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Nitrogen acquisition by two U. humidicola genotypes differing in biological nitrification inhibition (BNI) capacity and associated microorganisms

1 **Nitrogen acquisition by two *U. humidicola* genotypes differing in biological nitrification inhibition**

2 **(BNI) capacity and associated microorganisms**

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14 **Abstract**

15 Biological nitrification inhibition (BNI) has been considered a plant strategy to increase N use efficiency

16 by reducing N losses via N_2O emissions or nitrate leaching. However, recent studies have revealed no

17 difference in gross nitrate production among *Urochloa humidicola* genotypes with previously described

18 high- and low BNI capacity, and pointed towards a crucial role for microbial N immobilization. In the

19 current greenhouse study, we compared the ^{15}N acquisition by two *U. humidicola* genotypes (with high-

20 and low- BNI capacity) and their soil-associated microorganisms at four points in time after fertilization

21 (50 kg N ha^{-1}). Soil microorganisms slightly out-competed both genotypes during the first 24 hours after

22 fertilization and microorganisms associated with high-BNI genotype immobilized more N than microbes

23 associated to low-BNI plants. Nevertheless, by the end of the experiment, low-BNI plant genotype had

24 acquired more ^{15}N, despite higher N_2O emissions. Furthermore, higher ^{15}N root-to-shoot transfer was

25 observed in low-BNI plants, potentially indicating higher contribution of nitrate to plant N uptake. In

26 conclusion, our results confirm the higher importance of microbial N immobilization in high-BNI

27 genotypes, at least in the short-term. However, this did not result in higher N uptake by the high BNI

28 genotype during the first 3 weeks after fertilization as could be expected. Long-term field studies are

29 required to better understand the implications of direct (BNI *sensu stricto*) and indirect mechanisms

30 (including differences in rhizosphere microbial biomass, activity and composition between high and low

31 BNI genotypes) processes on plant N use efficiency, N storage in soil and N losses to the environment.

32 **Key words:** plant-microbe competition; ^{15}N tracing; tropical grasses; N acquisition

33 **Introduction**

34 Rapid or excessive microbial oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) is often related to

35 increased risk of N losses from agroecosystems owing to the high mobility of NO_3^- in the soil profile

36 and consequent N leaching, and to the release of nitrous oxide (N_2O) during nitrification and

37 denitrification. Thus, prevention of NO_3^- formation has been postulated as a strategy to increase N use

38 efficiency (NUE) thereby reducing negative environmental impacts of agriculture (Subbarao and

39 Searchinger 2021). The use of synthetic nitrification inhibitors including dicyandiamide (DCD) and 3,4-

40 dimethylepyrazole phosphate (DMPP) has been recommended to increase both crop yields and NUE

41 (Abalos et al. 2014), but their use under tropical climates and particularly by small farmers remains

42 limited due to their low persistence in soil and high price.

43   Nevertheless, the root exudates of certain plant species or landraces have been observed to

44 biologically suppress nitrification (Subbarao et al. 2009, 2015). In particular, soils with *Urochloa*

45 *humidicola* grasses (formerly *Brachiaria humidicola*) have been studied extensively for their low

46 nitrification rates and suppressed abundance of ammonia oxidizing microorganisms (Sylvester-Bradley

47 et al. 1988; Subbarao et al. 2009; Byrnes et al. 2017; Karwat et al. 2017; Nuñez et al. 2018). This

48 phenomena, termed biological nitrification inhibition (BNI), has been linked to lower N_2O emissions

49 and potential NO_3^- leaching losses (Byrnes et al. 2017). The suppression of ammonia oxidation by

50 *Urochloa* grasses is considered a strategy of plants directed to prevent leaching losses of NO_3^- from the

51 environment (Subbarao et al. 2007). The enhanced retention of N in the soil allows for higher plant N

52 uptake and thus should be translated into improved NUE (Subbarao et al. 2012). Karwat et al. (2017)

53 found increased N uptake by maize grown after *Urochloa* grasses, but the authors attributed the

54 improved N nutrition to mineralization of grass-root derived N in the deeper soil layers rather than

55 improved acquisition of ammonium-based fertilizer applied to the maize crop. Thus, besides root

56 exudation, root turnover is likely an important mechanism explaining effects of *Urochloa* grasses on

57 ammonia oxidation in soil (Karwat et al. 2017; Nakamura et al. 2020). Karwat et al. (2017) also

58 speculated that applied fertilizer was rapidly immobilized in the microbial biomass, which was later

59 confirmed by other studies demonstrating higher microbial N immobilization in the rhizosphere of high-

60 BNI *U. humidicola* genotypes in a field study (Vázquez et al. 2020). Therefore, the microbial N

61 immobilization seems to play a pivotal role in the higher N retention and reduced N losses of genotypes,

62 which are characterized by high BNI capacity.

63   Nitrogen is a limiting nutrient in the majority of tropical pastures. As both plant and soil

64 microorganisms are depending on N supply from soil, the competition for N may be particularly strong

65 in the rhizosphere (Kuzyakov and Xu 2013). Root exudates provide easily decomposable organic carbon

66 (C) which stimulates the growth of soil microorganisms, which in turn take up a substantial amount of

67 available N from soil. This short-term immobilization of N by microbial biomass is suggested as an

68 ecosystem process that reduces N losses (Cavagnaro et al. 2015). After the depletion of available N, the

69 growing microbial population produces extracellular enzymes to obtain additional N from soil organic

70 matter (SOM). Depletion of available C results in dieback of microorganisms which in turn serve as N

71 source for plant roots. Thus, based on the different lengths of life cycle between microorganisms and

72 plant roots and the unidirectional flow of N towards the roots, plants out-compete the microorganism in

73 the long-run (Dunn et al. 2006) as they turn the competition into mutualistic relation based on temporal

74 niche specialization (Xu et al. 2011) by regulating microbial activity *via* rhizodeposition (Kowalchuk et

75 al. 2002; Porazinska et al. 2003; Innes et al. 2004).

76   The initial lower capacity of plants to take up organic N and NH_4^+ in comparison to

77 microorganisms, contrast with the relatively small difference observed between the uptake ability of

78 roots and microorganisms in case of NO_3^- (Jackson et al. 1989; Kuzyakov and Xu 2013). Teutscherova

79 et al. (2019) suggested that BNI primarily promotes short-term microbial N immobilization in the root

80 zone by allowing and maintaining higher microbial biomass, which serves as a temporal storage of N,

81 rather than a simple retention of NH_4^+ in the soil for direct root uptake. Recent studies, however, found

82   contrasting results with regard to the afore mentioned rationale: Vázquez et al. (2020) reported a superior

83   NH_4^+immobilization in soils under high-BNI, compared to low-BNI, *Urochloa* grasses under field

84   conditions, whereas the opposite was found in a greenhouse experiment (Teutscherová et al. 2021b).

85   Neither of these studies detected differences in gross NO_3^- production between supposedly high- and

86   low-BNI genotypes. Both studies speculated that plants likely exert stronger influence on N dynamics

87   *via* N uptake by plants themselves as well as by their associated microorganisms, than by direct BNI

88   (*i.e.* the reduction of ammonia oxidation/nitrification rate directly induced by root exudates).

89   We therefore hypothesized that the relationship between plants and their associated rhizosphere

90   microorganisms strongly determines the final outcome of the N partitioning between plants and

91   microorganisms as well as the NUE of different *Urochloa* genotypes: the plant species or genotypes

92   with mutualistic relationships with their rhizosphere microorganisms will benefit from the temporal N

93   immobilization (Kuzyakov and Xu 2013). To the best of our knowledge, the influence of BNI on the

94   competitive ability of *Urochloa* genotypes *versus* their rhizosphere microorganisms has not been

95   addressed. Based on the constant higher proportion of immobile NH_4^+ respect to mobile NO_3^- resulting

96   from suppressed nitrification, higher initial N allocation to microbes could be expected in plant-soil

97   systems of high-BNI genotypes, further contributing to increase in microbial biomass in the rhizosphere.

98   Consequently, we tested the following hypotheses in a greenhouse experiment:

99   H1: Shortly after the fertilization, microbial N immobilization is higher in high-BNI genotype due to

100 higher initial microbial biomass (and likely soil biological activity observed in previous studies

101 (Teutscherová et al. 2019; Vázquez et al. 2020)) allowed by higher NH_4^+ availability. Hence, high-BNI

102 plants take up less N than low-BNI plants, where N mobility (in the form of NO_3^-) is higher due to higher

103 nitrification;

104 H2: At the later stages of the experiment, high-BNI plants benefit from the temporal storage of N in the

105 microbial biomass and acquire more N than low-BNI plants, where higher proportion of applied

106 fertilizer is lost due to NO_3^- formation and related N_2O emissions.

Materials and Methods

Soil and plant material

Soil (Andosol) was collected from a long-term experiment located at International Centre for Tropical Agriculture (CIAT) experimental station site near Popayan, Colombia (2º26'36''N, 76º57'17''W, 1760 m above sea level). Soil was collected from plots with two contrasting genotypes, which differ in their BNI capacity: *U. humidicola* CIAT 16888 (high-BNI) and *U. humidicola* CIAT 26146 (low-BNI), selected according to previously published results (Arango et al. 2014; Nuñez et al. 2018). Both soils contain a high amount of soil organic C and total N (120 g C kg^{-1} soil and 8 g N kg^{-1}) a low pH (pH 5.8) and loamy texture (Teutscherová et al. 2021b). The available (Bray-1) phosphorus contents were 1.06 and 6.61 mg P kg^{-1} in the high- and low-BNI soils, respectively. The soil microbial biomass C was 421 and 341 mg kg^{-1} and microbial biomass N was 68 and 61 mg kg^{-1} in high- and low-BNI genotypes, respectively. Soil collected from field plots of high-BNI and low-BNI genotypes was homogenized separately, air-dried and sieved (<5 mm) prior the establishment of the greenhouse experiment. Plastic pots (17 cm height, 18 cm diameter) were filled with two kg of soil and one tiller of each genotype (*U. humidicola* CIAT-16888 (high-BNI) and *U. humidicola* CIAT-26146 (low-BNI) was planted in each pot with the corresponding soil. More details on the soil properties and plant materials can be found in Teutscherova et al. (2021b) within the same special issue.

Experimental design

We prepared 38 pots for each genotype with its corresponding soil, yielding 76 pots in total. Treatments were assigned to pots following a randomized complete block design in the greenhouse with controlled temperature and humidity (Fig. S1). Plants were watered regularly and the moisture was gravimetrically adjusted to 60% of field capacity every 2-3 days. After six months, when all plants were well established and of comparable size, 24 pots from each genotype were fertilized with 50 kg ^{15}N ha^{-1} of enriched ammonium sulfate (36.8 mg N per kg, 10.3883 atom % ^{15}N), 16 pots were fertilized with the same amount of ^{14}N and 4 pots without fertilization were sampled on the same day. Six ^{15}N-labeled pots were destructively sampled 1, 3, 7 and 21 days after fertilization (DAF). To account for ^{15}N natural abundance of both genotypes, 4 pots fertilized with ^{14}N-fertilizer were sampled at each sampling date.

Sampling and analysis

One, 3, 7 and 21 days after fertilization, aboveground plant biomass was cut with scissors at the soil surface level and dried at 70°C until constant weight. Roots were carefully separated from the soil with tweezers and immediately washed with 0.05 mmol l^{-1} CaCl$_2$ solution and distilled water to remove all ^{15}N from the root surface. The roots from the pots fertilized with ^{14}N were scanned (Epson 10000xl scanner) and root length, root volume and root surface area were determined using the WinRHIZO root-scanning software (Regent Instruments Inc., Ottawa, ON Canada). All root biomass was then dried at 70°C until constant weight. Afterwards, all dried plant biomass samples were ball-milled and stored until the analysis of ^{14}N : ^{15}N ratio using continuous-flow isotope ratio mass spectrometry (NA 1108 elemental Analyzer, CE Instruments, Milano, Italy) coupled via ConFlo III open-split interface (Finnigan MAT, Bremen, Germany) to a delta S isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) at the University of Bayreuth.

Microbial biomass C and N were quantified using fumigation-extraction method as described by Vance et al. (1987). One subsample of fresh soil (15 g) was fumigated with ethanol-free chloroform for 24 hours and extracted with 0.5M K$_2$SO$_4$ (1:4 soil:extractant ratio). Another subsample was extracted directly without fumigation. Organic C was determined colorimetrically by measuring Cr^{3+} produced by reduction of Cr^{6+} (578 nm) after microwave digestion of both extracts (Speedwave four, Berghof, Eningen, Germany) at 135 °C for 30 min as previously described by Teutscherova et al. (2017). The concentration of N in the extracts was determined colorimetrically as N-NO$_3^-$ (Robarge et al. 2008) after alkaline persulfate oxidation (Cabrera and Beare 1993). The MBC and MBN were then 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. After the quantification of N concentration in the microbial biomass extracts, the ^{14}N :^{15}N ratio was determined in freeze-dried aliquot of both extracts (fumigated and unfumigated) by mass spectroscopy as described for plant biomass. Inorganic N was extracted and determined as described in (Teutscherová et al. 2021b).

Calculations and statistics

The ^{15}N atom% excess of shoot or root plant samples was calculated as described in Liu et al. (2016):

161   ^{15}N atom% excess sample $= ^{15}$N atom% sample $-\ ^{15}$N atom% control,   Eq. 1

162   where ^{15}N atom% sample was the ^{15}N atom% of the shoot or root samples fertilized with ^{15}N and the

163   ^{15}N atom% control was the ^{15}N atom% of the control plants fertilized with the equivalent amount of ^{14}N.

164   Then, the ^{15}N excess amount of shoot or root samples was calculated as:

165   ^{15}N excess amount (g pot^{-1})$= ^{15}$N atom excess sample (%) x N amount (mg pot^{-1}) x 100^{-1}, Eq. 2

166   where N amount sample was the amount of N stored in shoot or root biomass of each individual pot and

167   was calculated as:

168   N amount (mg pot^{-1}) = N concentration (mg g^{-1}) x sample biomass (g pot^{-1}) x 100^{-1},   Eq. 3

169   where N concentration was the N concentration of a shoot or root plant and the sample biomass was the

170   dry weight of the shoot or root of a single pot.

171   The ^{15}N microbial biomass was calculated as the difference in the amount of ^{15}N between the

172   fumigated and unfumigated soil samples. Briefly, the ^{15}N excess amount of soil K_2SO_4 extracts was

173   calculated as:

174   ^{15}N excess extract (mg L^{-1}) $= ^{15}$N atom extract (%) x N concentration (mg L^{-1}) x 100^{-1},   Eq. 4

175   where ^{15}N atom extract was the ^{15}N atom% of the K_2SO_4 extracts and the N concentration was the N

176   concentration of the corresponding extract. The same procedure was used for both fumigated and

177   unfumigated samples.

178   Then, the ^{15}N excess extract was expressed per pot of soil and the ^{15}N microbial biomass was

179   calculated as:

180   ^{15}N microbial biomass (mg pot^{-1}) $= ^{15}$N excess fumigated (mg pot^{-1}) - ^{15}N excess control (mg pot^{-1})

181   Eq. 5

182   The ^{15}N excess control was considered as the amount ^{15}N added which remained in soil (^{15}N

183   soil).

184   The ^{15}N recovery in shoot, root, microbial biomass and soil for each port was calculated as

185   follows:

186   ^{15}N recovery (%) = ^{15}N excess amount (g pot^{-1}) x 100 x ^{15}N excess applied^{-1} (g pot^{-1}),   Eq. 6

187   where ^{15}N excess applied^{-1} (g) was calculated as:

188   ^{15}N excess applied^{-1} (g) = ^{15}N excess fertilizer x N amount applied (g) x 100^{-1},   Eq. 7

189   where ^{15}N excess fertilizer was 10.3883% and the N amount applied per plot was 73.5 mg N per pot.

190   Finally, we estimated the ^{15}N losses per pot as:

191   ^{15}N losses = 100 - ^{15}N shoot recovery (%) - ^{15}N root recovery (%) - ^{15}N microbial biomass recovery (%)

192   - ^{15}N soil recovery (%)   Eq. 8

193   Additionally, the microbial ^{15}N uptake was calculated with the correction factor 0.54 accounting

194   for partial extraction of ^{15}N by the standard fumigation-extraction method (Brookes et al. 1985) because

195   of the potential relevance particularly at the later sampling dates.

196   **Statistical analysis**

197   After testing the normality and homogeneity of variance, data were analyzed using Generalized Linear

198   Model (GLM) considering plant genotype (low- or high-BNI) and time as fixed factors (n=6). The

199   differences between genotypes were considered significant at $p < 0.05$ level. All statistical analysis were

200   performed in SPSS 28.0 (IBM SPSS, Inc., Chicago, USA) software.

201   **Results**

202   **Soil N pools, soil pH and N losses**

203   The content of NH_4^+-N gradually decreased in the soil of both genotypes between 1 day after fertilization

204   (218 and 177 mg kg^{-1} in high- and low-BNI genotype, respectively) and the end of the experiment (21

205   DAF; 11 mg kg^{-1}). The soil NO_3^- content was not affected by plant genotype and increased with time (p

206   < 0.001) but remained low throughout the experiment, and decreased particularly 21 DAF in pots with

207 low-BNI plants, where the values were lower than before fertilization (Fig. 1). Soil pH in pots with low-

208 BNI was higher than in pots with high-BNI plants throughout the experiment (Fig. 1). While soil pH of

209 pots with low-BNI plants did not change in time (remained around 5.8), soil pH in pots with high-BNI

210 plants decreased from 5.8 to 5.6 during the 21 days after fertilization (Fig. 1).

211 One day after fertilization, 58 and 54% of applied $N-NH_4^+$ was recovered in soil K_2SO_4 extract

212 in high- and low-BNI genotypes, respectively. Nevertheless, these values dropped to 11 and 6% at the

213 end of the experiment in high-BNI and low-BNI plants, respectively. The final losses of applied ^{15}N

214 were not affected by plant genotype and accounted for 32.2 and 29.2% in high- and low-BNI plants,

215 respectively (Fig. 2).

Microbial biomass and N uptake

217 Soil microbial biomass N was consistently higher in high-BNI *U. humidicola* CIAT 16888 in

218 comparison to low-BNI CIAT 26146 (Fig. 3), except for the last sampling date (21 days after

219 fertilization), when MBN in both genotypes was comparable. The recovery of applied ^{15}N in microbial

220 biomass was also higher in high-BNI genotype than in low-BNI and gradually decreased in time. Soil

221 microorganisms out-competed plants for N immobilization only in the beginning of the experiment (Fig.

222 2, Supplementary material Fig. S2), when 14.7 and 11.7% of applied ^{15}N was recovered in microbial

223 biomass associated to high- and low-BNI plant genotype, respectively, which is indicated by the ratio

224 plant ^{15}N uptake / microbial ^{15}N uptake < 1 (Supplementary material Fig. S3). When microbial ^{15}N

225 recovery was calculated without correction, plants out-competed microorganisms already 3 days after

226 fertilization. When the correction factor was applied to account for not extractable ^{15}N, plants out-

227 competed microorganisms 7 days after fertilization.

Plant growth parameters and N uptake

229 While no difference was observed between plant genotypes in shoot biomass production (Fig. 1), high-

230 BNI plants produced more root biomass (Fig. 3) with superior root length, root surface area, mean root

231 diameter and root volume (Supplementary material Fig. S4) than low-BNI plant genotype. Plant ^{15}N

232 recovery gradually increased in time during the experiment with higher ^{15}N recovery observed in low-

233   BNI plants (59% of applied ^{15}N) than in high-BNI plants (48% of applied ^{15}N) (Fig. 2). While high-BNI

234   plants allocated acquired ^{15}N preferentially to root biomass (^{15}N recovery in root biomass higher in high-

235   BNI genotype, $p < 0.001$), low-BNI plants seemed to accumulate ^{15}N more in the shoots (higher shoot

236   ^{15}N recovery in low-BNI plants in comparison to high-BNI plants).

237   **Discussion**

238   **Short-term plant-microbe competition**

239   Microorganisms out-competed both plant genotypes for ^{15}N uptake only in the very beginning of the

240   experiment (within 24 hours after fertilization), which is indicated by the ratio of ^{15}N recovery in plants

241   and in microorganisms with values higher than one (Supplementary material Fig. S3). The higher initial

242   ^{15}N uptake after fertilization by soil microorganisms than by plant roots has been well documented and

243   is attributed to the N acquisition advantage of microbes, such as higher surface area to volume ratio,

244   greater spatial distribution and improved uptake affinities (Lipson and Näsholm 2001). Nevertheless,

245   the higher N acquisition of soil microorganisms compared to plants is thought to be short-term and

246   generally last hours to days (Kaye and Hart 1997; Hodge et al. 2000; Bardgett et al. 2003; Harrison et

247   al. 2007) before the plant N uptake exceeds that of microbes, which was also the case of both plant

248   genotypes in the present study.

249   Higher recovery of ^{15}N in microbial biomass was detected in pots with high-BNI than with low-

250   BNI plants, which confirms our first hypothesis. Similarly, soil under high-BNI plants contained higher

251   concentrations of NH_4^+ confirming the findings of Vázquez et al. (2020). Many previous studies have

252   observed the preference of microorganisms for NH_4^+ to other N forms, and microbes being better

253   competitors for NH_4^+ in comparison to plant roots in the short-term (Liu et al. 2016). It is therefore

254   plausible that the soil microorganims associated to high-BNI genotype benefit from the higher NH_4^+

255   concentration, which further enhances their competitive advantage as compared to plant roots, at least

256   in the short-term. A parallel study performed under identical study conditions revealed no difference in

257   gross NO_3^- production rate between the two studied genotypes (Teutscherová et al. 2021b). Therefore,

258   the direct BNI (*i.e.* production of root exudates that inhibit nitrification) is unlikely the sole driver of the

259   higher NH_4^+ soil content in high-BNI genotype. Furthermore, besides BNI by root exudates, nitrification

260   (conversion of rather immobile NH_4^+ to mobile NO_3^-) may be affected by other plant traits, such as

261   specific root length, root N concentration and plant affinity for NH_4^+ (Cantarel et al. 2015), particularly

262   under strong N limitation. The separation of BNI *sensu stricto* from the indirect effects of plants on NO_3^-

263   production remains to be addressed by future studies. Similarly, up to date no study has attempted to

264   reveal the role of heterotrophic nitrification in these tropical pastures as potentially affected by BNI,

265   which may clarify the encountered discrepancies in the literature.

266   Competition between plants and soil microorganisms for inorganic N strongly depends on root

267   density (Xu et al. 2011), as the higher proportion of plant tissues with supportive function (roots) respect

268   to the tissues with growth function (aboveground biomass) translates into higher competitive capacity

269   for water and nutrients. On the other hand, higher root density indicates faster turnover of fine roots and

270   root exudation, which may accelerate microbial-driven N cycling and N immobilization upon N input,

271   as also indicated by our results of superior ^{15}N recovery in microbial biomass of high-BNI genotypes

272   despite the high root density. Furthermore, the root turnover and the release of BNI compounds

273   (brachialactone and others) from root tissues may further contribute to enhanced N immobilization

274   (Karwat et al. 2017). Although the plants' competitive capacity may have been overestimated in the

275   present greenhouse experiment, similar differences in root density between genotypes were observed

276   under field conditions (Teutscherová et al. 2021a).

277   Ultimately, our results contradict a parallel study using the same soils and plants grown under

278   identical conditions, but a different methodological approach (Teutscherová et al. 2021b). While the

279   results of N pool dilution technique used in the study of Teutscherová et al. (2021b) indicated higher

280   microbial N immobilization in the low-BNI genotype, the opposite was detected in the present study,

281   where ^{15}N was quantified by direct measurement of ^{15}N amount excess in the microbial biomass using

282   the fumigation-extraction method. The discrepancy may also lay in the absence of living plant roots

283   during ^{15}N pool dilution technique (Teutscherová et al. 2021b). While the absence of plant N uptake is

284   one of the assumptions of the pool dilution method, the method also leads to a disruption and death of

285   fragile mycelia of soil fungi and of arbuscular mycorrhizal fungi (AMF) in particular. Therefore, the use

286 of pool dilution technique in Teutscherova et al. (2021b) likely underestimated the N immobilization by

287 fungi, including the AMF. The extensive mycelium of AMF can take up a substantial amount of N from

288 soil, which can either transferred to the host plants in exchange for C-rich compounds or can be used

289 for the growth and maintenance of the AMF mycelium. Thus, the higher microbial N immobilization of

290 high-BNI genotypes in the present study, together with the opposite trend detected with pool dilution,

291 may indicate higher importance of soil fungi in the microbial N immobilization in the presence of the

292 high BNI genotype. This assumption corresponds with field study results, where higher AMF root

293 colonization was observed in high-BNI genotype (including *U. humidicola* CIAT 16888 used in the

294 present study) in comparison to low-BNI *Urochloa* hybrid Mulato 1 (Teutscherova et al. 2019). On the

295 other hand, the tight relationship between high-BNI plants and their associated AMF can overestimate

296 N uptake by plant roots as part of the obtained N was likely supplied *via* AMF mycelium. The separation

297 of the AMF- and root-pathway of N uptake should be addressed in future studies to better understand

298 the mechanisms responsible for BNI.

299

300 **N use efficiency and implications for N cycling in tropical pastures**

301 During the 21 days after addition of fertilizer, the high- and the low-BNI genotype took up 48 and 59%

302 of applied N fertilizer, respectively, with no significant difference between plant genotypes. This means

303 that our second hypothesis of higher N uptake efficiency in high-BNI genotype was not confirmed.

304 Microbial ^{15}N recovery accounted only for 9 and 6% of applied ^{15}N fertilizer in high-and low-BNI plants,

305 respectively. Similar range of values was observed by Inselsbacher et al. (2010), detecting 45-80% of

306 initially applied ^{15}N in crop plants already one week after N fertilization, while only 1-10% of fertilizer

307 was recovered in soil microbes.

308   In contrast to our expectations (second hypothesis of higher NUE in high-BNI genotype), in our

309 short-term (3 weeks) greenhouse study, the high-BNI genotype had a lower NUE and no difference was

310 observed in the calculated amount of N losses. These findings are surprising as this genotype emitted

311 lower amount of N_2O according to direct measurements reported in a parallel study (Teutscherová et al.

312 2021b), considering that no leaching losses could occur during neither of these two (pot) experiments.

313 Nevertheless, the amount of N released as N_2 and other gaseous forms of N was not assessed and

314 warrants attention in future studies.

315 Except the 3-7 initial day(s), the ratio of ^{15}N recovery in microorganisms and in plants remained

316 below 1, indicating out-competition of microbes by plants (Fig. 3). The ^{15}N recovery in the microbial

317 biomass was primarily calculated without the correction factor to account for less extractable N forms,

318 assuming that all applied N could be extracted (Liu et al. 2016). While this likely stands true for the first

319 few days after the fertilization, the extractability of applied N likely decreased during the experiment

320 due to the microbially-mediated N transformation, which likely caused an underestimation of the

321 microbial ^{15}N recovery. Similarly, the formation of more complex N-compounds could lead to an

322 underestimation of ^{15}N recovered in soil, further contributing to the possible overestimation of calculated

323 ^{15}N losses under study conditions. It would be interesting, in future work, to investigate whether the

324 higher importance of AMF (Teutscherova et al. 2019) and increased microbial biomass in soil under

325 high-BNI genotypes, coincide with the formation of stable soil aggregates (Horrocks et al. 2019) and

326 higher organic matter stabilization, as a possible explanation for slightly higher SOC and total N content

327 in soil under this genotype. If so, this would lead to a higher underestimation of the amount of ^{15}N

328 recovered in soil in high-BNI genotypes in comparison to pots planted with low-BNI plants.

329 Plants can take up N from the soil both as NH_4^+ and NO_3^- and the N form taken up by roots can

330 alter rhizosphere pH (Raven and Smith 1976). While soil pH remained comparable throughout the

331 experiment in low-BNI plants, it considerably decreased in time in high-BNI genotype, which could be

332 attributed to the preferential uptake of NH_4^+ over NO_3^-. Furthermore, the rhizospheric soil acidification

333 further promotes BNI (Subbarao et al. 2007) feeding the positive feedback loop. Soil pH reduction could

334 be also caused by the accumulation of organic anions in the form of soil organic matter (Tang and Rengel

335 2003), which is also supported by higher microbial biomass of *U. humidicola* CIAT 16888 (high-BNI

336 genotype).

337 Ultimately, as a differences between gross NO_3^- production rates has repeatedly failed to be

338 detected (Vázquez et al. 2020), the influence of plant genotypes on soil NO_3^- content through difference

339 in NO_3^- reduction rather than in NO_3^- production remains a viable alternative. Higher biological activity

340 of high-BNI genotype, together with denser root system, can create more anaerobic sites, which may

341   give advantage to nitrate-reducing microorganisms and hence, reduce the N_2O emission by promoting a

342   complete denitrification to N_2. The assessment of other potential gaseous N losses deserve attention in

343   future studies.

344   Regardless the underlying mechanisms, there is ample evidence for the higher NO_3^-

345   accumulation in soil of low-BNI genotypes under field conditions (Subbarao et al. 2009), which could

346   be related not only to plant N uptake but also to the rate of root-to-shoot N transport within plant. After

347   N absorption from soil by plants, there are two alternative fates of NO_3^-: (i) immediate NO_3^- reduction

348   in the roots, and (ii) root-to-shoot transport and NO_3^- reduction in the leaves (Hachiya and Sakakibara

349   2017). The supply of NO_3^- to *Ricinus* plants resulted in higher N allocation to the shoots, likely because

350   of the high relative rate of NO_3^- transfer to the xylem (Schobert and Komor 1990). Furthermore, NO_3^-

351   also seems to enhance root-to-shoot transport of NH_4^+ and/or its assimilates (Kronzucker et al. 1999).

352   We observed higher ^{15}N recovery in shoots of low-BNI plants and in roots of high-BNI plants. This

353   could be related to the different N form taken up by different plant genotypes, in line with the higher

354   proportion of plant N uptake in NO_3^- form in low-BNI genotype (Kronzucker et al. 1999).

355   Correspondingly, the activity of nitrate reductase was detected to be almost exclusively present in plant

356   leaves and was closely related with potential nitrification activity (Karwat et al. 2019), which supports

357   our speculation. Regardless the environmental consequences of nitrification or N losses, the enhanced

358   root-to-shoot allocation of N in plant biomass is of high economic importance in pastures as it increases

359   the forage quality and hence, the animal productivity. On the other hand, higher preferential

360   accumulation of applied N in the root biomass may lead to higher stabilization and reduced losses in the

361   long-term.

362   **Conclusions**

363   In this study, we found superior short-term microbial ^{15}N immobilization upon N fertilization in soil

364   with high-BNI genotype than with low BNI capacity. The temporal out-competition of plants by their

365   associated microorganisms despite the greater root density of high-BNI plants is likely caused by higher

366   microbial biomass in high BNI genotype soil due to the long-term higher proportion of NH_4^+ in soil N

367   and linked lower N losses. Nevertheless, at the end of the fertilization experiment (21 DAF) the higher

initial microbial N immobilization had not (yet) translated into higher plant N uptake. Instead, our results indicate that high-BNI plant genotypes promote N immobilization by sustaining higher microbial biomass and activity as well as denser plant root system in comparison with low-BNI plants, which could translate into reduced N losses and higher N acquisition by these plants in the long-term. We therefore conclude that low soil NO_3^- content in these tropical pastures is at least partially caused by indirect effects of BNI (*i.e.* changes in soil microbiome, differences in N cycling due to the alteration of NH_4^+/NO_3^- ratio or availability of N etc.) rather than simply by nitrification reduction. More attention should be therefore paid to changes in the microbiome of different genotypes both under controlled as well as field conditions.

Conflict of interest

Authors declare no conflict of interest.

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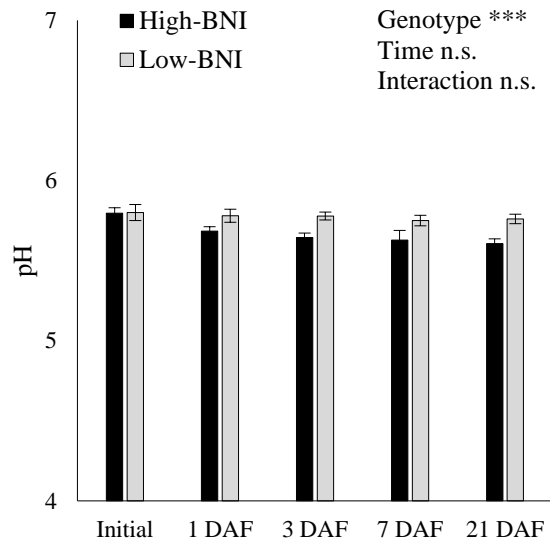
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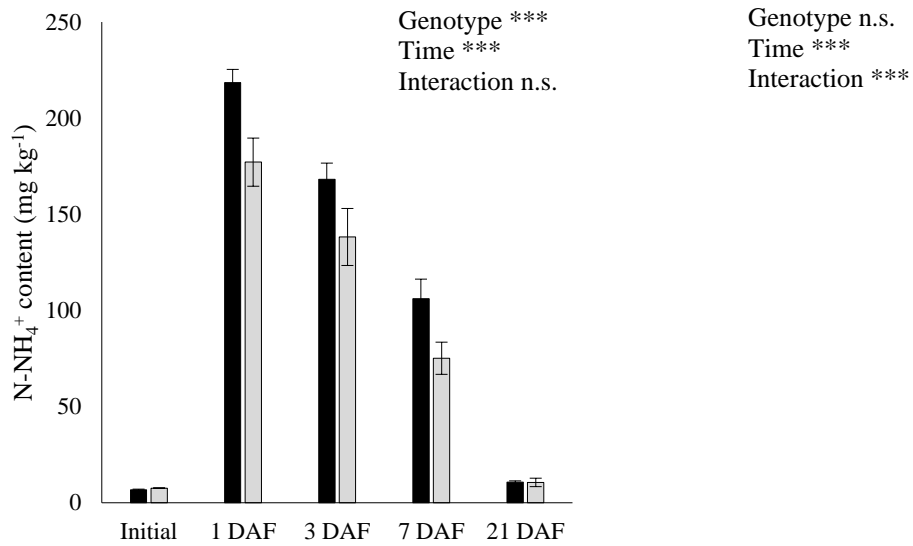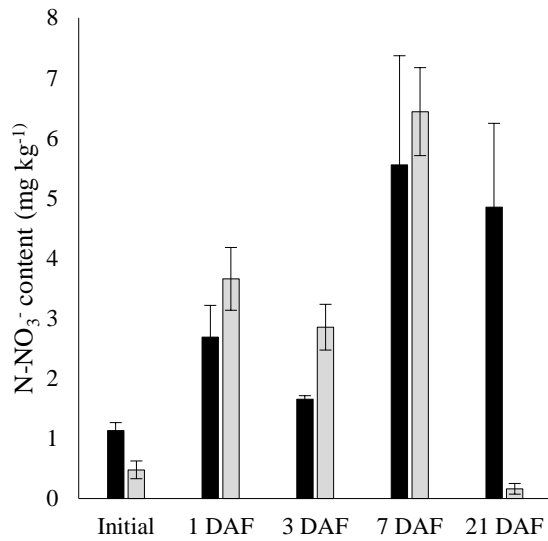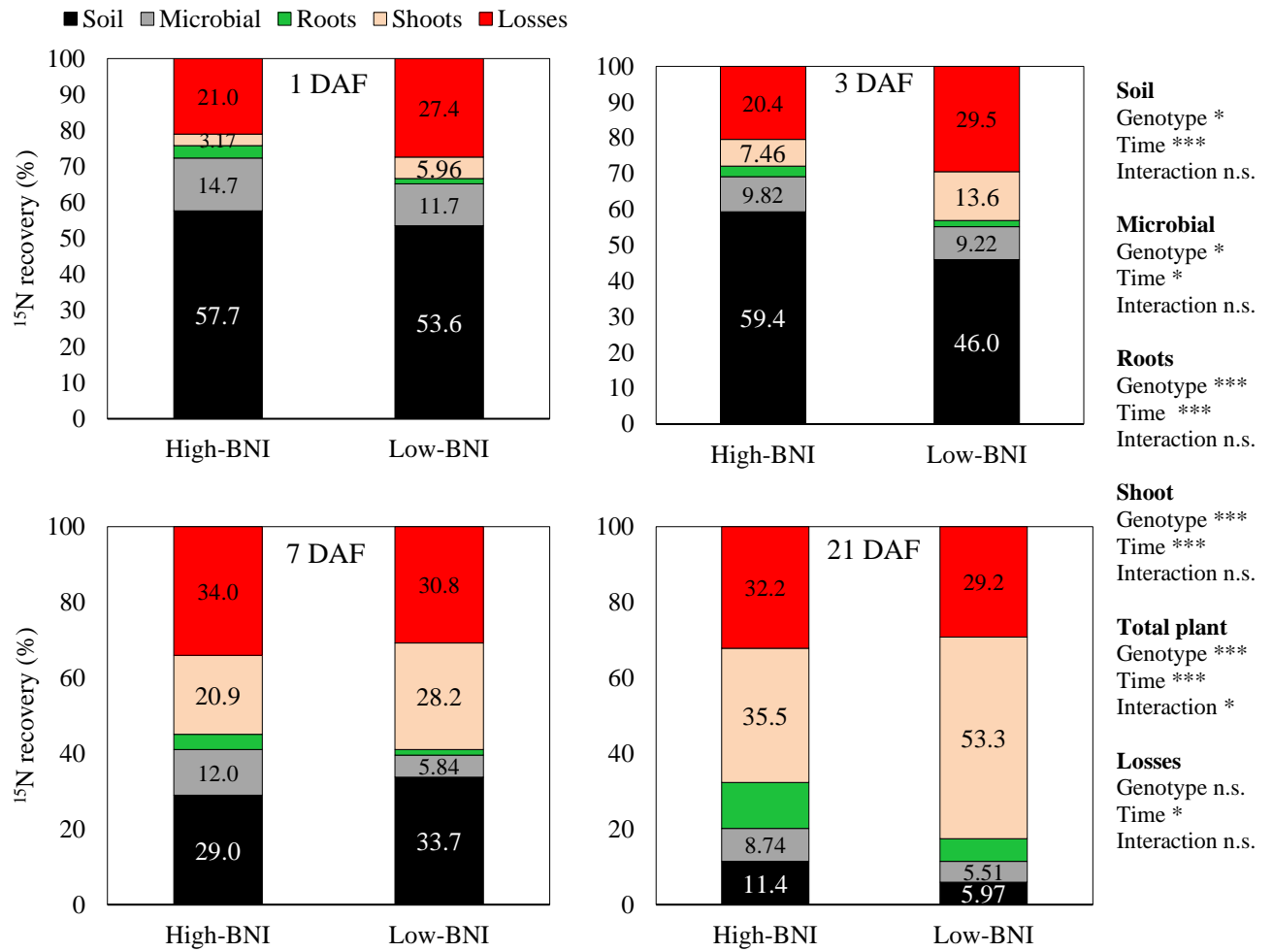
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Fig. 1. Soil pH and the contents of NH_4^+ and NO_3^- in *U.humidicola* CIAT 16888 (high-BNI) and *U. humidicola* CIAT 26146 (low-BNI) determined during the greenhouse experiment. Bars indicate SEM (n=6).
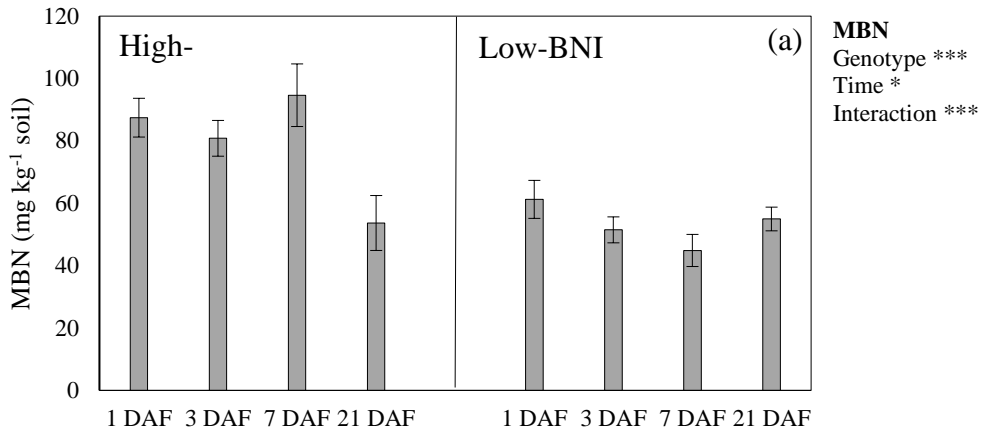
Fig. 2. The recovery (%) of applied N fertilizer in soil, microbial biomass, plant roots and shoots and calculated N losses from pots planted with *U. humidicola* CIAT 16888 (high-BNI) and *U. humidicola* CIAT 26146 (low-BNI). Mean values with standard error of the means can be found in Fig. S3 (Supplementary material).
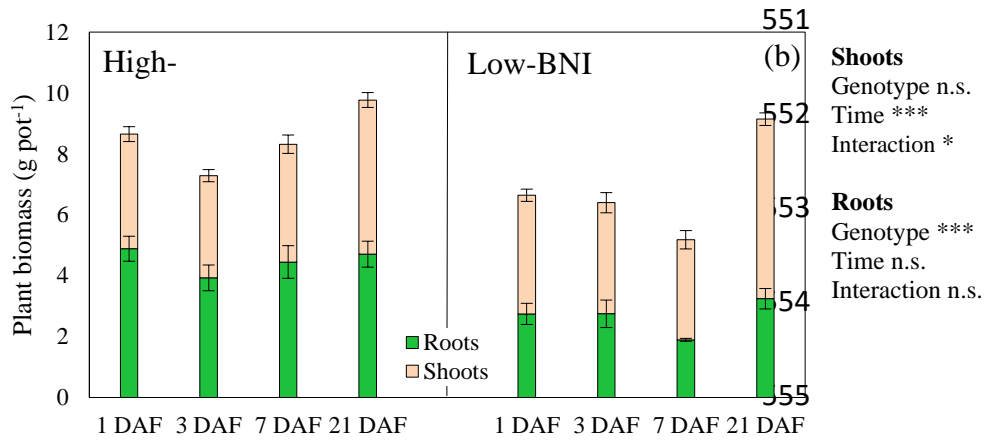
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Fig. 3. Microbial biomass N (a) root and shoot biomass production (b) of pots with *U. humidicola* CIAT 16888 (high-BNI) and *U. humidicola* CIAT 26146 (low-BNI). Mean ± standard error of the mean (n=6).

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