consequently, the presented research on ^{15}N natural abundance in *Bh* shoot tissue has been conducted under low pH soil aerobic conditions.

N isotopic fractionation could also occur during N transport within plants, and therefore different $\delta^{15}N$ values could be observed in different plant tissues. However, a significant correlation between $\delta^{15}N$ in main leaves and stolon leaves of the same *Bh* plant was measured by the author (data not shown). Consequently, it appeared that either the comparison of stolon or main leaves among different treatments (e.g. *Bh* genotypes) could deliver the same information. Generally, each transformation step of mineral N into finally assimilated organic N in plants discriminates against ^{15}N. This within-plant fractionation is especially crucial (for grasses as *Bh*) between root and shoot. This occurs since mineral N assimilation might take part in a different tissue as metabolized products (amino acids) are stored. For instance, NO_3^- first has to be reduced in two steps (by nitrate reductase and nitrite reductase) to NH_3 before it can be assimilated into glutamate and glutamine respectively by glutamine synthetase and glutamate synthase.

Nitrate reductase has been shown to cause ^{15}N:^{14}N fractionation when substrate (NO_3^-) to enzyme (NR) ratio was high (e.g. under high NO_3^- availability) (Mariotti et al. 1982). This enzymatic step is of importance when the site of NO_3^- reduction and NH_3 assimilation in the plant is not identical. It could be demonstrated for *Bh*, that NO_3^- reduction takes mainly part in the shoot (Chapter 4). NH_3 assimilation takes normally part in the roots and therefore high ^{15}N discrimination between shoot and root in *Bh* under high NO_3^- availability is suggested. Evidence for this appeared in an experiment related to Chapter 4 (Figure 5.2.1), where significant different ^{15}N values in root and shoots of *Bh* were observed under high NO_3^- nutrition. Furthermore, the observed $\delta^{15}N$ values in the two tissues of *Bh* indicated that ^{15}N discrimination between shoot and root is primarily driven by N availability and not by the N form (Fig 5.2.2). However, the information derived from this study is limited since plants were harvested already 72 hours after N addition. Therefore, a possible effect on root and shoot $\delta^{15}N$ due to expected decreasing substrate-to-enzyme-ratio over time is missing and straightforward conclusions could not be drawn.

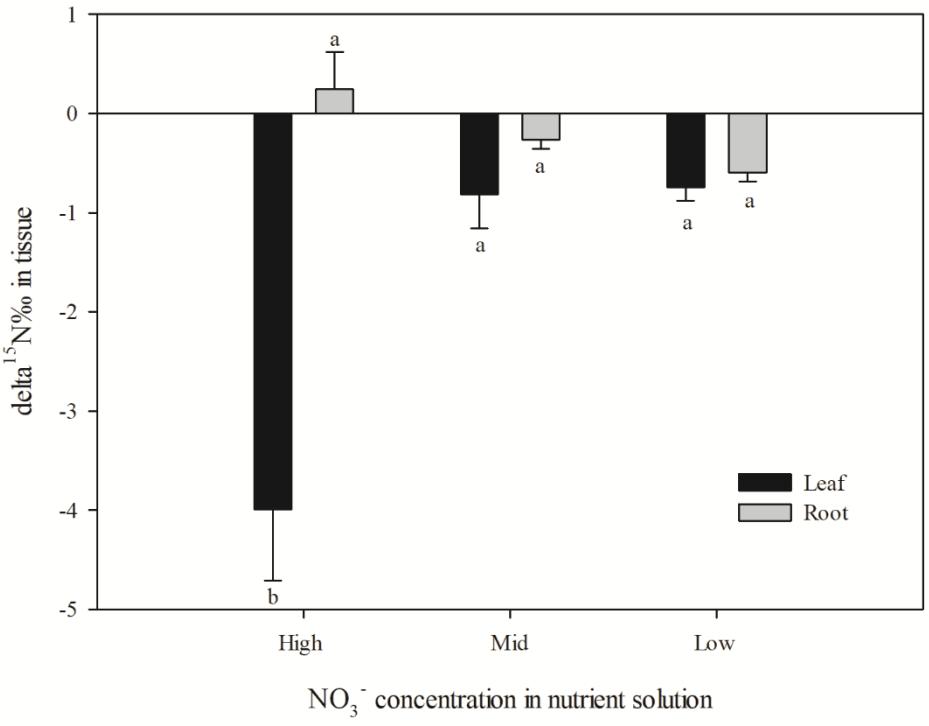

Fig 5.2.1 $^{15}N‰$ values in second last developed leaves and cumulative root samples of *Bh* (CIAT 679) corrected by the $^{15}N‰$ values of the respective N fertilizer signature. *Bh* has been grown in a sand-perlite mix, N has been fertilized in form of NO_3^- as nutrient solution. Treatments differ in terms of N-NO_3^- concentration in the applied nutrient solution. Respective $\delta^{15}N‰$ signal of the fertilizer: NO_3^- = 17.08. Bars with same letters are not significantly different.

