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Research article

Nitrate reductase activity in leaves as a plant physiological indicator of *in vivo* biological nitrification inhibition by *Brachiaria humidicola*

Hannes Karwat[a,b], Marc-André Sparke[a], Frank Rasche[a], Jacobo Arango[b], Jonathan Nuñez[b,1], Idupulapati Rao[b,2], Danilo Moreta[b], Georg Cadisch[a,*]

[a] Institute of Agricultural Sciences in the Tropics (Hans-Ruthenberg-Institute), University of Hohenheim, 70593, Stuttgart, Germany
[b] International Center for Tropical Agriculture (CIAT), Km 17 Recta Cali-Palmira, A.A. 6713, Cali, Colombia

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ABSTRACT

The tropical forage grass *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 reflects *in vivo* performance of BNI in soils. NRA was measured in 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. Potential soil nitrification rates (NRs) and leaf $\delta^{15}N$ values were used to verify *in vivo* BNI by the NRA assay in the field study. 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 reflectance of *in vivo* BNI performance. Additionally, leaf NRA data from the field study significantly correlated with simultaneously collected NRs and leaf $\delta^{15}N$ data. 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 was observed. Among Bh genotypes tested, leaf NRA was closely linked with nitrification activity, and consequently with actual BNI performance. It was concluded that NRA in leaves of Bh can serve as an indicator of *in vivo* BNI activity when complemented with established BNI methodologies ($\delta^{15}N$, NRs) under greenhouse and field conditions.

1. Introduction

Plants control soil nitrification via root exudation of nitrification inhibitors, a process termed biological nitrification inhibition (BNI) (Subbarao et al., 2015, 2006). BNI is induced by a wide range of forage species including *Brachiaria humidicola* (Bh) (Subbarao et al., 2007; Ishikawa et al., 2003; Sylvester-Bradley et al., 1988). So far, detection of BNI potentials in Bh genotypes relied solely on the application of a bioluminescence assay using a recombinant *Nitrosomonas europaea* (NE) strain (Subbarao et al., 2007, 2006). A modified assay using a non-modified NE strain along with a *Nitrosospira multiformis* (NM) strain has been published recently (O'Sullivan et al., 2017, 2016). Both methods rely on hydroponics in which plants have to be cultivated for root exudate collection. This procedure may lead to an overestimation of the actual BNI effect under environmental conditions. Alternatively, pasture grasses grown in soil may be transferred to distilled water for root exudate collection (Subbarao et al., 2006), but this practice also revealed a high risk of root damage. Hence, BNI might be obscured by either active or passive (e.g., root damage) release of BNI substances (Souri and Neumann, 2018). These results suggest clearly that nitrification inhibition by NE and NM *in vivo* may not reflect the actual BNI effect in the intact plant-soil system (Coskun et al., 2017). Recently, it was shown that low $\delta^{15}N$ in Bh shoot tissue indicated high BNI and consequently reduced long-term NO_3^- leaching losses (Karwat et al., 2018). Thus, alternative proxies for both pot and field studies need to be developed 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 of nitrate (NO_3^-).

* Corresponding author.
E-mail address: georg.cadisch@uni-hohenheim.de (G. Cadisch).
[1] Present address: Landcare Research, Gerald Street, Lincoln 7608, New Zealand.
[2] Present address: Plant Polymer Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA.

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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 and Masclaux-Daubresse, 2017). The responsible enzyme is nitrate reductase which catalyzes the reaction of NAD(P)H with NO_3^- to produce $NAD(P)^+$, nitrite (NO_2^-), and water (Evans and 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 high soil nitrification and increasing 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 and 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 or roots to approve corresponding NRA as an indicator 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 leaf NRA as an indicator for the detection of *in vivo* performance of BNI by selected field-grown Bh accessions and genotypes exposed to contrasting N fertilization regimes. Our research approach was guided by the main hypothesis that low soil NO_3^- availability resulting from strong *in vivo* BNI performance is reflected in low NRA in plant tissues.

