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Abstract:	In a field experiment in Palmira, Colombia, we studied mycorrhizal root colonization, phosphomonoesterase activities and P and N foliar content before and after N fertilization among different Brachiaria genotypes with demonstrated BNI capacity. Furthermore, we tested the potential nitrification rate (PNR) in soil in order to confirm the inhibition of nitrification of the selected genotypes and relate the BNI performance with P acquisition. We hypothesized that: (i) genotypes will differ in key variables related to P acquisition, and that there will be a positive correlation between (ii) AMF root colonization, P uptake and BNI activity, and (iii) between the activity of acid and alkaline phosphomonoesterase and BNI performance. Higher N immobilization one week after application of synthetic fertilizer (ammonium sulphate) and low PNR of Brachiaria humidicola CIAT 679 and CIAT 16888 confirmed that these genotypes have high BNI activity. Despite the relatively high soil P status, high affinity of Brachiaria grasses for AMF was observed at the study site: more than 60% of root length was colonized by AMF in high-BNI genotypes, versus 45% in low-BNI Brachiaria cv. Mulato. The N content of high-BNI genotypes was positively correlated with mycorrhizal root colonization suggesting uptake of NH4+ by AMF and its transfer to high-BNI genotypes

	and/or regulation of AMF colonization by P demand. Furthermore, increased activity of acid phosphomonoesterase (6.98 and 7.68 µmoles g-1 h-1 in high-BNI versus 5.20 in low-BNI genotypes) and the depletion of the most labile available P fractions in the rhizosphere of high-BNI genotypes (by 21-32%) suggest enhanced P uptake and P use efficiency. To the best of our knowledge this is the first study that explored relations between BNI and biotic factors affecting P acquisition. Our results highlight the importance of AMF in Brachiaria grasses even under high P availability, and warrant further studies including a larger number of different BNI genotypes that can elucidate biotic plant-soil interactions affecting nutrient use efficiencies in improved pastures under low and high P status.
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L. 22, "and N foliar";
Modified

L. 28, "activity of acid and alkaline phosphomonoesterase and";
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L. 49-50, "oxidation of NH4+ to NO3- is...the N cycle causing";
Modified

PLease indent L. 71,77, 245, 296;
Indented

Please add "exchangeable" before "NH4+" in the text;
Added where appropriate

L. 236-237, "phosphomonoesterase activities.";
Modified

Please complete the list of authors at L. 507, 513, 521, 539, 549, 554, 557, 565, 571, 574, 584, 590, 605;
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L. 513, after Miller please complete the list of editors and write at L.513-515, "Eds)...Madison, WI, pp";
Changed

L. 534-535, "(Eds)...Biochemistry. Academic Press, London, pp";
Completed

Footnotes of Table 1, "organic C...total N";
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Heading of Table 3, "genotypes, acid and alkaline phosphomonosterase activities and";
Modified

Legend of figure 3, "d) phosphomonosterase activities."
Modified

Differences in arbuscular mycorrhizal colonization and P acquisition between genotypes of the tropical Brachiaria grasses: Is there a relation with BNI activity?

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Abstract

In a field experiment in Palmira, Colombia, we studied mycorrhizal root colonization, phosphomonoesterase activities and P and N foliar content before and after N fertilization among different *Brachiaria* genotypes with demonstrated BNI capacity. Furthermore, we tested the potential nitrification rate (PNR) in soil in order to confirm the inhibition of nitrification of the selected genotypes and relate the BNI performance with P acquisition. We hypothesized that: (i) genotypes will differ in key variables related to P acquisition, and that

there will be a positive correlation between (ii) AMF root colonization, P uptake and BNI activity, and (iii) between the activity of acid and alkaline phosphomonoesterase and BNI performance. Higher N immobilization one week after application of synthetic fertilizer (ammonium sulphate) and low PNR of *Brachiaria humidicola* CIAT 679 and CIAT 16888 confirmed that these genotypes have high BNI activity. Despite the relatively high soil P status, high affinity of *Brachiaria* grasses for AMF was observed at the study site: more than 60% of root length was colonized by AMF in high-BNI genotypes, versus 45% in low-BNI *Brachiaria* cv. Mulato. The N content of high-BNI genotypes was positively correlated with mycorrhizal root colonization suggesting uptake of NH_4^+ by AMF and its transfer to high-BNI genotypes and/or regulation of AMF colonization by P demand. Furthermore, increased activity of acid phosphomonoesterase (6.98 and 7.68 μmoles g^{-1} h^{-1} in high-BNI versus 5.20 in low-BNI genotypes) and the depletion of the most labile available P fractions in the rhizosphere of high-BNI genotypes (by 21-32%) suggest enhanced P uptake and P use efficiency. To the best of our knowledge this is the first study that explored relations between BNI and biotic factors affecting P acquisition. Our results highlight the importance of AMF in *Brachiaria* grasses even under high P availability, and warrant further studies including a larger number of different BNI genotypes that can elucidate biotic plant-soil interactions affecting nutrient use efficiencies in improved pastures under low and high P status.

Key words: arbuscular mycorrhizae; tropical grassland; biological nitrification inhibition; phosphomonoesterase; *Brachiaria*

Introduction

Nitrification, the oxidation of NH_4^+ to NO_3^-, is the key step in the nitrogen (N) cycle causing significant losses of N from agricultural lands. Such losses occur in the form of NO_3^- leaching

to subsurface layers, as gaseous N_2O and N_2 resulting from denitrification or as N_2O formed during nitrification under aerobic conditions. The application of synthetic nitrification inhibitors has received wide attention owing to their potential to decrease greenhouse gas emissions by inhibiting ammonium monooxygenase (AMO), an enzyme that catalyzes the oxidation of ammonia to hydroxylamine (Subbarao et al. 2006). Such a reduction of nitrification can reduce soil acidification and the production of NO_3^- which is prone to losses. Low-nitrate environments have been observed naturally and are believed to be a result of the exudation of nitrification-inhibiting compounds by some plant species (Coskun et al. 2017a,b) after root contact with NH_4^+ (Subbarao et al. 2007). This mechanism, known as biological nitrification inhibition (BNI), has been documented in tropical forage grasses (*Brachiaria humidicola* (Rendle) Schweick.) (Subbarao et al. 2009; Nuñez et al. 2018) and is considered to be an adaptive strategy of plants to reduce N limitations. As a co-benefit BNI suppresses N_2O emissions from soils with application of synthetic fertilizers (Subbarao et al. 2009) and bovine urine patches (Byrnes et al. 2017).

The inhibition of nitrification alters the ratio of NH_4^+ and NO_3^- in the soil with direct or indirect impacts on different rhizosphere micro-organisms, thereby affecting the dynamics of other nutrients in the soil as well as nutrient uptake by plants (Britto and Kronzucker 2002; Boudsocq et al. 2012). While the assimilation of NH_4^+ by plants and soil microbes is energetically beneficial when compared to NO_3^-, given the low mobility of NH_4^+ ion, one may expect a negative impact on plant N uptake depending on soil conditions and rooting patterns. Plant nutrient uptake can be enhanced by the symbiosis with arbuscular mycorrhizal fungi (AMF), which are obligate symbionts that depend on C supply from the roots of the host plant in exchange for mineral nutrients, especially P, but also zinc (Zn), copper (Cu) and N (Clark and Zeto 2000). Given the higher competitive capacity of AMF for NH_4^+ (and other nutrients

with low mobility) when compared to plant roots (Pérez-Tienda et al. 2012), AMF may benefit from BNI especially in low nutrients environments.