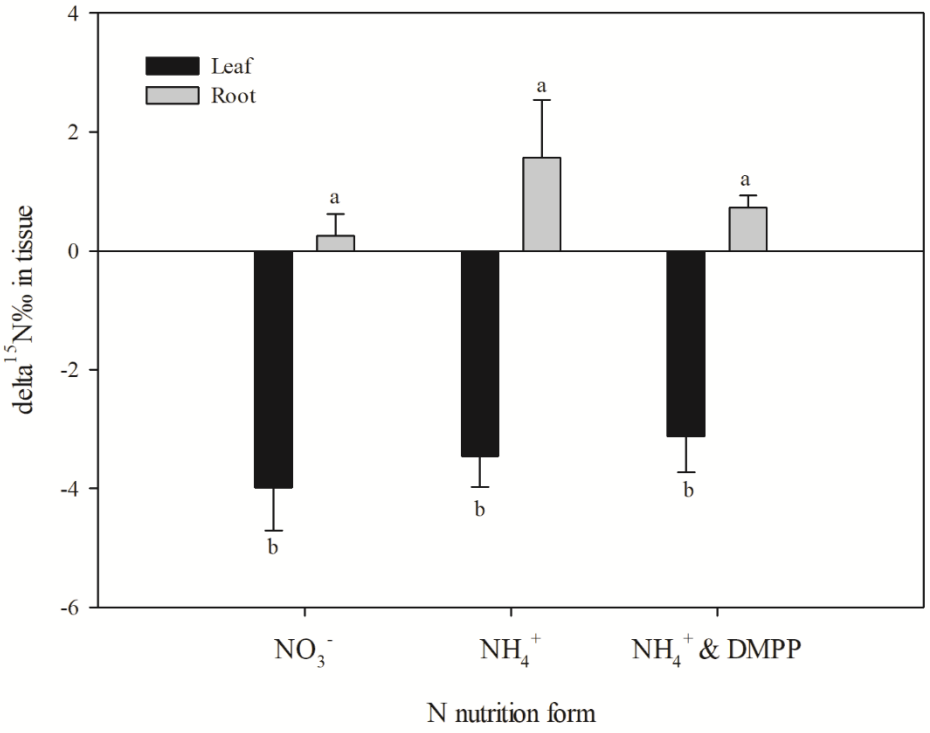

Fig 5.2.2 ^{15}N‰ values in second last developed leaves and cumulative root samples of *Bh* (CIAT 679) corrected by the ^{15}N‰ values of the respective N fertilizer signature. *Bh* has been grown in a sand-perlite mix, N has been fertilized in form of either NO_3^-, NH_4^+ or NH_4^++DMPP as nutrient solution. Respective δ^{15}N‰ signal of the fertilizer: NO_3^- = 17.08; NH_4^+ = -0.11; NH_4^+ & DMPP = -0.205. Bars with same letters are not significantly different.

Further complications of the ^{15}N natural abundance method in terms of indicating lower NO_3^- losses due to BNI theoretically appear for low BNI *Bh* genotypes or young *Bh* plants. The ^{15}N signature of the plant could be confounded under substantial nitrification activity by uptake of both, NH_4^+ and NO_3^- with respective different δ^{15}N values. Since it appeared that *Bh* is highly efficient in the uptake of both N forms (Castillo & Jackson, 1991), a mix of NH_4^+ and NO_3^- nutrition might display a major error source. To interpret the δ^{15}N values appropriately and link them to BNI reduced NO_3^- leaching, the experiment needs to be designed in a way that nitrification derived ^{15}N depleted NO_3^- is rather leached and that the grass cannot take it up. The complication appeared in terms of the fact that a significant BNI effect could not be confirmed in Chapter 4.3.1, since plants were simply too young or root density was too low to manifest a significant depressing effect on nitrification. However, in a later stage of the greenhouse trial sampling of *Bh* grass with a higher root density (when a possible BNI effect would have been more likely) leaching of NO_3^- could have been biased by high NO_3^- uptake by the larger rooting system. The

dilemma of insufficient rooting (BNI not dominant over gross nitrification in the pot) and excessive rooting (large plant uptake of NO_3^- and consequently biased trapped NO_3^- for quantification) is evident for all pot studies. However, in the field, soil depth is not limited and NO_3^- leaching is more likely as compared to depth limited systems, as e.g. pot studies. Therefore, using $\delta^{15}N$ values of *Bh* plants as indicator of long-term NO_3^- losses and BNI efficiency might rather be an appropriate tool for field studies.

A general question is when plant samples should be taken after N addition to reflect BNI in $\delta^{15}N$ shoot samples. NH_4^+ has been identified to trigger BNI substance release and is also necessary as substrate for nitrifiers. However strong discrimination against ^{15}N during uptake and assimilation has been observed by the author in some greenhouse and field studies, when $\delta^{15}N$ was monitored in sampled leaves (Egenolf et al. 2015). Consequently, it is suggested to wait until substrate-to-enzyme-ratio is more balanced, sometime after N application. On the other hand, ^{15}N signature of NO_3^- will increase when substrate is fully converted (Robinson et al. 2001). Complete conversion of substrate could occur e.g., in experiments with closed systems (e.g. nutrient solutions, pot studies with sand). At the point of full substrate conversion to product, the plant signature would theoretically be close or equal to the original substrate signature (Yoneyama et al. 2001) and isotopic ^{15}N:^{14}N discrimination by nitrification is masked. Although, the product will always be relatively ^{15}N reduced compared to substrate under constant subsequent supply or infinite substrate flow (Evans 2001), as for instance, in the field or pot studies due to soil buffer capacity.

From an agroecological point of view, enhanced N uptake and reduced NO_3^- losses from *Bh* pastures might be of minor importance. However, there is insufficient information concerning differences of NO_3^- leaching losses of N fertilized (enhanced or well-managed) *Bh* pastures compared to non N fertilized *Bh* pastures. Overall, *Bh* is very efficient in N uptake, has no preference for one mineral N form and a large root system. Even a low BNI type with an extensive root system could catch nitrification derived NO_3^- during leaching also from deeper layers, even under submergence. Therefore, speculating that BNI by *Bh* might play a major role for N uptake of the grass per se is doubtful, since even low BNI

accessions (e.g. CIAT 26146) have shown high N uptake and high biomass production in field and pot studies (Moreta et al., unpublished).