2. Material and methods

2.1. 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 of 100 mM phosphate buffer with a pH of 7.5, 30 mM KNO_3 and 5% propanol to simulated cytosolic plant cell conditions and 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 avoid interference with molecular oxygen (O_2). All operations were conducted under dimmed light to reduce photosynthetic activity (and consequently reduce O_2 production). A polystyrene isolating box filled with ice pads was used to diminish the metabolic activity and enzyme degradation in sampled 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 min 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 min 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^- are 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.

2.2. Experiment 1: NRA 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 of 800 µmol m^{-2} during a photoperiod of 12 h d^{-1}. Plants were irrigated daily with 100 mL of a nutrient solution according to Yoshida et al. (1976) for 30 d. N was provided either as NH_4^+ (to test for BNI, and as substrate for nitrification), 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 out 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 NR. The NRA baseline sample was collected 1 d before N fertilization. Sampling of leaves was conducted 12 h after plants were re-supplied with the respective N form and amount. Final harvest of leaf and root tissue was performed 72 h after N supply and NRA determination was conducted as described above.

2.3. Experiment 2: relationship among NRA and soil nitrification under different N forms in a contrasting 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 and photoperiod) was performed with 4 replications arranged in two blocks (α design) to perform a genotypic evaluation of their BNI potential using the NRA assay. The experimental pots (PVC-drainpipes Ø 11 cm × 100 cm) enabled 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 was 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 depths 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., 2009, 2006), were included as controls. Bh stolons were planted in August 2014 and frequently cut and fertilized with macro- and micro-nutrients. N was applied either as NH_4^+, NH_4^+ + DMPP or NO_3^- (cp. *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 a 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 d 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 h 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 and frozen immediately until NO_3^- was quantified photometrically (AutoAnalyzer 3/QuAAtro AQ2, SEAL Analytical, Southampton, UK). 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.

2.4. 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 m above sea level with a mean annual temperature of 26 °C and annual rainfall of 2933 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, 3 replicates each, to evaluate their BNI activity compared to CIAT Bh accessions. Each plot was 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 had not received 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 NH_4^+ 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), CIAT 679 (mid-high BNI control) and CIAT 26146 (low BNI control according to Nuñez et al., 2018, Rao et al., 2014; 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 NH_4^+ unfertilized subplots were used to determine the baseline NRA, whereas NRA determined in leaves from the NH_4^+ fertilized plots served to assess the effect of NH_4^+ fertilization on nitrification and consequently on NRA. Sub-samples of leaves (NH_4^+ unfertilized) collected at 11 DAF were also used for leaf $\delta^{15}N$ determination, described in detail by Karwat et al. (2018).

As a further indicator of contrasting soil nitrification patterns (after NH_4^+ fertilization) 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, transferred onto NO_3^- test strips and analyzed using Nitracheck 404 (both Merck Millipore, Billerica, USA).

For *in situ* soil NO_3^- monitoring, 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. Gravimetric determination of soil dry matter was conducted with one subsample, whereas another subsample was mixed in a plastic bottle 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 in yellow ionized form derived from alkalization with sodium salicylate using a microplate reader (BioTek Instruments).

To verify a direct link between NRA in Bh tissues and BNI activity, we determined potential soil nitrification rates (Nuñez et al., 2018; Karwat et al., 2018) influenced by contrasting BNI of the used Bh genotypes. Topsoil samples were collected before N application from the plots and air dried for 48 h and sieved (2 mm mesh size). Small stones and visible root residues were removed. Representative 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 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 NO_3^- was extracted before incubation start (basal), and after 5, 11, 14, 20 and 25 d (based on pretests) with 50 mL 1 M KCl. Soil NO_3^- was corrected by basal NO_3^- levels at each sampling time.

2.5. 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 h 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), d 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 $\alpha = 0.05$ 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 derived 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. In order to further corroborate the potential of NRA as *in vivo* BNI indicator, recently published leaf $\delta^{15}N$ and potential soil nitrification rate data (Karwat et al., 2018) were used for the linear regression analysis of *Experiment 3*.