Increased availability of N often results in enhanced uptake of P by the induction of several P-acquiring mechanisms, such as the release of enzymes or the enhancement of symbiosis with other soil biota. Although it is generally accepted that high levels of available soil P can have a negative impact on mycorrhizal symbiosis (Johnson 2010), Nouri et al. (2014) observed that AMF symbiosis was promoted under N-limitation regardless of P supply, suggesting that plants promote root colonization as long as at least one of the nutrients (N and/or P) is limiting. Similar results were obtained by Blanke et al. (2005), who found increased root colonization under N deficiency in P-rich soil. Thus, AMF may increase both N- and P-use efficiency in low-nitrification environments: increasing the uptake of less mobile NH_4^+, while enhancing the P use efficiency.

Altogether, AMF and plants with BNI ability seem to have a mutual interest in the retention of N in the NH_4^+ form, which is energetically beneficial for both symbionts. Nevertheless, possible effects of altered NH_4^+:NO_3^- ratio as a result of nitrification inhibition have not been addressed and the effects of BNI capacity on soil-plant interactions that may strengthen or modify nutrient coupling in agricultural soils have been unexplored: (i) the reduced mobility of NH_4^+ can stimulate the interaction between plant roots and AMF irrespective of P availability or, (ii) the increased availability of N (or longer lasting availability of N resulting from reduced N losses) may lead to enhanced plant-fungi interaction in order to increase plant P uptake.

The main objective of the present study is to evaluate the differences among three *Brachiaria* genotypes in a long-term experiment (14 years-old), on BNI, in AMF root colonization and spore abundance, acid and alkaline phosphomonoesterase activity, foliar and soil nutrient content and microbial biomass before and after N fertilization. We hypothesized

that *Brachiaria* genotypes will differ in their rhizosphere soil properties indicative of N and P acquisition strategies manifested as differences in AMF root colonization and the activity of acid and alkaline phosphomonoesterase. Furthermore, we tested the relation between changes in P acquisition strategies (enzyme activity and AMF colonization) and BNI performance, hypothesizing that higher P requirements will be found in high-BNI genotypes.

Materials and Methods

Experimental site

The study was conducted at the International Center for Tropical Agriculture (CIAT) headquarters at Palmira (3°30'7"N 76°21'22"W), Colombia, within a long-term field experiment comparing several forage grasses for their BNI ability over time (Subbarao et al. 2009). The altitude of the study area is around 1000 m.a.s.l, with mean annual precipitation of 894 mm and mean annual temperature of 24°C. The precipitation, minimum, maximum and mean temperatures during the experimental period (February and March 2017) are shown in Fig. 1. The soil was characterized as a Vertisol (Typic Pellustert) with a silty clay texture and a pH of 7.4 (Subbarao et al. 2009).

Treatments and sampling design

The experiment was established in 2004 in a completely randomized block design with three replicates per genotype, each with the plot area of 10 m^2. Three *Brachiaria* genotypes were selected based on their contrasting BNI activity described in previous studies (Subbarao et al. 2009; Byrnes et al. 2017) conducted on the same experimental field. Only three *Brachiaria* genotypes could be selected due to lack of long-term established field trials with a wider range of genotypes. *Bracharia* hybrid cv 'Mulato' produces less BNI compounds while *Brachiaria humidicola* CIAT 679 cv Tully (Bh 679) and *Brachiaria humidicola* CIAT 16888 (Bh 16888) showed high BNI activity that translated into low rate of nitrification in soil

(Subbarao et al. 2009; Byrnes et al. 2017). Every year since the establishment of the trial, all genotypes were cut 20 cm above the soil surface twice a year: first harvest between February and March and the second in August. After the harvest, the plots were fertilized with the equivalent to 48 kg N ha^{-1} as $NH_4^+$and 24, 8, 0.2 and 0.2 kg ha^{-1} of K, P, Zn and B, respectively. In the period 2010/17, the average value of dry weight of forage biomass harvested every six months was 9.99, 9.90 and 8.77 Mg ha^{-1} for Mulato, Bh 679 and Bh 16888, respectively. The amount of used fertilizer was calculated for maintenance of the trial. Nevertheless, the plants regularly showed symptoms of mainly N deficiency prior to each harvest and fertilization.

Within each experimental plot, a subplot of 1 m^2 was delimited for the present study. The first set of samples was taken on 17 February 2017, six months after the last fertilization when plants displayed N limitation manifested by reduced growth and yellow coloration of the aerial biomass. Subplots were fertilized on 8 March 2017 with ammonium sulphate (100 kg N ha^{-1}) in order to promote the BNI ability of plants (BNI compounds release is triggered when root is in contact with NH_4^+ (Subbarao et al. 2007)) while no other nutrients were supplemented. The second and third samplings were performed one week (15 March) and three weeks (30 March) after N fertilization, respectively.

During each sampling, ten soil cores (five cm in diameter, 10 cm in depth) were taken from each fertilized 1 m^2 subplot and soil samples along with plants and roots were immediately transported to the CIAT laboratory at the same location where they were pooled into one composite sample per subplot. Rhizosphere soil was separated from the bulk soil by gentle shaking off the soil not adhering to the root system. Fine fresh roots were hand-picked from the soil, washed and stored in ethanol (70%) until analysis of mycorrhizal root colonization. Rhizosphere soil was separated from the rest of the roots and sieved (2 mm), a subsample was air-dried for the analysis of soil chemical properties at CIAT laboratory. The

rest of the soil was stored at 4°C until the determination of microbial biomass and enzyme activity in the Laboratory of Soil Science in Technical University of Madrid, Madrid, Spain. Plant aboveground biomass was cut at 10 cm above the surface level before the first sampling, oven-dried, weighed and stored for foliar analysis. During the two consecutive samplings, ten newly emerging shoots were randomly selected from each subplot, oven-dried and stored for foliar analysis. Foliar analysis was performed in the Laboratory of Soil Science in Technical University of Madrid, Madrid, Spain.

Soil microbial biomass and phosphomonoesterase activity

The fumigation-extraction method was used for determining microbial biomass C (MBC) and microbial biomass N (MBN) (Vance et al. 1987). Briefly, 15 g of moist soil was fumigated with ethanol-free chloroform and organic C was extracted with 0.5 M K_2SO_4 (1:4) and determined colorimetrically by measuring Cr^{3+} produced by reduction of Cr^{6+} (578nm) after microwave digestion (Speedwave four, Berghof, Eningen, Germany) at 135°C for 30 minutes. The content of N was quantified by Kjeldahl digestion of the extracts followed by steam distillation (Bremner and Mulvaney 1982). Microbial biomass C and N were calculated as the difference between the C and N content in fumigated and non-fumigated samples, divided by 0.38 (Joergensen 1996) and 0.54 (Brookes et al. 1985), respectively. The values of the control, unfumigated, samples of MBC and MBN were used as extractable organic C (EOC) and extractable N (EN), respectively.

For determination of MBP, the content of P was extracted from fumigated samples and unfumigated controls with $NaHCO_3$ (1:4 w/v) and determined colorimetrically as described by Joergensen (1995).

The potential activity of acid and alkaline phosphomonoesterase was determined according to Tabatabai and Bremner (1969) by incubating fresh soil sample (0.5 g) with p-nitrophenyl phosphate and MUB buffer (pH 6.5 and 11, respectively).