5.5 Nitrate reductase activity as proxy to identify differences in terms of BNI after N addition

Discrepancies between BNI potentials of *Bh* genotypes assessed by the bioassay method (Subbarao et al. 2006b, 2009) and the BNI effect observed in incubated soil are known from other studies (Subbarao et al. 2006a; Nuñez et al. 2018). Furthermore, these methodological approaches to characterize BNI by *Bh* are either destructive or conducted under artificial environments, that hinder drawing of straightforward predictions on the real-time BNI performance. Furthermore, potential fluctuations of BNI expression of *Bh* in the field with these methods could only be observed with labor-intensive time-shifted sampling. Additionally, the soil incubation might confound a BNI effect by soil attached BNI substances and an N immobilization depressing effect on nitrification (Chapter 2). In Chapter 3 of this dissertation an approach was introduced to identify contrasting *in vivo* BNI expression under field conditions, with a minimal disturbance of the soil-plant system. This new proxy for BNI enables a real-time BNI assessment based on a well-adapted enzymatic assay known for decades already (Jaworski 1971). Leaf samples simply serve as the target tissue and the measured activity of the nitrate reductase activity (NRA) has been shown to be linked with BNI potentials (determined by the soil incubation method), soil nitrification indicators (soil NO_3^-) and NO_3^- plant status (NO_3^- in stem sap). The main advantage of the NRA assay compared to bioassays in hydroponics is, that it reflects the effect of BNI on the complete nitrifier populations in the soil, and not just on a few selected bacterial strains (Subbarao et al. 2006a; O'Sullivan et al. 2016, 2017). An advantage of the NRA method compared to soil incubations is that it does not alter soil microbial dynamics per se, since the root-soil system remains untouched. Enzymatic assays measuring NRA have been used by others to indicate potential NO_3^- assimilation (Barford & Lajtha 1992), or to subscript contrasting nitrification activities in tropical grassland savannas (Lata et al. 1999). The underlying theory is that BNI reduces NO_3^- derived by nitrification, and that in this regard less NO_3^- is taken up by the plants. Less NO_3^- uptake and assimilation is consequently reflected in respective low leaf NRA (Högberg et al. 1986; Högbom et al. 2002). The presented study (Chapter 3) not only contributes to BNI research with a new potential *in vivo* BNI proxy,

but it also delivered some valuable insights into the N assimilation pattern of *Bh*. One is, that *Bh* reduces NO_3^- mainly in shoots and not in roots. Higher *in vivo* NRA in sampled leaves was clearly related to higher NO_3^- soil and plant status (excess NO_3^- stored in vacuoles [Tegeder & Masclaux-Daubresse, 2017]) of the pre-sampled *Bh* grass. Contrasting *Bh* genotypes did not show significant different NRA per se under efficient synthetic nitrification inhibition (NH_4^++DMPP) or pure NO_3^- nutrition. However, different NRA patterns among the genotypes were finally observed under NH_4^+ application, and this was clearly related to contrasting soil nitrification patterns that consequently was due to different BNI patters of the *Bh* genotypes.

However, it could be speculated about certain limitations of the NRA method for further BNI detection. From the conducted research the presented NRA method could not detect expected small BNI differences between certain *Bh* genotypes. For instance, *Bh* CIAT 679 was classified as medium-BNI and CIAT 16888 as high-BNI (Subbarao et al. 2007a, 2009). However, in the case study of Chapter 3, classification with the NRA and the soil incubation method indicated that the two CIAT accessions did not differ in terms of BNI. However, the observed similar BNI expression of the 2 CIAT accession is in line with the observation by Nuñez et al. (2018). Therefore, either the NRA method and the methodological approaches by Nuñez et al. (2018) are lacking the necessary sensitivity to capture BNI differences of the two CIAT accessions, or it should be considered, that the used bioassay method by Subbarao et al. under- or overestimates the actual BNI of certain genotypes. Eventually, *Bh* genotypes have expressed BNI differently in the mentioned experiments of the different authors. This is evident from additional NRA data related to Chapter 3 (Table 5.3.1), which could be related to soil NO_3^- indicators. Therefore, it is suggested that the NRA assay was able to capture this genotypic BNI inconsistency. However, the particular factors that restricted the expression of the high BNI potential of the CIAT accessions in the greenhouse study could not be identified.

Table 5.3.1 Nitrate reductase activity (NRA in µmol NO_2^- g FW^{-1} h^{-1}) measured in leaves of different *Bh* genotypes sampled 9 days after N fertilization from a greenhouse and 8 days after N fertilization from a field experiment (cp. Chapter 3). SE = standard error of the mean. Different letters indicate significant differences among the means per row at $\alpha = 0.05$.

Bh genotype	Experiment 2 (Greenhouse)		Experiment 3 (Field)	
	NRA day 9 (µmol NO_2^- g FW^{-1} h^{-1})	SE	NRA day 8 (µmol NO_2^- g FW^{-1} h^{-1})	SE
CIAT 679	1.00^{b}	0.18	0.80^{cd}	0.28
CIAT 16888	1.31^{a}	0.11	0.40^{d}	0.07
Bh08-1149	0.60^{c}	0.11	1.11^{ab}	0.30
Bh08-0700	0.48^{c}	0.06	0.98^{ab}	0.22
Bh08-0675	0.30^{c}	0.03	1.38^{a}	0.18