3. Results

3.1. 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 h after N supply (Fig. 1). The N form influenced NRA in leaves ($p < 0.0001$), but not in roots ($p = 0.36$). 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. 2a) being higher in leaves sampled at 72 h after N fertilization compared to sampling at 12 h after N supply

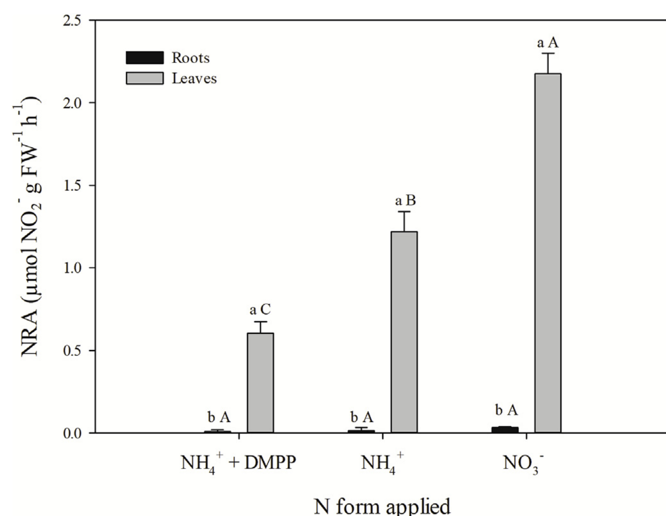

Fig. 1. Nitrate reductase activity (NRA) in root and leaf tissue of *Brachiaria humidicola* (accession CIAT 679) under 3 different nutritional N forms (*Experiment 1)*. Bars are means of 4 replications. Standard errors are given in error bars. Least square means that share a common letter are not significantly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

($p < 0.001$). The difference among the two sampling time points was most 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 ($p = 0.06$) (Fig. 2b).

3.2. 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 ($p < 0.05$) between NO_3^- in the topsoil at 2 DAF and NRA at 9 DAF was observed (Fig. 3a) for the 7 Bh genotypes of the pot trial (*Experiment 2*). The positive relationship ($p < 0.01$) between soil NO_3^- at 5 DAF and leaf NRA at 9 DAF was even stronger ($R^2 = 0.85$) (Fig. 3b). However, no significant correlation was detected for leaf NRA and soil NO_3^- measured at the same date.

NO_3^- measured in stems of the 6 Bh genotypes in the field (*Experiment 3*) correlated with NRA (Fig. 3c) measured at the same sampling date (11 DAF) ($p < 0.05$), but not when sampled at 3 ($p = 0.25$) or 8 ($p = 0.24$) 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). Analogous 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 ($p < 0.05$) measured in leaves 3 d later (11 DAF) (Fig. 3d).

3.3. Leaf NRA in Bh development 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 were similar in terms of the 2 different N fertilizer control treatments (Fig. 4, a and e). NH_4^+ + DMPP nutrition resulted in lowest NRA, whereas NO_3^- nutrition showed greatest NRA rates. NH_4^+ nutrition as presumed BNI trigger was reflected in higher NRA