Potential nitrification rate (PNR) was estimated by aerobic soil incubation with the methods described by Byrnes et al. (2017) and Karwat et al. (2017). The PNR assay has been demonstrated to be a useful method to test the BNI activity in soils under different *Brachiaria* genotypes (Nuñez et al. 2018). Briefly, three grams of homogenized air-dried soil (two days drying at room temperature) were weighted into 10-ml amber flask with one hole in the cap. Soil was amended with 0.8 ml of 27 mM ammonium sulphate to adjust the moisture content to 60% of water holding capacity. Ammonium and NO_3^- contents were extracted with 1M KCl (1:10 w/v) and determined after 4 and 12 days of incubation using the method described below for NO_3^- determination. The PNR was expressed as NO_3^- production per kilogram of soil per day and was calculated using [1] equation.

$$\text{PNR (mg } NO_3^- \text{ kg}^{-1} \text{ day}^{-1}) = [(NO_3^-{}_{t12}) - (NO_3^-{}_{t4})] / \text{days of incubation} \quad [1]$$

Where $NO_3^-{}_{t12}$ is the nitrate N content in the soil after 12 days of incubation and $NO_3^-{}_{t4}$ the nitrate N content after 4 days of aerobic incubation.

Mycorrhizal parameters

Fine roots (at least hundred 1 cm root segments) that were stored in ethanol were washed and cleared with 10% KOH for 30 minutes at 85°C and stained using ink-vinegar method (Vierheilig et al. 1998) by heating the cleared roots for five minutes at 80°C in ink-vinegar solution (5%). Stained roots were observed under microscope and percentage of AMF root colonization was calculated based on the 100 views (McGonigle et al. 1990) and the results reported as a percentage of AMF colonized root length. AMF spores were extracted from the soil using wet-sieving and decanting method followed by sucrose gradient centrifugation (Sieverding et al. 1991) and counted under stereomicroscope.

Soil chemical properties

The content of soil organic C was calculated following the procedure of Hoogsteen et al. (2015) after quantifying the soil organic matter by loss on ignition at 540°C . Soil pH and electrical conductivity were determined in soil suspension (1:2.5 w/v) after one hour shaking. Available P was extracted from air-dried soil with Mehlich III (1:10 w/v) (Mehlich 1984) and determined by ICP-OES (THERMO ICAP 6500 DUO).

Mineral N (N_{min}) was extracted with 1M KCl (1:10 w/v) from the fresh-sieved soil right after sampling. The content of NH_4^+ was determined using sodium salicylate method (Forster 1995) and NO_3^- was quantified colorimetrically after alkalinization with sodium salicylate as described by Karwat et al. (2017). The content of N_{min} corresponds to the sum of NH_4^+-N and NO_3^--N.

Soil P fractionation

Soil P was fractionated sequentially following a reduced methodology proposed by Hedley et al. (1982) using the following extractants with 0.5g of air-dried soil (<2mm): H_2O with anion exchange resin, 0.5M $NaHCO_3$ and 0.1M NaOH (Tiessen and Moir 1993). The inorganic P in the extracts was determined using the colorimetric molybdate-ascorbic acid method proposed by Murphy and Riley (1962) as described in Tiessen and Moir (1993). Total P in the NaOH and $NaHCO_3$ extracts was determined after the digestion of an extract aliquot with 0.6 g of potassium persulfate (Bowman 1989). In the NaOH and $NaHCO_3$ extracts, organic P was calculated as the difference between total P and inorganic P.

Foliar analysis

Foliar nutrients content was determined by acid digestion of 0.4 g of dried biomass with hydrogen peroxide (H_2O_2) and nitric acid (HNO_3) in microwave (Speedwave four, Berghof, Eningen, Germany) by heating to 145°C (10 min ramp) for five minutes followed by heating

to 190°C (five min ramp) for 10 min. Nutrient contents were then analyzed using ICP-OES (THERMO ICAP 6500 DUO). Nitrogen content was quantified using Kjeldahl apparatus.

Statistical analysis

Data were analyzed statically using SPSS 22.0 program (IBM SPSS, Inc., Chicago, USA) using Linear Mixed Model using plant genotype and sampling days as fixed factors, while each field plot was considered as a random factor in which time was nested as a repeated measurement. Several models with different covariance structure were carried out and the most appropriate fit was selected according to the lowest Akaike's information criterion. When a significant effect was detected ($p<0.05$), the LSD post-hoc tests ($p<0.05$) were used to test the differences between the different plant genotypes and sampling days. For identification of the main drivers of the foliar N, P and N:P contents, separate stepwise regressions were applied for each plant genotype separately. The stepping criteria employed for the entry and removal were based on the significance level of the F-value and set at 0.05. In addition, multiple stepwise regressions were used to analyze the effect of the measured soil parameters on the AMF root colonization, spore abundance and both phosphomonoesterase activities.

Results

Soil chemical properties

After thirteen years of the field experiment, different *Brachiaria* genotypes have had significant effects on soil properties. The soil under *Brachiaria* hybrid cv. Mulato had higher soil pH (by 0.7 units) when compared with both *Brachiaria humidicola* genotypes (Table 1). On the other hand, the rhizosphere soil of Mulato contained the lowest amount of organic C and TN.

The prevailing mineral N form under all three genotypes was NH_4^+, while NO_3^- contents remained low throughout the study period (Fig. 2) with no significant difference among genotypes before the application of N fertilizer. Nevertheless, a significant ($p<0.05$) difference was observed at one week after application of ammonium-based fertilizer (March 15th), when soil NH_4^+ content under Bh 679 increased to 83.8 mg NH_4^+-N kg^{-1} and under Mulato to 65.3 mg NH_4^+-N kg^{-1}, but remained low (29.9 mg NH_4^+-N kg^{-1}) under Bh 16888 genotype. Three weeks after fertilization (March 30th), soil NH_4^+ content under all genotypes was comparable. The NO_3^- content remained relatively low even after fertilization, but was slightly higher in Bh 16888 when compared to Bh 679 or Mulato ($p<0.05$). Potential nitrification rate observed during aerobic incubation with $(NH_4)_2SO_4$ was the highest in the low-BNI Mulato.

Soil microbial biomass

Microbial biomass C was affected by genotype (Table 2) and it was significantly higher in Bh 16888 when compared to Mulato or Bh 679. Although there was no significant effect of sampling date, the interaction revealed distinct pattern between all three genotypes: while under Mulato and Bh 679 the MBC content dropped one week after fertilization and raised again two weeks later, the MBC in fields under Bh 16888 increased slightly after fertilization reaching to values that were twice as high as the other genotypes (Table 2). The MBN under Mulato was steadily increasing during the study period raising from 73.6 mg kg^{-1} prior to fertilization to 134.3 mg kg^{-1} at three weeks after fertilization. On the other hand, both high-BNI genotypes followed similar trend increasing the values one week after fertilization but dropping the values significantly three weeks after NH_4^+ application (Table 2). Similarly to MBN, the content of MBP was only affected by the interaction between genotype and sampling date (Table 2). While the MBP was slightly decreasing in time under Mulato, in case of Bh 679 and Bh 16888 it reached the highest values at three weeks after N application.

While the MBC:MBN ratio was affected by genotype (the highest in Bh 16888) and by sampling date (the lowest at one week and the highest at three weeks after fertilization, respectively), the ratios of MBC:MBP and MBN:MBP were only affected by the interaction (Table 2). In both cases, the initial ratios observed were the highest in Bh 679 and the lowest in Mulato. During the study period, the ratios under Mulato were increasing continuously while under Bh 679 they were decreasing. Thus, at three weeks after fertilization, both MBC:MBP and MBN:MBP were the highest in Mulato fields and were the lowest in Bh 679 (Table 2).