Other morphological differences among contrasting BNI *Bh* genotypes e.g. different root systems, might also play a role in terms of secondary effects on nitrification activity. For instance, a genotype with a large root system might contribute to more organic C supply (in form of root residues) to the rhizosphere and foster N immobilization, which might negatively affect nitrification. The difference between the direct effect of BNI substances and a potential secondary effect on nitrification by root biomass could unlikely be detected by the NRA method. Plant NO_3^- uptake rate is also rate limited by the rooting size, e.g. if there are severe differences among the root systems among *Bh* genotypes. Consequently, this could also influence NO_3^- uptake amounts and ultimately *in vivo* NRA. Furthermore, enzyme activity in plants is generally dependent on the energy status of the plant, e.g. on photosynthesis. Two important environmental factors are primarily important for photosynthetic activity and linked metabolism such as enzyme synthesis. NRA is influenced by temperature (Fig 5.3.1) and radiation, and consequently comparisons of absolute NRA values can only be conducted among *Bh* candidates under similar environmental conditions. In case the method is used to screen for *in vivo* BNI in large field sites, factors affecting NRA, such as light and temperature, should be taken into account. For instance, trees or hedges might provoke heterogeneous shading of the trial and could therefore cause a decreasing effect on the

enzyme activity. However, it is possible to compensate for natural fluctuations due to heterogeneity, as demonstrated in the field study of Chapter 3: absolute NRA was corrected by gross-NRA (subtraction of the NRA under -N conditions from NRA under +N treatment) which results in the net effect of the N fertilizer treatment on NRA.

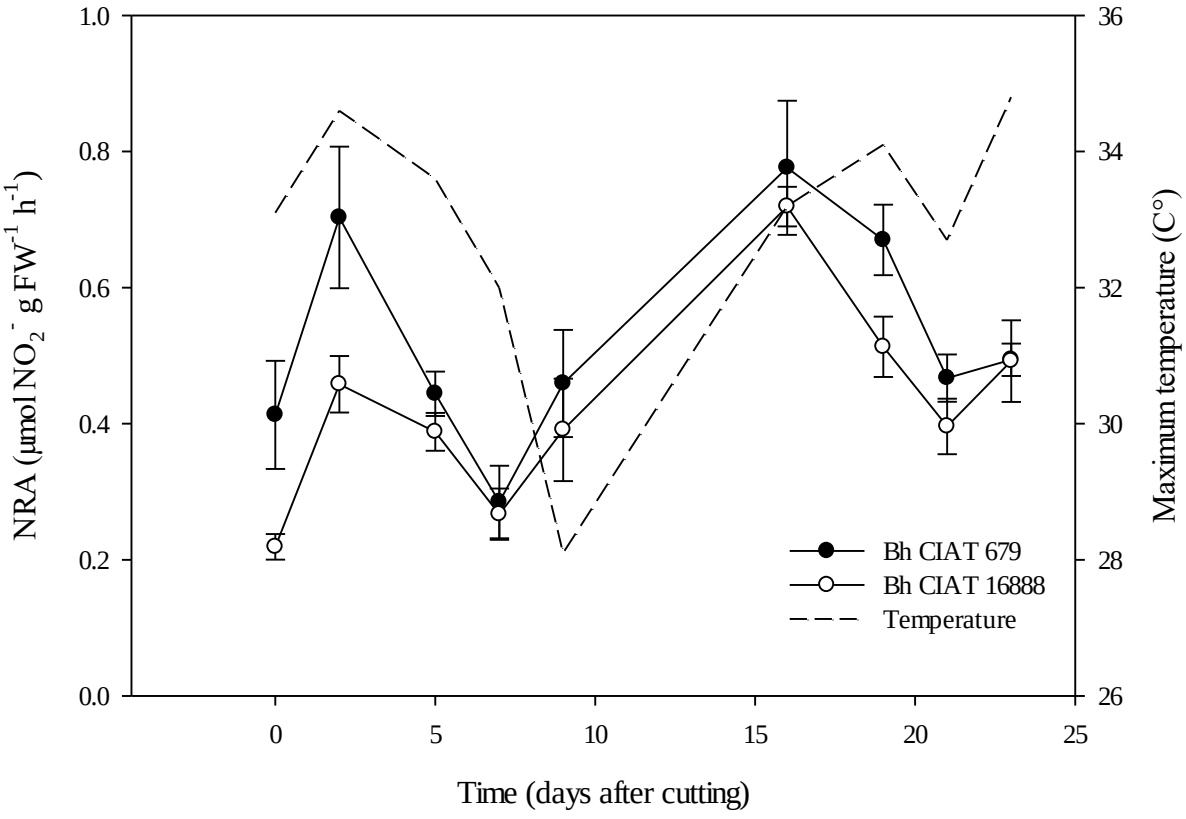

Fig 5.3.1 Nitrate reductase activity (NRA) in leaves of two *Brachiaria humidicola* (*Bh*) accession (CIAT 679 and CIAT 16888) and daily maximum temperature (in °C). Leaves were sampled frequently for NRA determination in July 2015 after cutting back the grass from a field site at CIAT, Colombia. The field trial was established on August 2004, on a Vertisol, pH 7.4. Data source: Sparke et al., 2016.

The NRA method depends on a representative uptake of nitrification derived NO_3^- to straightforward reflect soil BNI effects in the activity of leaf NR. Strong NO_3^- leaching could therefore be a potential biasing factor. To avoid this, the field study of Chapter 3 has been conducted at the beginning of the dry season to avoid a major bias by high NO_3^- leaching, that occurs mainly in the rainy season. On the other hand, plants enhance rooting under lower water availability and could enhance N uptake compared to

the dry season. Consequently, it should be tested if the NRA method would also be a suitable method in seasons or environments with high precipitation intensity, since this has so far not been tested.