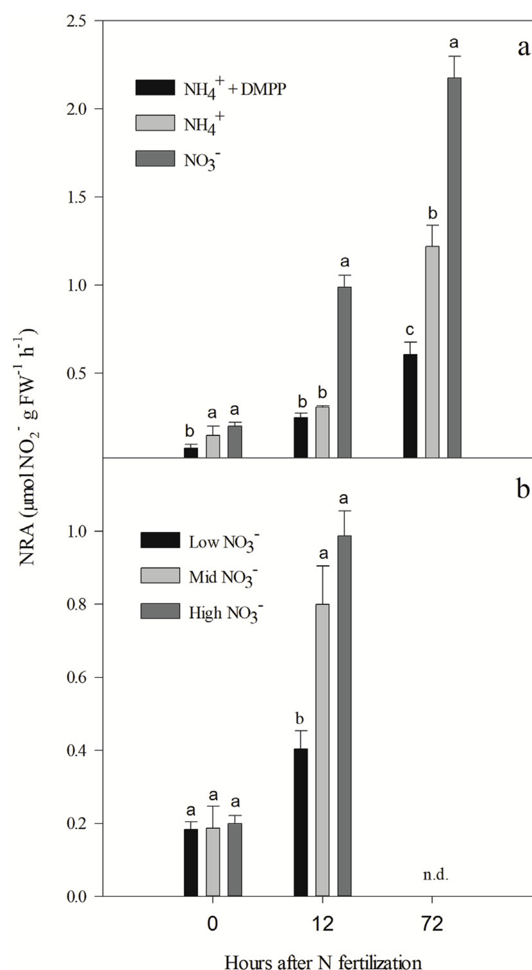

Fig. 2. Nitrate reductase activity (NRA) in leaf tissue of *Brachiaria humidicola* (accession CIAT 679) before N suppsly, at 12 h and 72 h after N fertilization (*Experiment 1)*. N was applied in 3 different nutritional N forms shown in Fig 2a. NO_3^- treatment included 3 different nutrient solutions (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.

values compared to 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 d after N supply (Fig. 4 c). In this case, NRA was higher in CIAT 679 than in the hybrid Bh08-675 at 5 and 9 d after fertilization for the NH_4^+ treatment. NO_3^- in the topsoil showed a similar trend as the NRA for the respective genotypes (Fig. 4, 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-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).

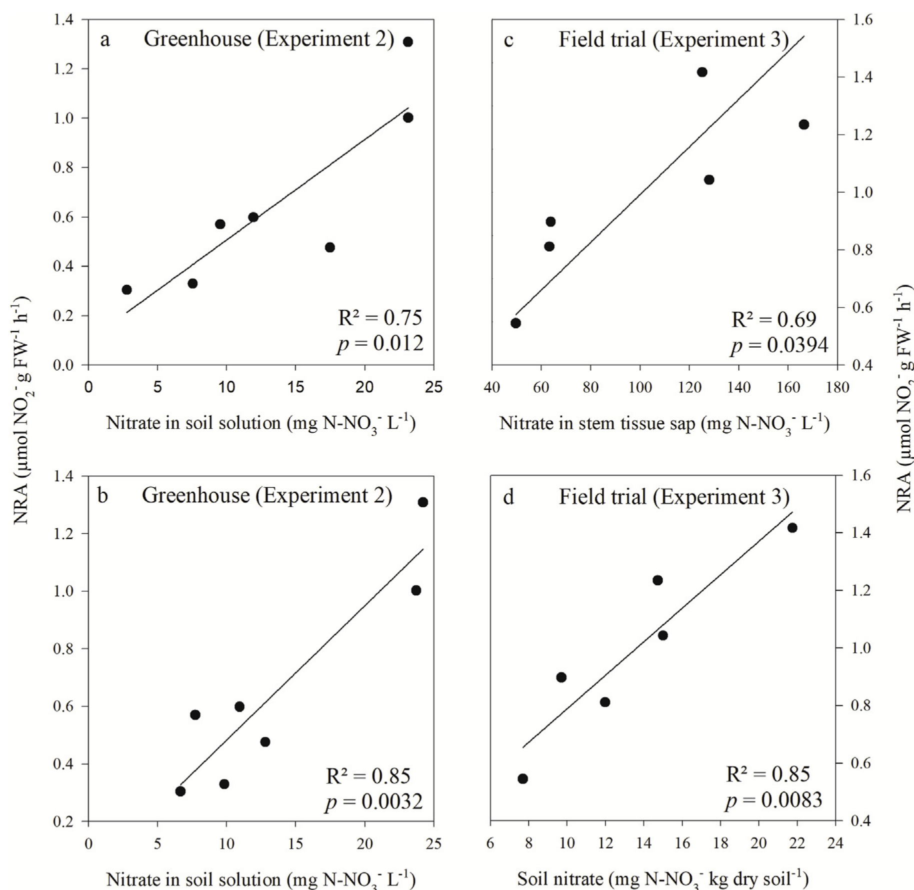

Fig. 3. Linear regression between nitrate reductase activity (NRA) in leaf tissue at 9 d after N-NH_4^+ fertilization (DAF) and NO_3^- measured in soil solution from the topsoil at 2 (a), 5 DAF (b) of 7 *Brachiaria humidicola* (Bh) genotypes. Sampling was conducted at 16 months after establishment of 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. Leaf NRA development of contrasting Bh genotypes under field conditions (Experiment 3)

NRA in leaves sampled in the field (Fig. 5) before N fertilization from 6 contrasting Bh genotypes (*Experiment 3*) depicted 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 increasing 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 ($p < 0.001$).

3.5. Relationship between leaf NRA, potential soil nitrification rates and leaf $\delta^{15}N$ of contrasting Bh genotypes from the field site (Experiment 3)

Regression analysis among net leaf NRA values (NRA 11 DAF – NRA 0 DAF) and potential soil nitrification rates (NRs) showed a significant ($p = 0.045$) linear relationship (Fig. 6). High BNI control CIAT 16888 was found on the lower end of the regression line, whereas low BNI accession CIAT 26146 had higher net NRA and higher NRs. Furthermore, a strong linear ($R^2 = 0.82$) relationship was evident ($p = 0.013$) among leaf NRA (11 DAF) and $\delta^{15}N$ in leaves sampled simultaneously of the respective Bh genotypes from the N unfertilized split-plots (Fig. 7). Genotypic effect for $\delta^{15}N$ in leaves among the 6 genotypes was significant ($p = 0.02$), whereas leaf $\delta^{15}N$ of low BNI (CIAT 26146) was significantly higher compared to leaf $\delta^{15}N$ of the high and mid-high BNI control accessions (Karwat et al., 2018).

4. Discussion

4.1. NRA measured in vivo in Bh leaves 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., 2017, 2006; Lata et al., 1999). To overcome this constraint, we demonstrated here that nitrate reductase activity (NRA) in Bh leaves serves as a plant physiological indicator of *in vivo* performance of BNI, particularly when combined with other BNI related proxies. This approach is substantiated through its combination of earlier assays that estimate BNI (Subbarao et al., 2009) or NRA (Macedo et al., 2013; Cazetta and Villela, 2004) in *Brachiaria* spp. The potential of NRA as a reliable indicator of differences in BNI was verified by strong correlations with NO_3^- in soil 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 (Tegeder and Masclaux-Daubresse, 2017; Li et al., 2013). This retarded reflection of nitrification derived NO_3^- in leaf NRA should be considered 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^- compared to tested Bh lines with low BNI but high NRA (i.e., Bh08 hybrids, CIAT 26146) (Nuñez et al., 2018). Furthermore, the assessed *in vivo* BNI of contrasting genotypes via the NRA assay was analogous to the BNI potentials observed with the plant $\delta^{15}N$ method used in the same field study (Karwat et al., 2018). On the other hand, we also observed contrasting levels of *in vivo* BNI performances via leaf

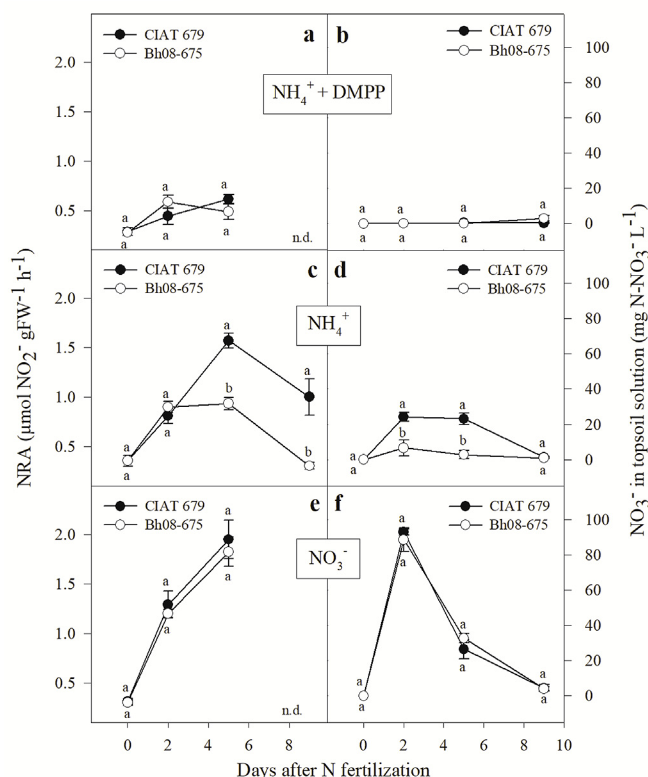

Fig. 4. Nitrate reductase activity (NRA) measured *in vivo* in leaf tissue (left) and NO_3^- (mg N-NO_3^- L^{-1}) in soil solution (right). The contrasting *Brachiaria humidicola* genotypes CIAT 679 (mid-high BNI control) and Bh08-675 hybrid (unknown BNI potential) of 4 replications (plants from randomized soil columns). N fertilizer was supplied in 3 different N forms. Leaf samples were collected before N supply, and at 2 d and 5 d 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*).

NRA along with correlated (nitrification derived) NO_3^- in soil solution between CIAT 679 and Bh08-675 in the greenhouse versus field study. In this case, our proposed NRA method (combined with soil NO_3^- data) suggests that CIAT 679 did not express its mid-high BNI potential under these experimental conditions. This clearly exemplified the complex and yet poorly understood nature of BNI expression in Bh germplasm (Subbarao et al., 2007; Miranda et al., 1996) under different conditions. 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). It could be thus assumed that different Bh genotypes release BNI substances of different composition and concentration (Subbarao et al., 2007) under varying environmental conditions. Here, BNI exudate fingerprinting of contrasting Bh genotypes combined with *in vivo* BNI efficacy using the NRA assay could provide a concerted assessment of BNI of a specific Bh genotype under certain edaphic conditions. Moreover, it is known that 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 and Hageman, 1969). Thus, it is necessary that assessment of absolute NRA values among different genotypes shall be performed in the field under similar environmental conditions to allow for a reliable and comparable determination of *in vivo* BNI in Bh. To compensate for natural fluctuations during sampling, our NRA values obtained in the field experiment represent net NRA (e.g. baseline corrected by subtracting NRA values of control plots without N addition).

Our field observations were 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 summary, *in vivo* BNI in the Bh accessions measured via NRA in the field was clearly related to observed BNI potentials in soil incubation assays and with $\delta^{15}N$ values.

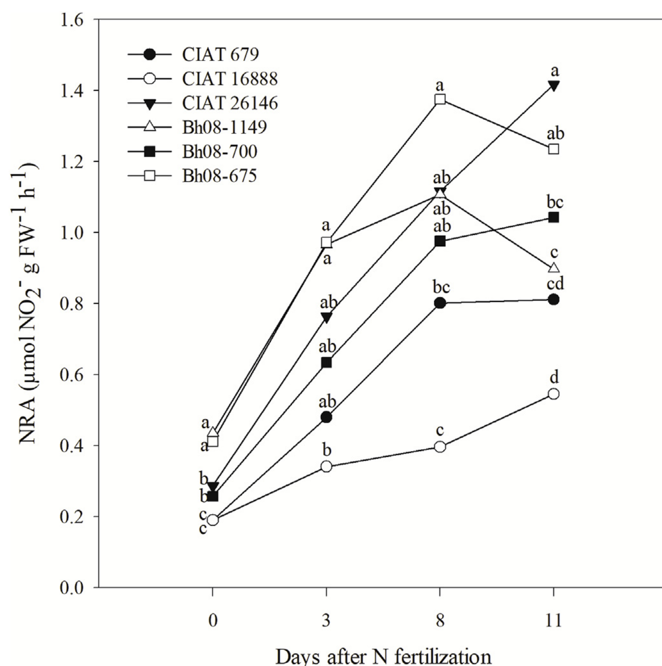

Fig. 5. Nitrate reductase activity (NRA) measured *in vivo* in leaf tissue over time of *Brachiaria humidicola* genotypes including CIAT 679 (mid-high BNI control), CIAT 16888 (high BNI control), CIAT 26146 (low BNI control) and 3 Bh08 hybrids (unknown BNI potentials). Samples were taken at 0, 3, 8 and 11 d 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 $\alpha = 0.05$ at equal DAF. Sampling was conducted at 27 months after establishment of the field trial (*Experiment 3*).

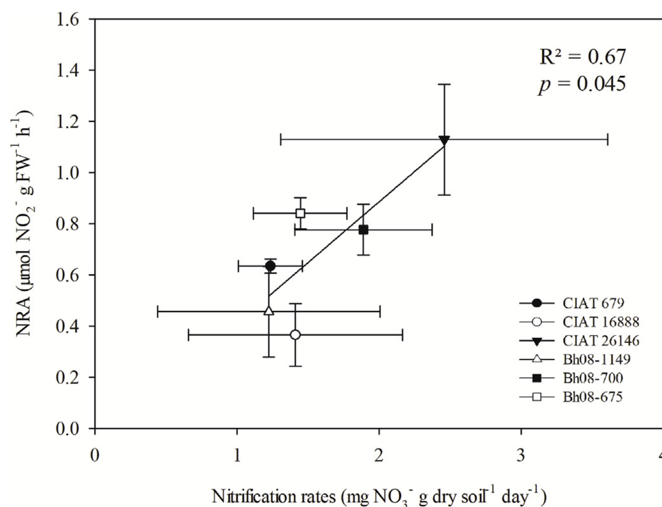

Fig. 6. Linear regression between nitrate reductase activity (NRA) in leaf tissue and nitrification rates (NRs) in incubated soil sampled from respective field plots. NRA values are expressed as net NRA increase (11 DAF – 0 DAF) after NH_4^+ fertilization. NRs values were adopted from Karwat et al. (2018). Six *Brachiaria humidicola* (Bh) genotypes were included: 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 for incubation was taken from replicated field plots (3 per genotype) before N fertilization.

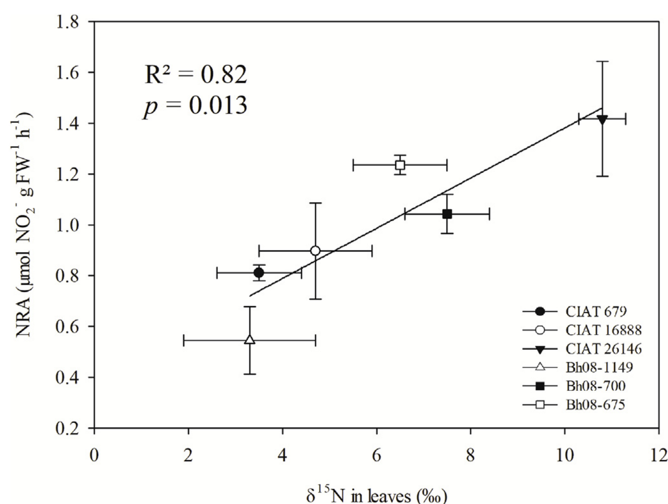

Fig. 7. Linear regression between nitrate reductase activity (NRA) in leaf tissue and leaf $\delta^{15}N$ (‰). Leaf samples for NRA determination were collected from NH_4^+ fertilized split-plots. Leaf samples for $\delta^{15}N$ measurement were sampled from the same main plots, but from the respective NH_4^+ unfertilized split-plot. $\delta^{15}N$ data were adopted from Karwat et al. (2018). Six *Brachiaria humidicola* (Bh) genotypes were included: 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*).