Phosphorus fractionation

Despite the relatively high available P content in soil, a significant effect of genotype was observed in the most available Pi fraction (Resin-Pi), which was decreased under-high BNI genotypes and this difference was most evident after N fertilization (Fig. 4). Despite the apparent decreasing trend in the less available Pi fractions (Bic Pi and NaOH-Pi) in Bh 679 and Bh 16888, this effect was not significant. Although there was no effect of genotype or sampling date observed in the H_2O-extractable P, the two following most-labile fractions were significantly higher in high-BNI *Brachiaria* grasses as well as the sum of total Po.

Foliar nutrient content

The foliar N content was significantly increased by N fertilization (Fig. 5), with the highest increase observed with Bh 679 where fertilization augmented the foliar N content from initial 0.86% to 3.36% at one week after fertilization. The foliar N content in Mulato and Bh 16888 increased much less after fertilization and reached values of 2.51 and 1.85, respectively. Unlike foliar N content, foliar P content was not affected by genotype and was significantly increased one week after fertilization, with significant positive correlation ($r=0.715$; $p<0.001$) between foliar N and P contents. The N:P ratio was the highest in Bh 679 but it was the

lowest in Bh 16888 and it significantly increased after the application of N fertilizer being the highest at one week after fertilization and the lowest before N fertilizer application (Fig. 5).

Stepwise variable elimination analysis revealed significant role of mycorrhizal parameters in foliar N content of both high-BNI genotypes, explaining 81% and 57% of the variability in Bh 679 and Bh 16888, respectively (Table 3). Similarly, the P uptake of Bh 679 also seemed to be related to spore abundance in soil which explained 59% of the variability. On the other hand, the foliar P content of Mulato was only negatively related to alkaline phosphomonoesterase activity which explained 54% of the variability.

Mycorrhizal parameters and phosphomonoesterase activity

The percentage of root colonization with AMF was influenced by the genotype, with higher colonization observed in both high-BNI treatments when compared to the low-BNI treatment Mulato (Fig. 3). Furthermore, this difference was strengthened after fertilization, when both high-BNI genotypes increased root colonization of AMF to values around 80% while the colonization of Mulato roots remained without significant change. The AMF spore abundance was the highest in the soil under Bh 679 and Bh 16888 and it increased with time in all three genotypes.

AMF root colonization was positively correlated with spore abundance ($r=0.464$, $p<0.05$) and with acid phosphomonoesterase activity ($r=0.397$, $p<0.05$) and negatively correlated with PNR ($r= -0.652$, $p<0.001$) (Fig. 3). Nevertheless, in the stepwise variable elimination analysis root colonization was better explained (67%, $p<0.001$) by soil pH, Bic-Po and exchangeable NH_4^+ content. Both exchangeable NH_4^+ and Bic-Po explained also 60% of the variability of spore abundance in the soil (Table 4). Acid phosphomonoesterase activity was positively correlated to related to soil organic C content and to negatively correlated to MBP and PNN (Table 4).

Discussion

Potential nitrification rate and soil nitrogen dynamics

In agreement with previous studies (Subbarao et al. 2009; Byrnes et al. 2017; Karwat et al. 2017; Nuñez et al. 2018) the potential net nitrification rate was strongly suppressed by both Bh genotypes resulting in negative values, indicating slight immobilization of NO_3^- or losses due to denitrification. Considering the preference of soil microorganisms for NH_4^+ over NO_3^- and high availability of exchangeable NH_4^+ in the N fertilized soil, immobilization of NO_3^- is less likely but could possibly occur in microsites where NH_4^+ was exhausted. Nevertheless, soil under both Bh 679 and Bh 16888 contained significantly higher total organic C content as well as EOC, suggesting that immobilization of N due to increased availability of C-rich substrate for heterotrophs could be an additional mechanism of these tropical grasses to lower N losses and to improve N-use efficiency by increasing organic matter input to the soil and root exudation. In field, rapid immobilization of ^{15}N in applied fertilizer in *Brachiaria* pastures has been observed and the potential role of higher organic C content in *Brachiaria* fields in the stimulation of denitrification activity has recently been suggested (Karwat et al. 2017). Despite the rapidly increasing body of literature focusing on BNI impact on nitrifiers, other groups affecting the N balance and N losses in the soil, such as denitrifiers or immobilizing heterotrophs, have received less attention.

One week after the field fertilization with ammonium sulphate, higher exchangeable NH_4^+ content in high-BNI plots was expected along with low amount of NO_3^-, which was, however, not detected. The content of exchangeable NH_4^+ and NO_3^- in plots under Bh 679 was more similar to contents under Mulato, the low-BNI genotype. Such a discrepancy between NO_3^- production under laboratory conditions and field observations could be the result of the direct effect of plant roots, which can have significant impact on dynamic N transformations in the rhizosphere through preferential uptake of N from NO_3^- or NH_4^+ forms

or root exudates of variable nature. The exchangeable NH_4^+ content in the rhizosphere soil of Bh 16888 before fertilization was slightly but significantly higher when compared to Bh 679. As the release of BNI compounds is triggered by root contact with NH_4^+, more exudation of such compounds from roots of Bh 16888 could be expected, which is in line with previous studies (Subbarao et al. 2009). Furthermore, the rhizosphere of Bh 16888 contained considerably larger pool of both EOC and MBC, which are considered labile C pools. Rapid microbial turnover and active microbial biomass in the rhizosphere could serve as a temporal sink of N. Indeed, the microbial immobilization of applied N seemed to be higher in Bh 16888 when compared to other *Brachiaria* genotypes as reflected in higher MBN at one week after fertilization. Thus, maintaining higher microbial biomass in the rhizosphere of Bh 16888 may be an additional strategy to enhance nutrient uptake due to the maintenance of a microbial 'safety-net' for capturing and temporal storage of N in the root zone.

The foliar N content of all three *Brachiaria* grasses increased rapidly after the fertilization suggesting a strong limitation of plant growth due to low N availability before fertilization. Furthermore, the N content in the aboveground biomass strongly correlated ($r=0.766$; $p<0.001$) with the exchangeable NH_4^+ content of soil, indicating preferential uptake of NH_4^+ over NO_3^- by *Brachiaria* grasses. The highest relative increase of foliar N content was observed in Bh 679 at one week after fertilization when N content in the leaves of Bh 16888 was detected to be the lowest of all three genotypes, which could be at least partly explained by temporal N immobilization in the rhizosphere of Bh 16888. Clearly, differences in other traits (besides BNI capacity) and their direct or indirect impacts on nutrient transformations deserve more attention as certain discrepancies between genotypes were detected.

AMF colonization as an indication of higher P and/or N requirements

Although the AMF root colonization may fluctuate in time due seasonal changes (increasing precipitation after N fertilization), the root colonization of low-BNI Mulato did not change in time while the colonization of both high-BNI genotypes increased between the first and the second sampling (Fig. 3). Thus, the differences between genotypes could be observed based on the different responses to N application under the same environmental conditions. Thus, at least under the circumstances (after N fertilization accompanied by increased rainfall) and within the time frame of our short-duration experiment, higher AMF colonization of both high-BNI genotypes confirms our hypothesis of higher AMF symbiosis of high-BNI genotypes.