For *Bh* no preference for either NO_3^- or NH_4^+ could not be detected (Castilla & Jackson 1991) but in terms of suitability of the NRA assay to screen for BNI in other plants, a potential N form preference need to be taken into account. If there is a preference of a plant species for NO_3^-, e.g. as for *Sorghum bicolor* (Li et al. 2013), it could still be speculated that this would not influence the uptake of NO_3^- per se, since the rate limiting step is NO_3^- availability in the soil. The latter depends mainly on the nitrification activity which is dominated by BNI. Therefore, successful BNI *in vivo* characterization of plants with NO_3^- preference should be tested. In contrast, NH_4^+ preference might constitute a potential bias of the NRA. A plant privileging NH_4^+ would only under NH_4^+ shortage feed on NO_3^- and therefore NRA would not indicate soil NO_3^- availability. It could therefore be hypothesized that the NRA assay might not be a suitable BNI screening tool for e.g. *Oryza sativa* (Li et al. 2013) which prefers NH_4^+ over NO_3^-. For further testing, it is suggested to determine the tissue where NR is primarily active. For instance, for *Zea mays* NRA has been shown to be 7 times higher in leaves as in roots (Murphy & Lewis 1978), and it would therefore be suggested to use shoot (leaves or stems) as target tissue.

5.6 Alternative methods to investigate BNI applicability and accuracy

Overall alternative methods for BNI detection and characterization with a minimum of disturbance of the plant-soil-microbe-system would be beneficial to capture fluctuations, triggers and impact of BNI efflux. Some of the methods discussed in this dissertation could be further developed.

The observation of mineral N in the soil incubation studies used in the case studies of this dissertation could not distinguish between the primary effect of BNI substances on nitrification and a secondary effect of fine roots as C source increasing NH_4^+ immobilization thereby constraining nitrification. This is a known effect as for instance it was shown that nitrification in incubated soil was inhibited by simply adding glucose (as C source treatment) that increased N uptake by heterotrophs (Nardi et al. 2013). The use of ^{15}N labeled NH_4^+ and its subsequent detection in microbial organic N would be an option to get insights into gross mineralization rates (Miranda et al. 1994; Urquiaga et al. 1998) while the use of $^{15}NO_3^-$ could inform about gross nitrification. Furthermore, soil N dynamics are altered by drying and

crushing and mixing the soil (Schimel et al. 1989). Consequently, combined with ^{15}N labeled N, the use of intact soil cores should be contemplable (Davidson et al. 1991) and might deliver further insights into N dynamics altered by BNI.

Another method that has shown potential for further testing was the measurement of taken up pre-assimilated NO_3^- (Chapter 3) in *Bh* stems. Prior to its reduction and assimilation NO_3^- is generally accumulated in vacuoles of roots and shoots of plants (Li et al. 2013; Tegeder & Masclaux-Daubresse 2017). In the presented case study (Chapter 3) NO_3^- in stem sap was accomplished by squeezing out stem samples and followed by NO_3^- quantification with test stripes. The detected NO_3^- levels in stems of contrasting *Bh* genotypes were significantly linked to soil NO_3^- indicators and the NRA of leaves samples. It is therefore assumed that there is an inverse relationship between BNI and NO_3^- in tissue contents. However, the method needs further standardization in terms of the target tissue and the representative amount that is sampled. The potential advantages would be the easy handling and relatively cheap equipment. Furthermore, the method is also non-invasive in terms of the plant-soil system and would allow in-field BNI characterization.

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Summary

Nitrogen (N) losses from agroecosystems harm the environment via increased nitrate (NO_3^-) amounts in water-bodies and nitrous oxide (N_2O) emissions to the atmosphere. NO_3^- is the product of the soil metabolic microbial process nitrification. Bacteria and archaea oxidize ammonium (NH_4^+) to NO_3^- under aerobic conditions. Furthermore, under mainly anaerobic conditions, microbial denitrification reduces NO_3^- to gaseous N forms. In particular, the herby produced potent greenhouse gas nitrous oxide (N_2O) negatively affects the atmosphere. Synthetic nitrification inhibitors have been shown to lose their efficacy under humid tropical conditions shortly after application and are furthermore too costly for smallholders in the tropics. The tropical forage grass *Brachiaria humidicola* (Rendle) Schweick (Bh) has been shown to reduce soil microbial nitrification via root derived substances. Therefore, biological nitrification inhibition (BNI) by Bh might contribute to reduction of N losses from agroecosystems.

Whereas N losses from well managed tropical pasture systems are expected to be rather low, crops cultivated under high N fertilization are characterized by high N losses and low N use efficiency. Rotation of *Brachiaria* pastures with crops such as rice, maize or soybean are common. However, the role of a potential residual BNI by Bh on a subsequent (non-BNI) crop system has so far not been investigated. Genotypic differences in terms of BNI within Bh have been reported. Furthermore, there is evidence that higher BNI potentials of recently developed Bh hybrids exceed the BNI effect of Bh standard cultivars. However, BNI potentials evaluated by hydroponic based methods of certain Bh genotypes could not consequently be confirmed in the field. The development of new BNI indicators for use under field conditions could contribute to close the gap between assessed BNI potentials determined under controlled conditions and BNI efficiency in the field. BNI might reduce nitrification derived NO_3^- formation and could consequently reduce NO_3^- leaching losses. However, reduced NO_3^- leaching by effective BNI has not been demonstrated yet.

The present doctoral thesis aimed at assessing the potential of the actual BNI by Bh, as well as the residual BNI effect with new developed methodologies. The overall research was based on the following major objectives: (1) characterization of the residual BNI effect by Bh on recovery of N by subsequent cropped maize (*Zea mays* L.) under different N fertilization rates; (2) investigate if low enzymatic nitrate

reductase activity (NRA) in leaves of Bh is linked to reduced NO_3^- nutrition by effective BNI; (3) identify a possible link between plant $\delta^{15}N$ of Bh and the BNI effect of different Bh genotypes on nitrification, plant N uptake and NO_3^- leaching losses. The overall objective was to use and test new methodologies with a minimum of disturbance of the plant-soil system, to characterize BNI of different Bh genotypes in greenhouse and field studies.

The methodological approach comprised a combination of greenhouse based pot and field experiments. Bh plant material included accessions developed by the International Center for Tropical Agriculture (CIAT) with known BNI capacity, as well as newly developed Bh hybrids by CIAT with unknown BNI potentials. Soil incubation studies were used to monitor the influence of BNI on N dynamics such as nitrification, mineralization and N immobilization. Synthetic nitrification inhibitors, e.g. dicyandiamide (DCD) or 3,4-dimethylpyrazole phosphate (DMPP), were used as controls to BNI. Monitoring of N_2O emissions was conducted with the closed chamber method with subsequent gas sample analysis with a gas chromatograph. Furthermore, two different stable N approaches were used: the ^{15}N natural abundance method, and the ^{15}N dilution method where plant and soil samples were measured with a continuous-flow isotope ratio mass spectrometer (IRMS). An enzymatic assay based on the activity of the enzyme nitrate reductase (NR) in leaves of Bh has also been tested.

The first research study focused on the investigation of a potential residual BNI effect of a converted long-term Bh pasture on subsequent maize cropping, where a long-term maize monocrop field served as control. The residual BNI effect was characterized in terms of enhanced maize grain yield, total N uptake and ^{15}N (labeled) fertilizer recovery. Furthermore, the impact of residual BNI effect on soil N dynamics was investigated. The residual BNI effect was confirmed for the first maize crop season after pasture conversion on the basis of lower nitrification in incubation soil, higher total N uptake and higher maize grain yields. However, the residual BNI effect did not result in higher ^{15}N fertilizer uptake or reduced ^{15}N fertilizer losses, nor in reduced N_20 emissions. Applied N was strongly immobilized due to long-term root turnover effects, while a significant residual BNI effect from Bh prevented re-mineralized N from rapid nitrification resulting in improved maize performance. A significant residual Bh BNI effect was evident for less than one year only.

In the second research study it was the aim to verify the potential of nitrate reductase activity (NRA) as a proxy for the detection of *in vivo* performance of BNI by selected Bh accessions and genotypes grown under contrasting fertilization regimes. NRA was detected in Bh leaves rather than in roots, regardless of NO_3^- availability. Leaf NRA correlated with NO_3^- contents in soils and stem sap of contrasting Bh genotypes substantiating its use as a proxy of *in vivo* performance of BNI. The leaf NRA assay facilitated a rapid screening of contrasting Bh genotypes for their differences in *in vivo* performance of BNI under field and greenhouse conditions; but inconsistency of the BNI potential by selected Bh genotypes was observed.

The third research study emphasized to link the natural abundance of ^{15}N (δ) in Bh plants with reduced NO_3^- losses and enhanced N uptake due to BNI. Increased leached NO_3^- was positively correlated to rising $\delta^{15}N$ in Bh grass, whereas the correlation between plant N uptake and plant $\delta^{15}N$ was inverse. Long-term field cultivation of Bh decreased nitrification in incubated soil, whereas $\delta^{15}N$ of Bh declined and plant N% rose over time. $\delta^{15}N$ of Bh correlated positively with assessed nitrification rates in incubated soil. It was concluded that decreasing $\delta^{15}N$ of Bh over time reflects the long-term effect of BNI linked to lower NO_3^- formation and reduced NO_3^- leaching, and that generally higher BNI activity of Bh is indicated by lower $\delta^{15}N$ plant values.

Within the framework of this thesis, a residual BNI effect by Bh on maize cropping could be confirmed for one season due to the combined methodological approaches of soil incubation and ^{15}N recovery. The development of the NRA assay for sampled Bh leaves was validated as a rapid and reliable method linked to the actual soil nitrification after NH_4^+ fertilizer supply. Consequently, the assay could be used for both greenhouse and field studies as BNI proxy. The possibility to perform several sampling intervals using the same plants allowed to detect fluctuations of BNI without major disturbance of the studied plant-soil environment. This methodological advances in methodology show a clear advantage in terms of real-time BNI performance monitoring in comparison to commonly used hydroponic-root exudation studies. The gathered data from the third study indicated that decreasing $\delta^{15}N$ of Bh over time reflects the long-term effect of BNI linked to lower NO_3^- formation and reduced NO_3^- leaching, and that generally higher BNI activity of Bh is indicated by lower $\delta^{15}N$ plant values. Consequently, it was

suggested that $\delta^{15}N$ of Bh could serve as an indicator of cumulative NO_3^- losses. Overall, this doctoral thesis suggests the depressing effect on nitrification by Bh might be a combined effect by BNI and fostered N immobilization. Furthermore, BNI by Bh might be altered by different factors such as soil type, plant age and root morphology of the genotypes. Finally, future studies should consider that Bh genotypes express their respective BNI potential differently under contrasting conditions.

Zusammenfassung (German summary)

Stickstoff(N)-Verluste von Agrarökosystemen schädigen die Umwelt einerseits durch erhöhte Nitrat(NO_3^-)-Auswaschung in Gewässern, und andererseits durch Emissionen von Lachgas (N_2O) in die Atmosphäre. NO_3^- ist das Produkt des bodenmetabolischen mikrobiellen Prozesses der Nitrifikation. Bakterien und Archaeen oxidieren Ammonium (NH_4^+) unter aeroben Bedingungen zu NO_3^-. Darüber hinaus reduziert die mikrobielle Denitrifikation, welche vorwiegend unter anaeroben Bedingungen stattfindet, NO_3^- zu gasförmigen N-Formen. Insbesondere das hierbei produzierte hochpotente Treibhausgas N_2O hat negative Folgen für die Atmosphäre. Synthetische Nitrifikationshemmer verlieren ihre Wirksamkeit unter feuchten, tropischen Bedingungen kurz nach der Anwendung, und sind außerdem für Kleinbauern in den Tropen zu teuer. Das tropische Futtergras *Brachiaria humidicola* (Rendle) Schweick (Bh) reduziert nachweislich die mikrobielle Nitrifikation im Boden durch Abgabe von Wurzelexsudaten. Daher könnte die biologische Nitrifikationshemmung (BNI) durch Bh zur Verringerung von N-Verlusten aus Agrarökosystemen beitragen.

Während N-Verluste aus gut bewirtschafteten tropischen Weidesystemen eher von geringer Bedeutung sind, ist der Kulturpflanzenanbau, welcher hohe N-Düngungsmengen einsetzt, durch hohe N-Verluste und niedrige N-Nutzungseffizienz gekennzeichnet. Rotationen von *Brachiaria*-Weiden mit Nutzpflanzen wie Reis, Mais oder Sojabohne sind generell in den Tropen verbreitet. Die Rolle eines potentiellen BNI-Folgeeffekts durch Bh in einem nachfolgenden (Nicht-BNI) Kultursystem wurde jedoch bisher noch nicht untersucht. Von genotypischen Unterschieden innerhalb von Bh in Bezug auf BNI wurde berichtet. Darüber hinaus gibt es Hinweise darauf, dass höhere BNI-Potentiale von kürzlich entwickelten Bh-Hybriden den BNI-Effekt von Bh-Standardsorten übersteigen. Jedoch konnten BNI-Potentiale bestimmter Bh-Genotypen, die durch nährlösungsbasierte Versuche bestimmt wurden, nicht unter bodenbasierten Topf- und Feldversuchen stringent bestätigt werden. Die Entwicklung neuer BNI-Indikatoren für den Einsatz unter Feldbedingungen könnte dazu beitragen, die Diskrepanz zwischen den unter kontrollierten Bedingungen bestimmten BNI-Potentialen und der BNI-Exprimierung im Feld zu verstehen. Die Hypothese, BNI könnte die nitrifikationsbasierte NO_3^- Bildung reduzieren, und folglich die Verluste durch NO_3^-Auswaschung verringern, wurde jedoch noch nicht überprüft.

Die vorliegende Doktorarbeit zielte darauf ab, das Potential des tatsächlichen BNI-Effekts durch Bh, sowie den verbleibenden BNI-Effekt nach Umbruch des Bh Systems, mit neu entwickelten Methoden zu bewerten. Die gesamte Forschungsarbeit basierte auf den folgenden Hauptzielen: (1) Der Charakterisierung des verbleibenden BNI-Effekts durch Bh auf die N-Aufnahme durch nachfolgenden Maisanbau (*Zea mays* L.) unter verschiedenen N-Düngungsraten; (2) Der Untersuchung, ob eine niedrige enzymatische Nitratreduktase-Aktivität (NRA) in den Blättern von Bh mit einer verringerten NO_3^- Ernährung durch einen effektiven BNI-Effekt verbunden ist; (3) Der Identifikation einer möglichen Verbindung zwischen der $\delta^{15}N$ Pflanzensignatur von Bh Hybriden und deren BNI-Wirkung auf Nitrifikation, Pflanzen-N-Aufnahme und NO_3^-Auswaschungsverluste. Das übergeordnete Ziel bestand darin, neue Methoden mit einer minimalen Störung des Pflanzen-Boden-Systems zu verwenden und zu testen, um den BNI-Effekt verschiedener Bh-Genotypen in Gewächshaus- und Feldstudien zu charakterisieren.

Der methodische Ansatz umfaßte eine Kombination von gewächshausbasierten Topf- und Feldversuchen. Das verwendete Bh Versuchspflanzenmaterial beinhaltete Akzessionen mit bekannter BNI-Kapazität, die vom Internationalen Zentrum für tropische Landwirtschaft (CIAT) entwickelt wurden, sowie neu entwickelte CIAT-Bh-Hybriden mit noch unbekannten BNI-Potentialen. Bodeninkubationsstudien wurden verwendet, um den Einfluß von BNI auf die N-Dynamik wie z.B. auf die Nitrifikation, die Mineralisierung und die N-Immobilisierung zu untersuchen. Synthetische Nitrifikationsinhibitoren, wie z.B. Dicyandiamid (DCD) oder 3,4-Dimethylpyrazolphosphat (DMPP), wurden als Kontrollen für BNI verwendet. Die Überwachung der N_2O-Emissionen wurde mit der geschlossenen Kammermethode und anschließender Gasprobenanalyse durch einen Gaschromatographen durchgeführt. Darüber hinaus wurden zwei verschiedene stabile N-Isotopenansätze verwendet: die natürliche ^{15}N-Abundanzmethode und die ^{15}N-Verdünnungsmethode, bei der Pflanzen- und Bodenproben mit einem Isotopenverhältnis-Massenspektrometer mit kontinuierlichem Fluß (IRMS) gemessen wurden. Ein enzymatischer Test, der auf der Aktivität des Enzyms Nitratreduktase (NR) in Blättern von Bh basierte, wurde ebenfalls durchgeführt.

Die erste empirische Studie konzentrierte sich auf die Untersuchung eines möglichen Folgeeffekts durch BNI einer umgebrochenen Langzeit-BH-Weide auf anschließenden Maisanbau. Als Kontrolle wurde

ein Mais-Monokultur-Langzeitfeld verwendet. Der BNI-Folgeeffekt wurde im Hinblick auf erhöhte Maisertragsausbeute, Gesamt-N-Aufnahme und ^{15}N-Düngemittelrückgewinnung charakterisiert. Darüber hinaus wurde der Einfluß des verbleibenden BNI-Effekts auf die N-Dynamik untersucht. Der verbleidende BNI-Effekt wurde für die erste Maiserntezeit nach Bh-Weideumbruch auf der Basis von geringerer Nitrifikation in Inkubationsstudien, höherer Gesamt-N-Aufnahme und höheren Maiserträgen bestätigt. Der BNI-Folgeeffekt führte jedoch weder zu einer höheren ^{15}N-Düngeraufnahme oder reduzierten ^{15}N-Düngerverlusten, noch zu reduzierten N_20-Emissionen. Applizierter N wurde aufgrund von langfristigen Wurzelumsatzeffekten stark immobilisiert, während ein signifikanter BNI-Folgeeffekt durch Bh die schnelle Nitrifizierung des remineralisierten N verhinderte, was zu einer verbesserten Maisleistung führte. Ein signifikanter BNI-Folgeeffekt war jedoch nur für weniger als ein Jahr nachweisbar.

In der zweiten Forschungsstudie wurde das Ziel verfolgt, das Potential der Nitratreduktaseaktivität (NRA) als Indikator für den aktuellen BNI-Effekt ausgewählter Bh-Akzessionen und Genotypen, die unter kontrastierenden Düngungsregimen getestet wurden, zu verifizieren. NRA wurde in Bh-Blättern, jedoch nicht in Wurzeln nachgewiesen, unabhängig von der NO_3^- Verfügbarkeit. Die Blatt-NRA korrelierte mit NO_3^- Gehalten im Boden und im Presssaft von Bh-Halmen von kontrastierenden Bh-Genotypen, was seine Verwendung als Proxy des *in-vivo* BNI-Effekts belegte. Der Blatt-NRA-Test erleichterte ein schnelles Screening von kontrastierenden Bh-Genotypen in Bezug auf Unterschiede des *in-vivo* BNI-Effekts unter Feld- und Gewächshausbedingungen. Es wurde jedoch eine Inkonsistenz des BNI-Potentials innerhalb der ausgewählten Bh-Genotypen beobachtet.

Die dritte Forschungsstudie untersuchte, ob die natürliche Abundanz von ^{15}N (δ) in Bh-Pflanzen mit reduzierten NO_3^- Verlusten und einer verstärkten N-Aufnahme durch BNI verknüpft werden kann. Erhöhte NO_3^- Auswaschung war positiv mit steigendem $\delta^{15}N$ in Bh korreliert, während die Korrelation zwischen Pflanzen-N-Aufnahme und $\delta^{15}N$ in der Pflanze negativ war. Die Langzeit-Feldkultivierung von Bh verringerte die Nitrifikation in inkubierten Bodenproben, wohingegen $\delta^{15}N$-Werte in Bh abnahmen und die Pflanzen-N% im Laufe der Zeit anstiegen. $\delta^{15}N$-Werte in Bh korrelierten positiv mit Nitrifikationsraten in inkubierten Bodenproben. Schlußendlich konnte angenommen werden, dass die $\delta^{15}N$-Abnahme über die Zeit in Bh den BNI-Langzeiteffekt in Verbindung mit geringerer NO_3^- Bildung

und reduzierter NO_3^- Auswaschung widerspiegelt, und dass eine allgemein höhere BNI-Aktivität von Bh durch niedrigere $\delta^{15}N$-Pflanzenwerte angezeigt wird.

Im Rahmen dieser Doktorarbeit konnte ein verbleibender BNI-Effekt durch Bh auf darauffolgenden Maisanbau aufgrund der kombinierten methodischen Ansätze der Bodeninkubation und der ^{15}N-Rückgewinnung für eine Saison bestätigt werden. Zudem wurde die NRA-Blatt-Methode entwickelt, welche eine schnelle und zuverlässige Analysemethode für die tatsächliche Bodennitrifikation nach NH_4^+-Düngung darstellt. Folglich könnte der Test sowohl für Gewächshaus- als auch für Feldstudien als BNI-Proxy verwendet werden. Die Möglichkeit, mehrere Probenahmen unter Verwendung derselben Pflanzen durchzuführen erlaubt es, Fluktuationen von BNI ohne größere Störung der Pflanzen-Boden-Umgebung nachzuweisen. Diese Fortschritte in der Methodik zeigen einen deutlichen Vorteil in Bezug auf den Echtzeit-BNI-Effekt im Vergleich zu den üblicherweise verwendeten Wurzelexudationsstudien, welche auf künstliche Nährlösungsversuche basieren. Die gesammelten Daten aus der dritten Studie zeigen, dass eine Abnahme von $\delta^{15}N$-Werten in Bh über die Zeit den BNI-Langzeiteffekt in Verbindung mit geringerer NO_3^- Bildung und reduzierter NO_3^- Auswaschung widerspiegelt. Zudem wird eine allgemein höhere BNI-Aktivität von Bh durch niedrigere $\delta^{15}N$-Pflanzenwerte angezeigt. Daraus läßt sich folgern, dass $\delta^{15}N$ von Bh als Indikator für kumulative NO_3^- Verluste dienen kann. Insgesamt legt diese Dissertation nahe, dass die hemmende Wirkung auf die Nitrifikation durch Bh ein kombinierter Effekt von BNI und einer verstärkte N-Immobilisierung sein könnte. Darüber hinaus könnte der BNI-Effekt von Bh durch verschiedene Faktoren wie Bodenart, Pflanzenalter und Wurzelmorphologie der Genotypen variieren. Ebenfalls sollten zukünftige Studien berücksichtigen, dass Bh-Genotypen ihr jeweiliges BNI-Potential unter gegensätzlichen Bedingungen unterschiedlich exprimieren.