4.2. N form and availability influence in vivo NRA in Bh leaves

BNI activity reduces soil nitrification and hence alters the ratio of plant available NH_4^+-to-NO_3^- ratio 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 confirms that NRA of Bh is strongly coupled to NO_3^- nutrition of *Brachiaria* (Macedo et al., 2013; Cazetta and Villela, 2004) as has been also observed in other plant species (Andrews, 1986; Beevers and Hageman, 1969). Castilla and Jackson (1991) reported that hydroponic systems, where N was supplied as NH_4^+ in conjunction with NO_3^-, forced Bh to take up both N forms, without any preference for either mineral N form. This is of importance for linking *in vivo* NRA with BNI since preferential NH_4^+ uptake over that of NO_3^- might not allow the distinction of medium and high BNI candidates. This would be particularly the case when substrate (i.e., NH_4^+) availability exceeds plant demand. Thus, the NRA assay might not be a suitable BNI screening tool for e.g. *Oryza sativa* with its high affinity to NH_4^+ (Li et al., 2013; Pariasca-Tanaka et al., 2010).

The observed close relationship between NRA of the incubated leaves and soil NO_3^- content combined with plant NO_3^- uptake indications revealed that *in vivo* NRA was assessed based on the presampled 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 already before N addition in the field study. Such genotypic differences might be accentuated under long-term field conditions with a corresponding accumulation of potential BNI related substances in the soil. However, under low N availability other factors, such as microbial immobilization of mineral N due to decomposition of organic residues with a high C-to-N ratio (Karwat et al., 2017) might interfere and thus result in a less clear relation of NRA with BNI. Accordingly, it is suggested to further investigate the applicability of the NRA assay as a BNI indicator for different Bh genotypes in long-term extensively managed systems with traditionally low N availability.

5. Conclusions

Our leaf NRA assay was verified as a plant physiological BNI indicator for Bh applicable for greenhouse and field studies. For Bh, NRA was validated as a rapid and reliable method being linked to the actual soil nitrification after NH_4^+ fertilization. The possibility to perform several leaf sampling intervals using the same plants allowed the detection of contrasting BNI patterns of selected Bh genotypes without major disturbance of the studied plant-soil environment. Furthermore, we integrated the determination of *in vivo* BNI potentials by the NRA assay with respective BNI long-term effects indicated by the recently introduced leaf $\delta^{15}N$ method (Karwat et al., 2018) of the same Bh genotypes in the same field study. Thus, we propose a combination of NRA with other methods to verify actual links with BNI. In addition to the commonly used hydroponics-based root exudation studies to determine BNI potentials, this methodological advancement represents a novelty for reliable real-time BNI performance monitoring of important crops (e.g., sorghum, wheat) when relying primarily on nitrate nutrition under natural conditions (O'Sullivan et al., 2016; Sun et al., 2016; Subbarao et al., 2013; Zakir et al., 2008).

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 by Bh. A delay occurred between increasing NO_3^- availability in the soil and its reflection in *in vivo* leaf NRA and should be considered when determining the suitable time for sampling of leaf tissue. Our results confirmed the high BNI potential in Bh CIAT 16888 and CIAT 679 accessions (Karwat et al., 2018; Rao et al., 2014; Arango et al., 2014; Subbarao et al., 2009, 2006) grown in field. However, their potential could not be always expressed under greenhouse conditions. Accordingly, future studies shall elucidate synergistic effects of edaphic and biochemical origin that potentially alter *in vivo* expression of BNI, apart from the known BNI triggers (NH_4^+ and low pH). This would also enhance the understanding of the dynamic relationship between *in vivo* BNI expression and N supply from soil to plant (Coskun et al., 2017).

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Author contributions

H.K., M.-A.S. and G.K. planned and designed the research. M.-A.S., J.N. and D.M. conducted the field work. H.K. and M.-A.S. conducted the greenhouse studies and analyzed the data. J.A. and I.R. supervised H.K. and M.-A.S. during their research activities in Colombia. G.K. and F.R. supervised H.K. and M.-A.S. during their research activities in Germany. H.K. wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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