AMF root colonization has been widely used as an indicator of enhanced P requirements of plants. The outcome of the symbiosis between higher plants and AMF depends on large amount of both biotic and abiotic variables (Yang et al. 2016) with N:P stoichiometry being the key factor determining the benefit of exchange between both symbionts (Johnson et al. 2015). While BNI is considered to be a plants strategy to increase N availability, enhanced demand for other nutrients could be expected in high-BNI plants. Both high-BNI genotypes of *B. humidicola* presented higher AMF root colonization when compared to low-BNI Mulato. Nevertheless, the evaluation of biomass production of all genotypes should be included in the future studies to determine whether enhanced P requirements originate from improved N use efficiency or from higher biomass production, which was not determined in the present study due to the limitation of the short duration of the experiment. Nevertheless, no significant differences in annual biomass production among different genotypes have been observed in the long-term evaluation of the same field trial (data reported in the Material and Methods section).

The increased P requirements of high-BNI genotypes manifested in higher AMF colonization, was also reflected in relative depletion of labile P pools in the rhizosphere of high-BNI genotypes when compared to low-BNI Mulato. Although the amounts of the most labile inorganic P pool (Pi-resin) and total Pi pool were comparable in all three genotypes in the first sampling, significant differences between (low-BNI) Mulato and high-BNI genotypes were detected in the subsequent samplings, which could indicate higher P uptake by high-BNI plants.

Significant correlation ($r=0.553$, $p<0.001$) was also found between foliar N content and AMF spore abundance in the soil. Although the extension of AMF in the soil and its capacity to reach to zones that are more remote from the plant roots depends rather on the extent of mycelium growth, the spore abundance could serve as a proxy of fungal biomass to some extent. On the other hand, intensive sporulation could be a sign of stressful conditions. In many tropical grasslands, the nutrients are scarce and in the majority of cases these are applied in the form of cattle urine/dung depositions in high concentrations in small patches. We observed the increase of spore abundance under all three genotypes in time, which could be related to temporal competition for nutrients between plant roots and AMF. Enhanced sporulation could also be an adaptive strategy of AMF as higher number of AMF propagules in soils may offer the fungi faster reaction to the subsequent N additions. The uptake of NH_4^+ (with low mobility) in the soil could therefore be mediated by the mycorrhizal symbiosis, as already suggested (Corrêa et al. 2015). Indeed, mycorrhizal parameters explained 81% and 57% of foliar N content variability in Bh 679 and Bh 16888, respectively. Higher importance of mycorrhizal symbiosis in N rather than P uptake has been suggested (Atul-Nayyar et al. 2009) and N deficiency has been found to promote symbiosis even in P-rich soils (Blanke et al. 2005). Nevertheless, in the Mulato genotype no such effect was detected and mycorrhizal symbiosis seems to have had a lower effect on nutrient uptake by low-BNI Mulato.

On the other hand, higher abundance of AMF in the rhizosphere of high-BNI genotypes could also affect the abundance and activity of other soil biota, including NH_4^+-oxidizers (Amora-Lazcano et al. 1998; Cavagnaro et al. 2007; Veresoglou et al. 2011). It has been speculated that AMF preference for NH_4^+ could lead to a reduction of the abundance of less-competitive NH_4^+-oxidizers (Bollmann et al. 2002). Thus, lower NO3- production rates observed previously (Subbarao et al. 2009; Byrnes et al. 2017) as well as in the present study could be caused by BNI capacitiy and/or by the abundance of AMF being an important NH_4^+ sink.

In agreement with our hypothesis, higher AMF root colonization was found in high-BNI genotypes when compared to low-BNI Mulato, where soil PNR was much higher. Furthermore, the positive correlation between the foliar N content and mycorrhizal root colonization of high-BNI genotypes indicates increased availability of N as a result of BNI and uptake of NH_4^+ by AMF followed by transfer of N to the host plant and/or enhanced P demand as a result of enhanced N uptake and the role of AMF in P nutrition even in soils relatively high in P.

Soil phosphomonoesterase as an indication of P requirements

Soil phosphomonoesterases origin from wide range of sources ranging from root exudation or are produced by soil biota in order to hydrolyse ester-phosphate bonds for releasing phosphate available to plants and soil microorganisms (Quiquampoix and Mousain 2005). Thus, the phosphomonoesterase activities in soil could be used as an indicator of P requirement of plants or associated soil biota.

On the other hand, alkaline phosphomonoesterase has not been observed to be released by plant roots (Joner and Jakobsen 1995) and is therefore considered to be produced by soil bacteria and other soil microorganisms. Unlike in high-BNI Bh genoytpes, the P foliar content of low-BNI Mulato could only be statistically explained by alkaline phosphomonoesterase

(54%, $p<0.05$). Nevertheless, the correlation between foliar P content and the activity of alkaline phosphomonoesterase was negative, suggesting that P stress did not reflect in enhanced P uptake by plant roots. Under some circumstances microbial communities could compete with the plant for P in the rhizosphere as a strong negative correlation ($r= -0.686$, $p<0.05$) was found between alkaline phosphomonoesterase activity and bicarbonate-extractable inorganic P, which is the second most available inorganic P fraction.

Although generally considered being dependent on soil pH (Acosta-Martínez and Tabatabai 2000), we found strong effects of soil organic C content, MBP and PNN, which together explained 79% of acid phosphomonoesterase variability in the stepwise regression analysis. The dependence of acid phosphomonoesterase on organic C content has been observed previously (Margalef et al., 2017) as well as its relation with the type of root exudate stimulating microbial activity (Renella et al. 2007; Nannipieri et al. 2008). The higher activity of acid phosphomonoesterase confirms our hypothesis related to enhanced P requirement of high-BNI genotypes as a consequence of improved N uptake.

Due to the lack of established BNI trials with a wider range of *Brachiaria* genotypes, only three genotypes could be included in the present study. Nevertheless, the obtained results indicate high dependency of *Brachiaria* on AMF which could play a crucial role in N and P management with implications for nutrient losses reduction, regardless of the soil P content. Furthermore, the differences between the two selected high-BNI genotypes indicate that the variability in other traits (besides BNI ability) among *Brachiaria* genotypes deserves further attention in the future BNI studies.

Conclusions

This study aimed to reveal, for the first time, possible relationships between BNI by tropical *Brachiaria* pasture grasses and arbuscular mycorrhizal fungi in the rhizosphere as well as to

understand the relative underlying mechanisms. We observed high mycorrhization of high-BNI *Brachiaria* grasses in P-rich soil, which was further stimulated by the application of ammonium-based fertilizer. Such an increase of root colonization (only in high-BNI genotypes, no difference observed with low-BNI Mulato) seemed to be related to enhanced uptake of both N and P from the rhizosphere. Furthermore, the mycorrhizal symbiosis in *Brachiaria humidicola*, known for its strong suppressive effect on nitrification in soil, seemed to be driven by N limitations rather than P limitations, at least in the soil type under study. Since NH_4^+ is the primary N source of *Brachiaira* grasses, the possible role of AMF in the uptake of rather immobile NH_4^+ deserves more attention. In addition, the rhizosphere of highly mycorrhizal high-BNI genotypes had higher acid phosphomonoesterase activity and reduced the most available P fractions, which could be interpreted as increased uptake requirements of plant-microbe associations when compared to low-BNI Mulato. The potential impacts on nutrient use efficiencies in agroecosystems deserve more attention in the future studies. Selection of a wider range of soil types including more P limited soils and inclusion of more genotypes is needed to gain a better insight into the relationship between BNI and AMF symbiosis. Furthermore, this study reveals patterns which need further and more robust confirmation by testing a wider range of low- and high-BNI germplasm accessions and hybrids in order to identify other possible traits (besides BNI ability) which influence *Brachiaria*-AMF interactions.

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Fig 1

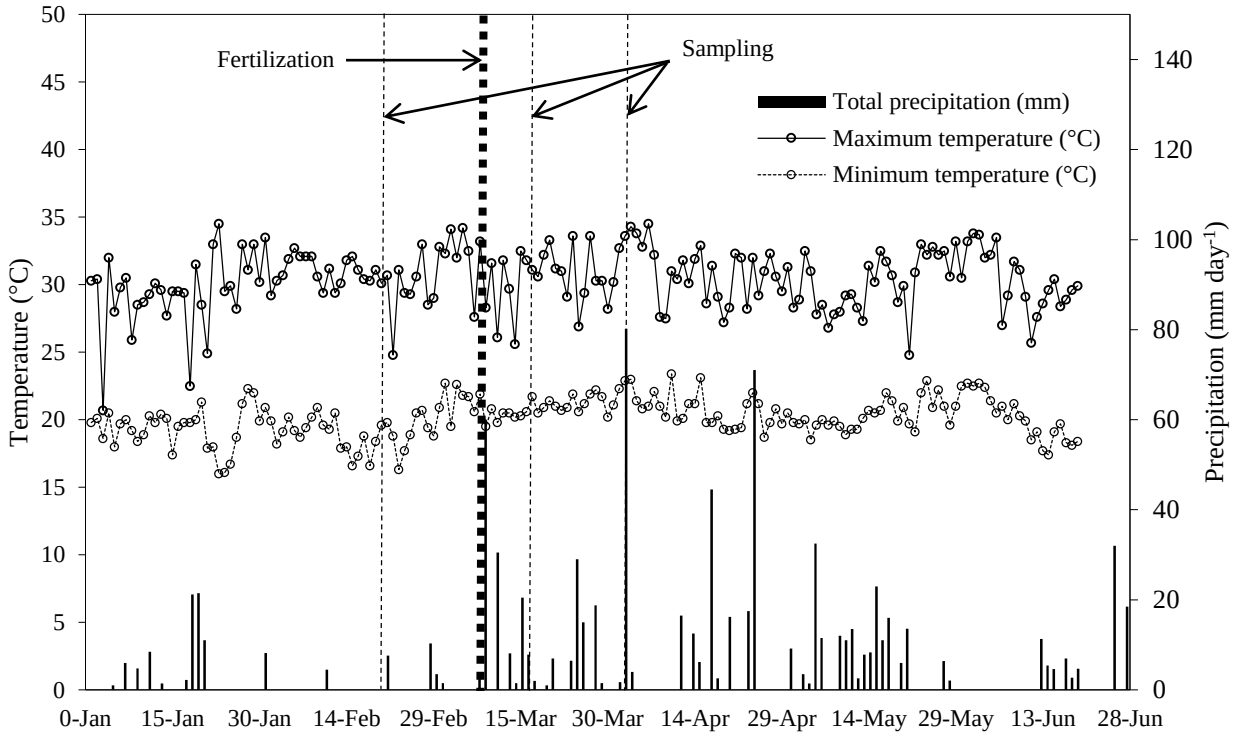

Fig. 1 Maximum and minimum temperatures and precipitation between January 2017 and June 2017 at the experimental site of CIAT-Palmira, Colombia.

Fig 2

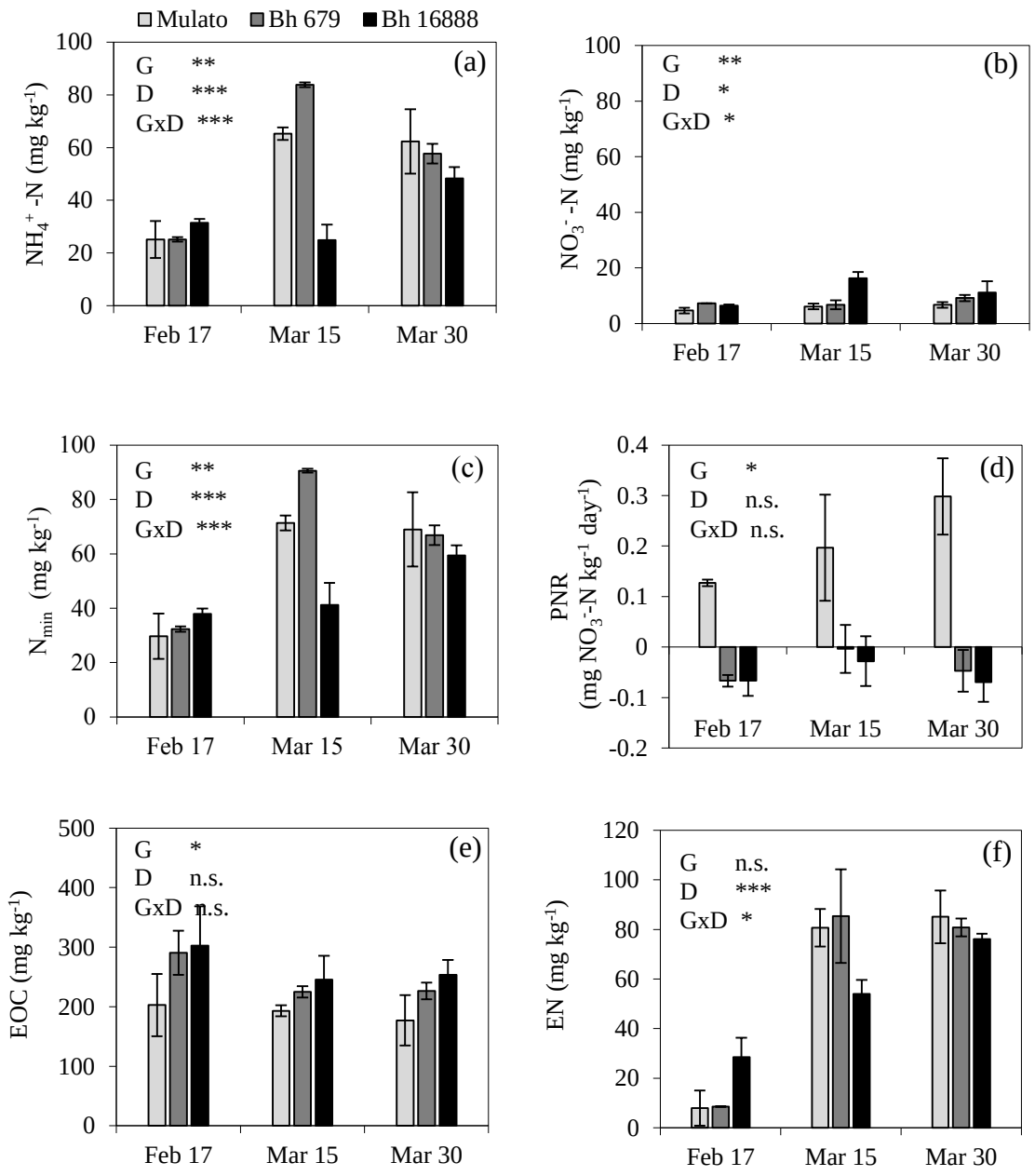

Fig. 2 Effects of genotype (G) and sampling date (D) on ammonium (a), nitrate (b), total inorganic N content (c), potential nitrification rate (d), extractable organic C (d) and extractable N (f). Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be medium-high BNI genotypes. Bars indicate standard error of the mean (n=3). * indicates statistically significant effect at $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 3

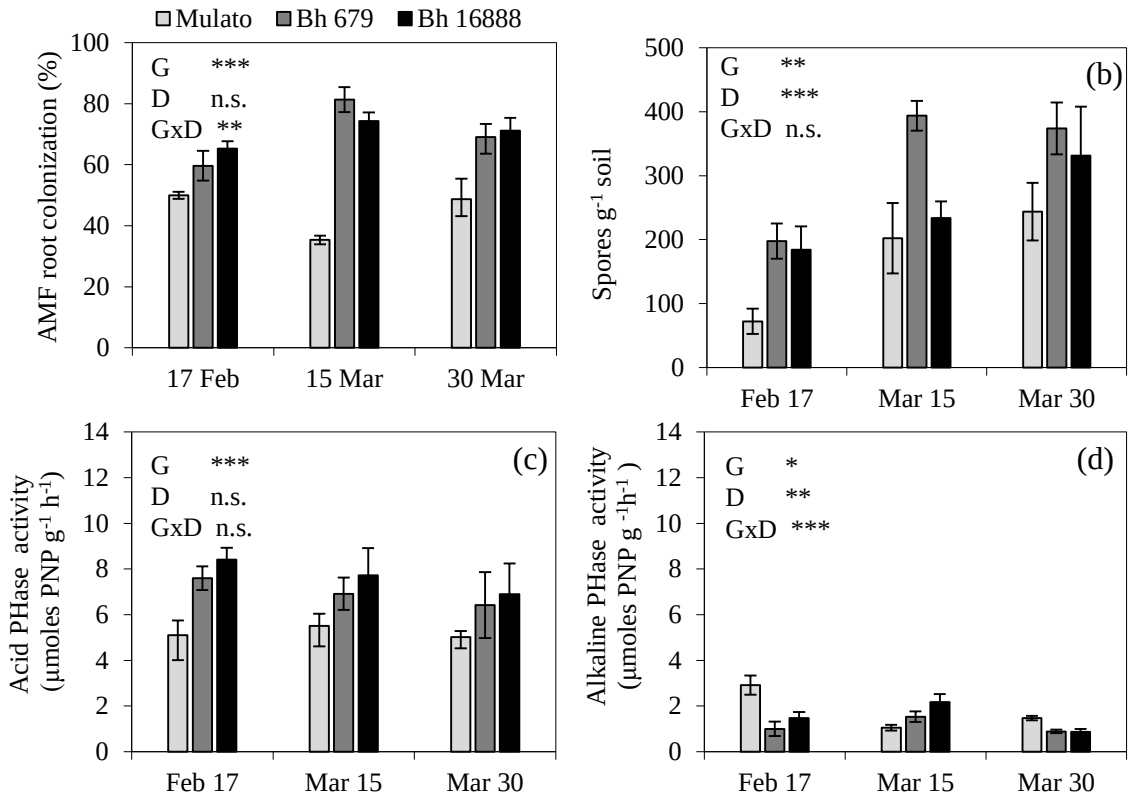

Fig. 3 Effects of genotype (G) and sampling date (D) on root mycorrhizal colonization (a), AMF spore abundance (b), acid (c) and alkaline (d) phosphomonoesterase activities. PHase, phosphomonoesterase. Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be the medium-high BNI genotypes. Bars indicate standard error of the mean (n=3). * indicates statistically significant effect at $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 4

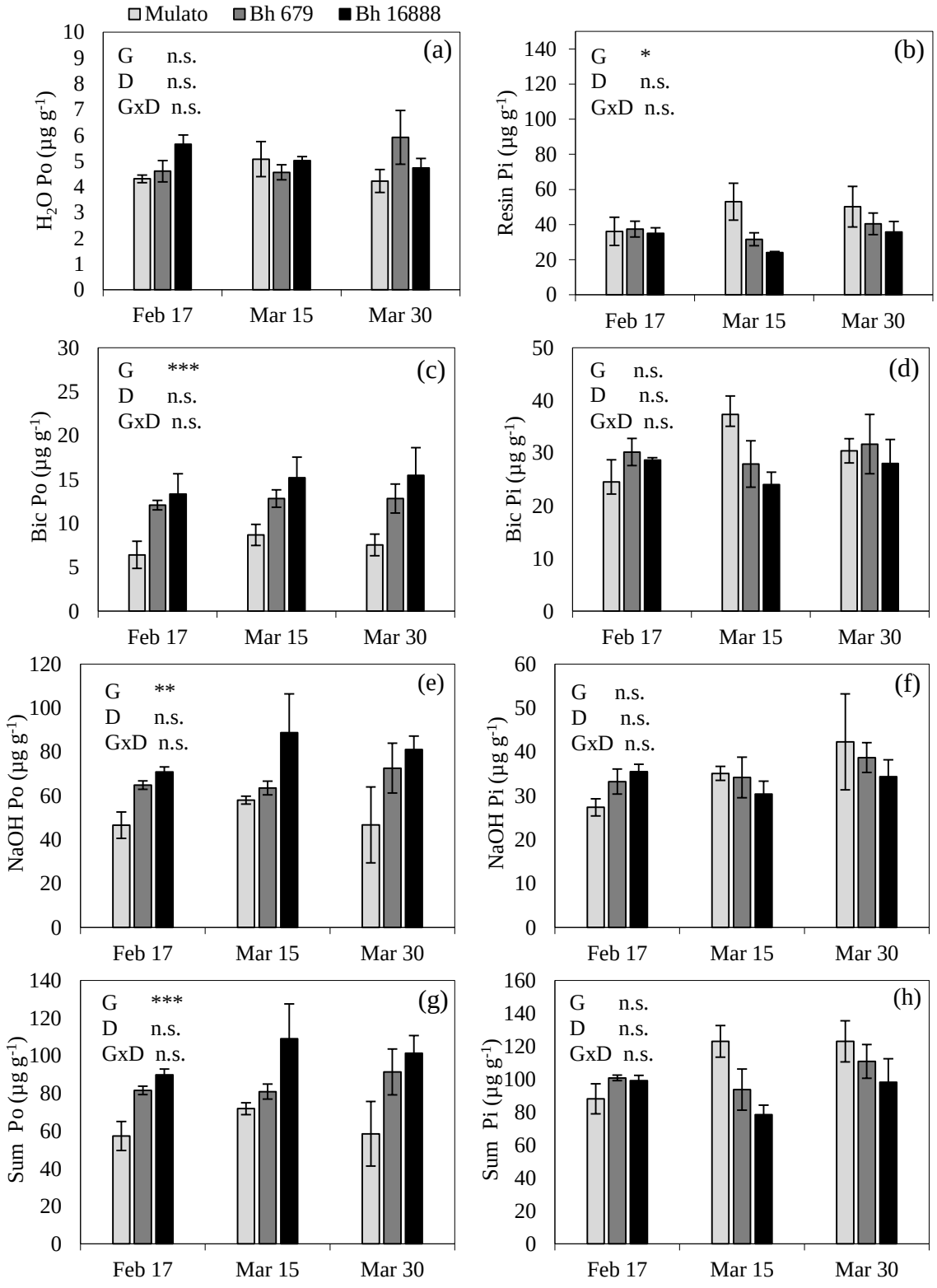

Fig. 4 Effects of genotype (G) and sampling date (D) on water-extractable organic P (a), resin-extractable inorganic P (b), Na_2CO_3-extractable organic (c) and inorganic (d) P, NaOH-extractable organic (e) and inorganic (f) P and the total sum of organic (g) and inorganic (h) P fractions. Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be the medium-high BNI genotypes. Bars indicate standard error of the mean (n=3). * indicates statistically significant effect at $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 5

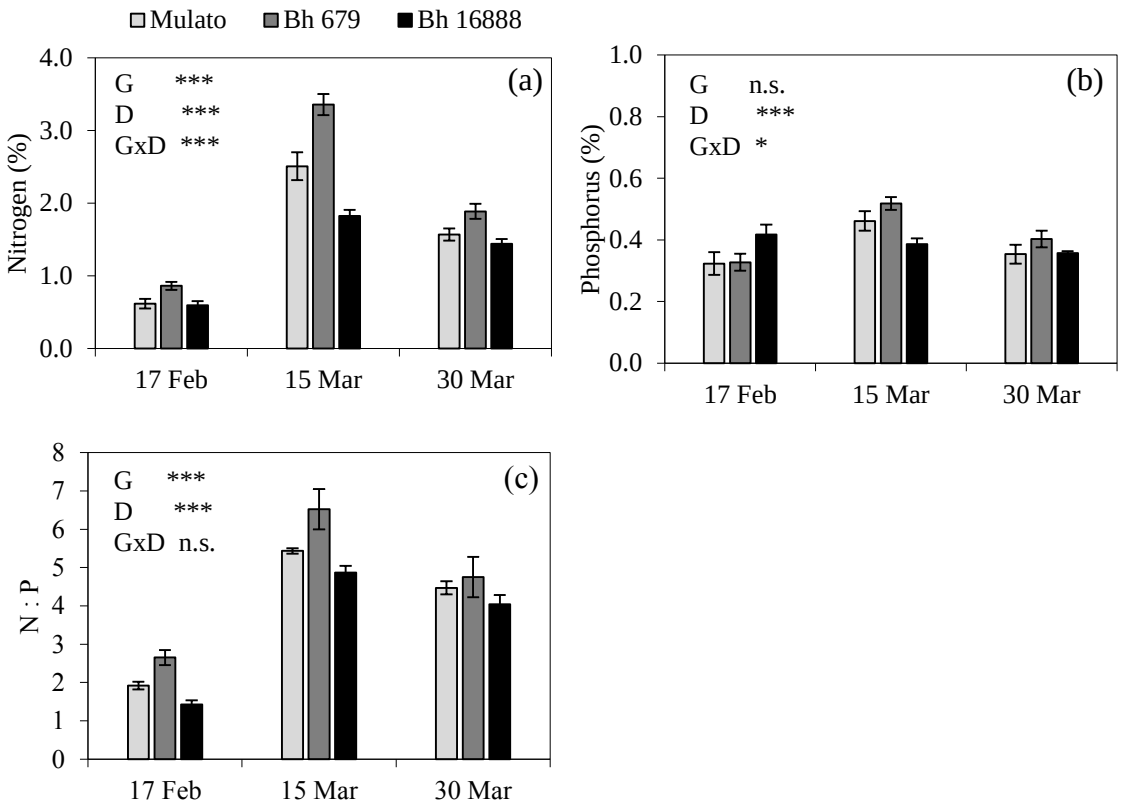

Fig. 5 Effects of genotype (G) and sampling date (D) on foliar N (a) and P (b) content and N:P ratio (c). Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be the medium-high BNI genotypes. indicate standard error of the mean (n=3). * indicates statistically significant effect at $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 1

Rhizosphere soil (0-10 cm) chemical properties as influenced by the growth of three different *Brachiaria* grasses that were established in 2004 at Palmira, Colombia (CIAT Headquarters). Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be the medium-high BNI genotypes.

	$pH_{(H2O)}$	EC ($\mu S\ cm^{-1}$)	C_{org} (%)	TN (%)	P_{av} ($mg\ kg^{-1}$)
Mulato	6.26±0.24a	263±49a	2.39±0.26b	0.17±0.01b	57.60±5.70a
Bh 679	5.54±0.10b	249±12a	3.49±0.22a	0.19±0.01a	44.60±2.73b
Bh 16888	5.66±0.10b	304±19a	3.75±0.26a	0.22±0.01a	39.78±2.73b

C_{org}, soil organic C; TN, total N; P_{av}, Mehlich III extractable P

Means±SE. Different letters indicate significant difference ($p<0.05$).

Table 2

The effects of plant genotype (G) and sampling date (D) on soil microbial biomass C (MBC), microbial biomass N (MBN), microbial biomass P (MBP) and their ratios. Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be the medium-high BNI genotypes.

	MBC	MBN	MBP	MBC:MBN	MBC:MBP	MBN:MBP
	(mg kg^{-1})					
17th February						
Mulato	375.12	73.59	135.18	5.22	3.20	0.66
679	493.58	85.35	41.57	5.78	15.60	2.77
16888	551.04	98.75	103.23	6.33	5.96	1.13
15th March						
Mulato	303.72	107.85	109.81	2.75	2.70	0.98
679	347.23	105.49	62.67	2.38	5.02	2.04
16888	678.65	112.33	74.30	6.24	10.98	1.82
30th March						
Mulato	585.81	134.32	96.20	4.34	7.14	1.55
679	396.80	44.07	141.05	10.00	2.91	0.33
16888	588.63	65.60	159.90	11.76	4.01	0.41
G	***	n.s.	n.s.	*	n.s.	n.s.
D	n.s.	n.s.	n.s.	**	n.s.	n.s.
GxD	*	*	**	n.s.	*	*

* indicates difference at $p<0.05$, ** at $p<0.01$, *** at $p<0.001$; n.s. not significant

Table 3

Multiple regression analysis for identification of the relationships between foliar P and N contents of three *Brachiaria* genotypes, acid and alkaline phosphomonoesterase activities and mycorrhizae parameters. The values are constants or coefficients in the fitted equation $Y=a+bx_1+cx_2+dx_3...$ where Y is the foliar nutrient content and x_1, x_2, x_3 … are the independent variables. Mulato represents the low-BNI genotype while Bh 679 and Bh 16888 are considered to be the medium-high BNI genotypes.

	Constant	Root Colonization	Spore density	Acid phosphomonoesterase	Alkaline phosphomonoesterase	R^2	p-value (model)
Foliar P content							
Mulato	4936.35	-	-	-	-630.70	0.54	*
Bh 679	20003.20	-	6.71	-	-	0.59	*
Bh 16888	-	-	-	-	-	-	n.s.
Foliar N content							
Mulato	-	-	-	-	-	-	n.s.
Bh 679	-32030.73	491.74	55.79	-	-	0.81	**
Bh 16888	-36409.99	704.09	-	-	-	0.57	*
Foliar N:P ratio							
Mulato	6.72	-	-	-	-1.54	0.82	***
Bh 679	0.51	-	0.01	-	-	0.55	*
Bh 16888	-9.47	0.18	-	-	-	0.54	*

$p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 4

Multiple regression analysis for the identification of the main soil parameters controlling AMF root colonization, spore abundance and acid and alkaline phosphatase activity. The values are constants or coefficients in the fitted equation $Y=a+bx_1+cx_2+dx_3...$ where Y is the dependent variable and x_1, x_2, x_3 … are the independent variables

	Constant	SOC	pH	Bic-Pi	Bic-Po	MBP	NH_4^+	PNN	R^2	p-value (model)
Root Colonization	243.71		-25.85	-1.50			0.21		0.67	***
Spore abundance	-74.08				14.69		3.22		0.60	***
Acid PHase	2.67	0.65				-0.01		-3.51	0.79	***
Alkaline PHase									-	n.s.

Discarded parameters ($p<0.05$) were MBC, MBN, EOC, EN, NO_3^-, Nt, H_2O-Po, Resin-Pi, NaOH-Po and NaOH-Pi.

SOC Soil organic matter (Loss of ignition at 540ºC); *Bic-Pi* bicarbonate-extractable inorganic P; *Bic-Po* bicarbonate extractable organic P; *MBP* microbial biomass P; *PNN* potential nitrification rate; *PHase* phophomonoesterase activity. $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant.