Nitrogen preference across generations under changing ammonium nitrate ratios

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17**Abstract**

18*Aims*: Nitrogen (N) in natural environments is typically supplied by a mixture of ammonia

19(NH_4^+) and nitrate (NO_3^-). However, factors that underlie either NH_4^+ or NO_3^- preference,

20and how such preference will change across generations remain unclear. We conducted a

21series of experiments to answer whether: (i) NH_4^+:NO_3^- ratio is the driving factor for plant

22N preference, and (ii) this preference is consistent across generations.

23*Methods*: We conducted both: (i) field observations (as a proxy for parent or P generation)

24and (ii) greenhouse experiments (the first generation or F1 and the second generation or

25F2) using corn and soybean grown under different NH_4^+:NO_3^- ratios.

26*Important findings*: Both corn and soybean had the physiological plasticity to prefer either

27NH_4^+ or NO_3^- depending on NH_4^+:NO_3^- ratios, and this plasticity was consistent across

28generations. Corn, however, showed a stronger preference towards NO_3^- while soybean

29showed a stronger preference towards NH_4^+. While both plants would try to make use of

30the most available form of N in their growing medium, plant species, physiological

31characteristics (e.g., maturity) and plant nutrient status also determined the extent of N

32uptake. From the evolutionary and productivity perspective, this plasticity is beneficial,

33allowing plants to effectively acquire available N particularly in a changing climate.

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35Keywords: climate change, filial, isotope, parent, plasticity

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Introduction

Plants require a considerable amount of available nitrogen (N) to support their growth and productivity. Since N is consumed by plants in greater quantity than other macronutrients, it most often limits plant growth (Crawford *et al.*, 1998; Wang *et al.*, 2010). Nitrate (NO_3^-) and ammonium (NH_4^+) commonly serve as the primary sources of N for plant growth (Craine et al., 2015), and both ions can be actively absorbed into root cells, even at low external concentrations (Glass *et al.*, 2002). In general, most plants require a mixture of NH_4^+ and NO_3^- rather than a single form of N (George, 2014), especially considering that excessive concentrations of NH_4^+ can be toxic to most plant species.

Numerous studies have been conducted to determine plant N preference (NH_4^+ or NO_3^-) under various growing environments and with different plant species (Criddle *et al.*, 1988; Hageman, 1984; Schortemeyer *et al.*, 1996; Smith *et al.*, 1990). Although NO_3^- uptake requires more energy than NH_4^+ because plants need to first absorb NO_3^- against a steep electrochemical gradient before reducing it to NH_4^+ (Britto *et al.*, 2013), most plants, particularly agricultural crop species generally prefer NO_3^- to NH_4^+. In these plants, NH_4^+ toxicity is usually observed when it serves as the sole source of N or when it is present in excessive quantity (external NH_4^+ concentration > 0.1 to 0.5 mmol/L) (Britto *et al.*, 2002). Ammonium toxicity is generally manifested in a reduction of growth, but the threshold at which the symptoms become visible differs widely among plant species (Britto and Kronzucker, 2002). Agricultural plants that are most sensitive to NH_4^+ toxicity include potato (*Solanum tuberosum* L.), barley (*Hordeum vulgare*), sugar beet (*Beta vulgaris* L.), citrus species, and sage (*Salvia officinalis* L.) (Britto and Kronzucker, 2002).

61   Plant preference towards NO_3^- or NH_4^+, however, may not be constant across plant

62 species but may vary with changing physiological phase and environmental conditions

63 (BassiriRad *et al.*, 1997; Britto and Kronzucker, 2013; Cui *et al.*, 2017; Smith *et al.*, 1990).

64 In a study of N absorption by tomato (*Solanum lycopersicum* L.), for example, 50% of

65 plant N was absorbed as NH_4^+ rather than NO_3^-, even though NH_4^+ only represented 10% of

66 the available N (Glass *et al.*, 2002). In contrast, NH_4^+ fertilization of pearl millet

67 (*Pennisetum glaucum* (L.) R. Br) under conditions that increased NH_4^+ absorption over

68 NO_3^- (i.e., higher NH_4^+: NO_3^- ratio) negatively affected its growth and development (Smith

69 *et al.*, 1990). Similarly, preference for either NH_4^+ or NO_3^- has been reported in natural

70 settings. In the dry, NO_3^--rich landscape, plants tend to prefer NO_3^- while in the wet, NH_4^+-

71 rich landscape, plants tend to prefer NH_4^+ (Houlton *et al.*, 2007; Wang *et al.*, 2011). Since

72 N-source preference varies within a wide range of overlapping environmental and

73 physiological factors, the concept of N preference may only be valid for certain plant

74 species, during specific developmental stages, or under definite soil conditions (Britto and

75 Kronzucker, 2013). Further, the heterogeneous distribution of soil nutrients in soils can

76 potentially affect N preference (Glass *et al.*, 2002).

77   In a recent study, Wang and Macko (2011) argued that N uptake preference is

78 ultimately determined by the relative abundance of NH_4^+ and NO_3^- in the plants' original

79 growing habitat. Indeed, several past studies indicated that N preference was strongly

80 affected by the dominant N form in soil solution, including for different crop species such

81 as barley, oat (*Avena sativa* L.), alfalfa (*Medicago sativa* L.), common vetch (*Vicia sativa*

82 L.) and pearl millet, (Cui *et al.*, 2017; Smith *et al.*, 1990). Therefore, the relative

83concentration of NH_4^+ and NO_3^- ions could be an important factor in determining plant N

84preference. Although plants can also use N in the form of organic N (e.g., amino acid),

85direct evidence that organic N contributes significantly to plant N nutrition remains scarce,

86in addition to the dependence of many crop plants on inorganic N sources and subsequent

87prominent role of these sources in many arable soils (Näsholm *et al.*, 2008).

88   Based on the observation that the second generation of wild, native African grasses

89exhibited a similar N uptake preference as the parent plants did in the field, even when the

90N sources (i.e., NH_4^+: NO_3^- ratios) were changed, Wang and Macko (2011) suggested a

91'memory' effect of the N preference phenomenon (i.e., consistent preference). So far, there

92has been no study that tested whether this 'memory' effect also existed for agricultural

93plants. Agricultural plants can be considered as introduced or domesticated, non-native and

94non-specialized species that have experienced a significant extent of genetic modifications.

95Agricultural plants have also been grown across different environmental conditions,

96highlighting the needs for conducting this study that aimed to understand the consistency

97of plant N preference across generations (i.e., parent or P generation, the first generation or

98F1 and the second generation or F2) experiencing variations in N speciation (expressed as

99the NH_4^+ and NO_3^- ratio). The plant species that were used are corn (*Zea mays* L.) and

100soybean (*Glycine max* L.) since they represent: (i) two of the most important agricultural

101crops on earth, and (ii) two major plant functional types: non-N_2-fixing C_4 and N_2-fixing C_3

102plants. The understanding will be crucial for increasing N-use efficiency and food

103resilience under a changing climate. For example, elevated CO_2 concentration could alter

104the root uptake capacity of different N forms (BassiriRad *et al.*, 1997), while the increasing

105 frequency of drought could lead to NH_4^+ dominance resulting from reduced activity of

106 nitrifiers (Hartmann *et al.*, 2013).

107

108 **Materials and methods**

109 **Plant growth**

110 We obtained the publicly available and fully genome-sequenced corn and soybean seeds

111 from the United States Department of Agriculture (USDA) in Iowa, United States of

112 America (USA). In our experiment, these seeds were considered the first filial (F1)

113 generation of plants. Under the assumption that the parent (P) generation of these F1 seeds

114 was grown in typical farm conditions in the Midwest region of USA, we used corn and

115 soybean crops grown in an agricultural field near Indianapolis, Indiana, USA as P

116 generation. We acknowledge that these corn and soybean plants were not the actual P

117 generation and therefore they were only regarded as the proxy for the P generation. We,

118 however, argue that this would not affect our results because both seeds have been used as

119 the parent material of numerous corn and soybean varieties (Grant *et al.*, 2008; Stojaković

120 *et al.*, 2005). Soybean is self-pollinating and all of the recessive genes in the corn seed

121 have been have been eliminated, ensuring that there is no genetic deterioration of the

122 offspring. The F1 seeds were grown in the greenhouse and the seeds obtained from these

123 F1 plants were considered the second filial (F2) generation. These F2 seeds were

124 germinated in the greenhouse and F2 plants were grown in the greenhouse until treatment.

125   The field (P) experiment was conducted when corn and soy bean plants were fully

126 established in the field. Twelve corn and twelve soybean plants were selected randomly as

127treatment plants. These plants were tagged according to their treatment ($^{15}NH_4NO_3$

128treatment or $NH_4^{15}NO_3$ treatment). In addition, four corn and four soybean plants were

129randomly selected as control. The F1 generation seeds were germinated in a greenhouse

130using commercial potting soils. On the 4th week after planting (WAP), after washing the

131roots to remove the remaining potting soils, each of the 20 soybean and 20 corn plants was

132individually transferred into pots (one plant per pot; pot surface diameter 27.5 cm)

133containing silica sand. Commercial slow-release fertilizer (Osmocote Smart-Release®)

134containing 15% N (8.4% as NH_4^+-N and 6.6% of NO_3^--N) was applied to each pot as the

135only source of N. Detail nutrient content of Osmocote Smart-Release® is available in the

136Supplementary Table S1. The amount of fertilizer applied (5 g and 10 g per pot for soybean

137and corn, respectively) was equivalent to 127.1 kg ha^{-1} N for soybean and 254.2 kg ha^{-1} N

138for corn (surface area of pot: 0.059×10^{-4} ha). This fertilization rate is approximately

139equivalent to the recommended rate for corn growing in the field (212.8-268.8 kg N ha^{-1})

140(Oberle *et al.*, 1990). While N addition is generally not made to field-grown soybean crops,

141application of this slow-release N fertilizer was needed to support plant N nutrition in the

142N-poor sand medium. Slow-release fertilizer was used instead of common N sources such

143as urea or anhydrous ammonia due to the lack of organic matter and reactive surfaces in

144the sand medium that could retain nutrients during the course of the experiment.

145Commercial inoculants (Burpee Booster, Warminster, PA) were used at the time of planting

146to induce root nodulation in soybean. Dicyandiamide (DCD) solution (equivalent to 4.5 kg

147DCD ha^{-1} for soybean and 9 kg ha^{-1} for corn) was applied weekly to prevent nitrification

148during the growth of the F1 generation plants. The rate of DCD application was within the

149range of the recommended values (1-5%) (Azam *et al.*, 2003), but it was applied more

150frequently than DCD half-life (i.e., between 18 and 25 days at 20°C) (Di *et al.*, 2005) to

151ensure that nitrification is effectively inhibited and that mineral N remains in the NH_4^+

152form. Conserve® and Endeavor® insecticides were also applied periodically to prevent

153flower thrips (*Frankliniella occidentalis*) and aphids outbreak.

154^{15}**N labelling**

155Both $^{15}NH_4NO_3$ and $NH_4^{15}NO_3$ were selected to provide an equal molar contribution of

156NH_4^+ and NO_3^- to the plants, so that isotopic fractionation should have a minimal effect on

157the observed $\delta^{15}N$ signature patterns. The $^{15}NH_4NO_3$ was used for the NH_4^+ treatment while

158the $NH_4^{15}NO_3$ for NO_3^- treatment. For field labelling (P generation), 19.52 mg of $^{15}NH_4NO_3$

159was combined with 8.64 g of NH_4NO_3 and dissolved in 480 ml of deionized water. The

160procedure was repeated for $NH_4^{15}NO_3$. In the field, six pairs of soybean plants were tagged

161and applied with ^{15}N labeling during two consecutive non-rainy days (4 and 5 August

1622015). The same procedure was also applied for six pairs of corn plants. A much higher

163field enrichment (i.e., equivalent to 24, 000‰ or 40-times higher than the amount in

164greenhouse experiment) was designed to ensure that a traceable isotope signal could be

165detected due to a relatively higher uncertainty (more variability) in the field. Meanwhile,

166the application duration (one injection per 24 h interval and 48 h in total) was also

167designed to minimize biases caused by: (i) the potential N transformation processes in the

168plant medium (Wang and Macko, 2011) and (ii) rapid but oscillating plant uptake since

169studies have shown that plants could respond to nutrient addition within hours of

170application (Jackson *et al.*, 1990; Rabie *et al.*, 1980; Wang and Macko, 2011; Wang *et al.*,

1712006).

172   For greenhouse labeling (F1 and F2 generation), a much smaller amount of

173NH_4NO_3 (0.216 g) was used in combination with 0.488 mg $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$

174(equivalent to 600‰), although the same amount of deionized water (480 ml) was used for

175each $^{15}NH_4{}^+$ and $^{15}NO_3{}^-$ treatment. For both F1 and F2 generations, watering was stopped

176approximately 24 hours before labeling. In each generation, six pairs of soybean plants

177were labeled with $^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$ on two consecutive days. A total of 16 plants

178(12 labeled and 4 control) were then harvested 24 hours after the second day of labeling

179and washed to remove any potential labels that remained on the plant surface. The plants

180were then dried in an oven (60˚C) until constant weight and then homogenized separately

181for the leaves, stems, roots and pods. The same procedure was also applied for the 20 F1

182corn plants. No corn plants produced seeds at the time of harvest. The remaining four

183plants (after 16 being used for treatments) were grown until maturity to produce F2 seeds.

184   For each day of application, 240 ml of the $^{15}NH_4NO_3$ solution was given to six

185soybean plants; each plant received 40 ml of the $^{15}NH_4{}^+$ treatment. Similarly, 240 ml of the

186$NH_4{}^{15}NO_3$ was given to different six soybean plants receiving $^{15}NO_3{}^-$ treatment, resulting in

187a total of six pairs of soybean plants for $^{15}NH_4{}^+$ and $^{15}NO_3{}^-$ treatment. This solution was

188added in the morning around the soil surface (i.e., approximately 1 cm from the stem base)

189as carefully as possible to avoid touching any of the plant parts. A similar solution was also

190prepared and given to six pairs of corn plants. The same procedure was repeated on the

191following day for both soybean and corn.

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192   For F1 plants, out of the 20 soybean or 20 corn plants, 12 were treated with ^{15}N-

193enriched N at 10 WAP and harvested. Four of the remaining plants were also harvested at

19410 WAP as control while the rest were left to produce seeds. These seeds were then

195harvested upon maturity and considered as F2 generation. The same planting procedure as

196the F1 generation was then repeated to test whether the N preference was carried over to

197the F2 generation. The only exception was that the F2 generation plants were planted in a

198NO_3^--N-rich medium to reflect the environmental changes between F1 and F2 generation

199(Fig. 1), by not applying DCD. Different measures, however, had to be applied for corn

200plants since they failed to produce seeds in the sand medium. Ten additional F1 corn plants

201were then planted in the commercial potting soil medium and treated with the same amount

202of slow-release fertilizer as those grown in the sand under the same time frame (i.e., 4

203WAP). We assumed that the initial soil nutrient content in the soil potting mix had been

204consumed during the first four week period, which was equal to the germination period of

205the plants before being transferred to the sand. These soils were also tested for their NO_3^-

206and NH_4^+ content (Electronic Supplemental Material Fig. S1), following the same

207procedures for the measurement of NO_3^- and NH_4^+ content in the silica sand. The seeds

208produced from this soil medium was considered the F2 generation of the corn plants.

209**Harvesting and laboratory analyses**

210All plants were then harvested 24 hours after the second day of isotopic labeling, and

211thoroughly washed to remove any labeled salts that potentially remained on the plant

212surface. We also took four unlabeled plants that were used as control. The plants were

213chopped into pieces, then dried to constant weight in an oven (60˚C), and different plant

214 parts (leaves, stems, roots and pods for soybean only) were ground (homogenized) into a

215 fine powder.

216   Plant parts were analyzed separately to identify plant organs where most of NO_3^- or

217 NH_4^+ assimilation occurs. These organs were selected in light of the general knowledge that

218 NO_3^- (~33%) is reduced in corn roots, partially stored in the stalk (inside the pith cells) and

219 assimilated in the leaves where the bulk of enzymes for NO_3^- assimilation are located

220 (Hageman 1984). Pods were also used to indicate NO_3^- preference but only for soybean as

221 pods are considered as active sinks for newly metabolized NO_3^--N (Rabie *et al.*, 1980). In

222 contrast, NH_4^+ preference was only indicated by the difference between $^{15}NO_3^-$ and $^{15}NH_4^+$

223 in the roots since soil-derived NH_4^+ is assimilated exclusively in plant roots and is not

224 transported to the xylem (Pearson *et al.*, 1993). Although both F1 and F2 generations of

225 soybean produced nodules, we did not separate the nodules from the roots because a

226 previous study has shown that among all plant parts, the abundance of ^{15}N was the least in

227 nodules (order: root > stem > leaf > pod > nodule) (Rabie *et al.*, 1980).

228 **Soil sampling and analysis**

229 To monitor the temporal variation of NO_3^- and NH_4^+ concentration in the growing medium,

230 planting medium samples were taken on the 5, 6, 8, 10 WAP from five-randomly selected

231 pot for plant growing in the greenhouse, including the soil used to grow seed-producing F2

232 corns. The NO_3^- and NH_4^+ concentration of the field soils (0-5 cm), however, was only

233 measured before isotope application (i.e., 4 August 2015) since there have been consistent

234 findings (e.g., across 35 sites) that NO_3^- concentration in agricultural soils is generally

235 higher than NH_4^+ (Crawford and Glass, 1998). All sand and soil samples were extracted

236 with water (soil:water ratio = 1:2). Extract was analyzed for NH_4^+ using method microplate

237 reader (Sims *et al.*, 1995) and for NO_3^- using EPA method 353.1 on an Aquachem Konelab

238 20 photometric analyzer (EST Analytical, Fairfield, OH).

239 Isotope analysis

240 The homogenized biomass of all treatments was each weighed into tin capsules (between 1

241 and 2 mg) for isotopic analysis. The nitrogen isotope ($\delta^{15}N$) composition was measured on

242 an elemental analyzer (ECS 4100, Costech Analytical) connected under continuous helium

243 flow to an isotope ratio mass spectrometer (Delta V Plus, Thermo Scientific). By

244 convention, the $\delta^{15}N$ values were corrected and reported in units of ‰ relative to

245 atmospheric N_2. The working reference materials are USGS 40 ($\delta^{15}N$ = -4.52‰) and USGS

246 41 ($\delta^{15}N$ = 47.57‰). Reproducibility of these measurements was approximately ±0.2‰.

247 Statistical analysis

248 A student t-test was used to compare plant medium NH_4^+ and NO_3^- concentration.

249 Meanwhile a one-way ANOVA was used to evaluate the significance of the differences

250 between $^{15}NH_4^+$ and $^{15}NO_3^-$ detected in different plant parts for all species across

251 generations, followed by a Tukey *post hoc* test (at $P = 0.05$ significance level). All analyses

252 had equal sample sizes. To facilitate the visualization of the results, the degree and forms

253 of N uptake preference at each generation were summarized for both field (P generation)

254 and greenhouse settings (F1 and F2 generation), but only when the difference between

255 $^{15}NH_4^+$ and $^{15}NO_3^-$ was significant. The degree and form of N uptake preference were

256 indicated by differences between the $\delta^{15}N$ values of $^{15}NH_4^+$ and $^{15}NO_3^-$ either in root (for

257 NH_4^+ and NO_3^- preference) or in leaves, stems and pods (for NO_3^- preference) (Wang and

258Macko, 2011). If the difference ($^{15}NH_4^+$-$^{15}NO_3^-$) was positive, the plant preferred NH_4^+; if

259negative, the plant preferred NO_3^-. A value of 0 indicated that no significant preference was

260detected (Wang and Macko, 2011).

261

262**Results**

263**$NH_4^+$and NO_3^- in planting medium**

264Nitrate concentration was higher in the corn than in the soybean field although the

265NH_4^+:NO_3^- ratio was considered similar in both fields (Fig. 1a). During the F1 generation,

266DCD application successfully suppressed nitrification, resulting in higher NH_4^+:NO_3^- ratios

267compared to the field condition (Figs. 1b-c). The slow-release fertilizer also provided an

268almost constant supply of N during the experiment for both corn and soybean (Figs. 1b-e).

269While the planting medium during F2 generation provided a slightly higher amount of

270NH_4^+ and NO_3^- compared to the field condition, the NH_4^+:NO_3^- ratio was considered similar

271for the field and F2 generations (Figs. 1a, 1d-e).

272   The low field soil NO_3^- and NH_4^+ concentration (i.e., between 1 and 2 mg kg^{-1} soil)

273towards the end of the growing season (August 2015) was likely caused by a combination

274of plant uptake and nutrient leaching due to an excessive amount of rainfall in July 2015

275(i.e., 333 mm), which was considered as the wettest July recorded in the history of this area

276according to the National Weather Service (2015). For comparison, the recent (2013-2014)

277soil NO_3^- and NH_4^+ concentrations (0-20 cm) in Indiana range between 2 and 5 mg kg^{-1} soil

278with the NH_4^+:NO_3^- ratio towards the end of the growing season is 3.36:4.45, while the 30-

279year average of July rainfall is 97 mm (Scott, 2015).

280 Field (P generation) N preference

281 When the field (P generation) soil was dominated by NO_3^-, our isotopic results showed that

282 both corn and soybean preferred NO_3^- than NH_4^+ (Figs. 1 and 2). However, greater $^{15}NO_3^-$

283 signal was observed in corn than in soybean. A significantly higher uptake of $^{15}NO_3$ was

284 only found in soybean leaves while it was apparent in corn roots, stems and leaves (Fig. 2).

285 F1 generation N preference

286 In contrast, when NH_4^+ was the dominant N species in the growth medium (i.e., F1

287 generation) (Figs. 1b-c), both corn and soybean exhibited a strong preference for NH_4^+,

288 indicated by significantly higher $^{15}NH_4^+$ than $^{15}NO_3^-$ signal, particularly in the roots.

289 Soybean preference for NH_4^+ was relatively higher than with corn (Figs. 3 and 5). A higher

290 $^{15}NH_4$ uptake was observed in root, stem, leaf and pod of soybean plant parts while it was

291 only apparent in corn roots and stems (Fig. 3).

292 F2 generation N preference

293 When the planting medium was dominated by NO_3^- during the F2 generation (Fig. 1d-e),

294 the NH_4^+ preference observed during F1 generation was reversed (Fig. 4). A more

295 significant $^{15}NO_3^-$ preference was found in corn with a significantly higher $^{15}NO_3^-$ content

296 found in all corn parts, but for soybean, it was only found in the pod (Figs. 4 and 5). This

297 finding was therefore similar to the field (P generation) where we found greater $^{15}NO_3^-$

298 uptake when the soil was dominated by NO_3^- (Figs. 1, 2, and 4).

299

300 Discussion

301 Factors regulating N preference

302Unlike the results of Wang and Macko (2011), the 'memory' effect (i.e., consistent

303preference) across the three generations of agricultural crops was not found in this study

304(i.e., soybean and corn), likely because these species are non-specialized species that grow

305in non-specific niche or habitat. Instead, corn and soybean were able to shift their N

306preference in each generation to the more abundant form of inorganic N available in the

307soil. Some cereal species, such as barley and oat show a similar trend of shifting preference

308following changes in the soil $NH_4^+:NO_3^-$ ratio (Cui *et al*., 2017). Shoot NO_3^- assimilation

309also generally increases as external NO_3^- concentration increases for annual legumes and

310non-legume species (Andrews, 1986). In forest ecosystems, tree species, Hinoki cypress

311(*Chamaecyparis obtusa* Endlicher), has flexibility in absorbing either NH_4^+ or NO_3^-,

312depending on availability (Takebayashi *et al.*, 2010). Similarly, functionally diverse plant

313groups show inherent flexibility to absorb different N forms by consistently preferring the

314more abundant form of inorganic N in the soil (Houlton *et al*., 2007). Our results provide

315further evidence that plant preference for a given N form (NH_4^+ or NO_3^-) may shift and the

316shift is mainly determined by the abundance and availability of each form, as has been

317indicated by several other studies (Cui *et al*., 2017; Houlton *et al*., 2007; Kronzucker *et al.,*

3181997; Smith *et al*., 1990; Wallander *et al*., 1997; Wang and Macko, 2011). We suggest,

319however, that there is a threshold for $NH_4^+:NO_3^-$ ratio before such preference changes and

320the threshold is related to, for example, the precipitation gradient in natural system (Wang

321and Macko, 2011). Higher $NH_4^+:NO_3^-$ ratio in the wetter end of the gradient due to high

322decomposition rate (more ammonium availability) but high NO_3^- leaching leads to plant

323 preference towards NH_4^+ (Wang and Macko, 2011). There is a switch, however, to NO_3^-

324 preference as $NH_4^+:NO_3^-$ ratio becomes lower at the drier sites (Houlton *et al.*, 2007).

325   While some plants can switch their nutrient uptake preference depending on

326 nutrient availability (i.e., $NH_4^+:NO_3^-$ ratio), strong preference towards a specific N form has

327 been observed in some plant species. Since NH_4^+ uptake and its assimilation in general are

328 considered to be energy efficient, one can assume that NH_4^+ will be the preferred N form,

329 at least under equimolar $NH_4^+:NO_3^-$ scenarios. However, past studies have shown that, in

330 some species, NH_4^+ was not always the preferred N form to support plant growth (Errebhi

331 *et al.*, 1990; Smith *et al.*, 1990). In a pearl millet study, Smith *et al.* (1990) shows that

332 when present in an equimolar ratio, NO_3^- is preferred over NH_4^+. However, when the

333 $NH_4^+:NO_3^-$ ratio is increased to 3:1, the plant was forced to absorb more NH_4^+, but overall

334 N uptake was reduced resulting in lower yield (Smith *et al.*, 1990). Similarly, Errebhi *et al.*

335 (1990) find that corn experiences a reduction in dry weight and cation absorption (K^+, Ca^+,

336 Mg^{2+}) when provided with an equimolar amount of NH_4^+ and NO_3^-. The same finding,

337 however, is not observed at low (i.e., <1) $NH_4^+:NO_3^-$ ratios. In contrast, high NO_3^-

338 concentration is found to have an inhibitory effect on nodule growth and activity in several

339 legumes species, including soybean, white clover (*Trifolium repens* L.), and pea (*Pisum*

340 *sativum* L.), by reducing nitrogenase enzyme, which is not observed in soil with high NH_4^+

341 concentration (Bollman *et al.*, 2006; Streeter, 1985; Svenning *et al.*, 1996). Bean

342 (*Phaseolus vulgaris* L.) dry weight is also not significantly different between different

343 $NH_4^+:NO_3^-$ ratios which varied from 0:11, 1:10, 2:9 and 1:1 (Errebhi and Wilcox, 1990).

344   Apart from species, the shift of plant N preference is also affected by plant

345 physiological maturity, either under a changing (Cui $et\ al.$, 2017) or constant (Smith $et\ al.$,

346 1990) NH_4^+: NO_3^- ratio. Smith $et\ al.$ (1990) and Cui $et\ al.$ (2017) both find that during their

347 early growth phase, plants prefer NH_4^+ over NO_3^-, but such preference is reversed during

348 late growth phase, which could be related to: (i) NH_4^+ detoxification and/or (ii) the

349 reduction of carbohydrate available associated with the beginning of reproductive phase.

350 This pattern of juvenile NH_4^+ preference and mature plant preference for NO_3^- is found to

351 be quite consistent across different cereal species, including rice ($Oryza\ sativa$ L.), pearl

352 millet, barley and oat (Cui $et\ al.$, 2017; Errebhi and Wilcox, 1990; Smith $et\ al.$, 1990).

353 Therefore, the same tendency for NO_3^- preference by mature plants would explain the

354 strong NO_3^- preference in corn plants near harvest time (10 WAP; Figs. 2, 4).

355   Plant N preference can also be linked to the need of growing plants to maintain

356 their anion or cation balance (von Wirén $et\ al.$, 1997), and is therefore highly time

357 dependent (Rabie $et\ al.$, 1980). An increase of NO_3^- uptake rate in corn was observed that

358 experiencing N deficiency only within a few hours after N addition (Rabie $et\ al.$, 1980; von

359 Wirén $et\ al.$, 1997), although the rate could decline after a prolonged exposure to NO_3^-.

360 This result suggests that there is a potential continuous feedback from the plant since plant

361 NO_3^- uptake will again increase if NO_3^- availability in the soil declines (von Wirén $et\ al.$,

362 1997). Similar increase of NO_3^- uptake, however, is not observed with NH_4^+ (von Wirén et

363 $al.$, 1997). The latter finding was consistent with our F1 generation observation (Fig. 3)

364 where there was a relatively low NH_4^+ uptake for corn despite it was grown under N-

365 limited conditions (as indicated by its failure to produce seed). Based on potential risk of

366NH$_4^+$ toxicity, it is suggested that certain plants (i.e., corn in our study) will avoid NH$_4^+$

367accumulation, likely because the rate of NH$_4^+$ assimilation is generally lower than its

368uptake (von Wirén *et al.*, 1997).

369**Physiological mechanisms of N preference**

370Plant species preference towards NO$_3^-$ or NH$_4^+$ can vary from one species to another. While

371most cereal species seemed to prefer NO$_3^-$ over NH$_4^+$, except for those grown in acid soils

372such as rice which showed preference towards NH$_4^+$ (Zhao *et al.*, 2013), legumes have

373shown greater flexibility in regards to both forms of N, although there was a tendency

374towards NH$_4^+$ preference (Figs. 2-5). In our experiment, greater NH$_4^+$ incorporation (almost

375double) than NO$_3^-$ was shown within 48 hours after treatment into soybean roots (Fig. 3),

376consistent with a study by Ohyama *et al.* (1989). In soybean, NH$_4^+$ is actively incorporated

377into amides, much of which remains in the roots (Weissman, 1972). Stronger preference to

378NH$_4^+$ in soybean may also be caused by the long-term effect of N assimilatory products

379(i.e., amino acids) since NO$_3^-$ uptake can be inhibited by phloem-translocated amino acids

380such as alanine, glutamic acid, aspartic acid, arginine and asparagine (Muller *et al.*, 1992).

381   Although the presence of mycorrhiza was not checked in our study, in its natural

382habitat, close association with mycorrhiza likely accounts for the preferential uptake of

383NH$_4^+$ by soybean. Mycorrhiza has been known to absorb NH$_4^+$ and transfer a substantial

384amount of N to the host plant (Kobae *et al.*, 2010). Mycorrhiza has also been shown to

385enhance NH$_4^+$ but not NO$_3^-$ absorption rates (Kronzucker *et al.*, 1997), likely because there

386are two NH$_4^+$ uptake sites for the mycorrhizal plants: the root-soil interface and the

387hyphae-soil interface (Marschner *et al.*, 1994). The recent discovery of membrane protein

388(nod26) confirmed the increase in permeability of root membrane to NH_4^+, allowing

389transport of fixed N_2 in the form of NH_3 from the symbiosome (Hwang *et al.*, 2010). Of the

39016 NH_4^+ transporter genes found in the soybean genome, five were induced by mycorrhiza

391(Kobae *et al.*, 2010).

392   On the other hand, the relatively fewer NH_4^+ transporters found in the corn genome

393could cause the lower affinity for NH_4^+ observed in corn (Figs. 3 and 5). So far only two

394NH_4^+ transporter genes have been isolated from the corn genome: ZmAMT1;1a and

395ZmAMT1;3 (Gu *et al.*, 2013). These transporters were based on High Affinity Transport

396Systems in corn roots which allowed NH_4^+ ions to be absorbed according to the

397electrochemical gradient (Gu *et al.*, 2013). As NH_4^+ concentration in plant cells increased,

398a reduction in NO_3^- uptake capacity was observed (George, 2014, MacKown *et al.*, 1982)

399since NH_4^+ would cause membrane depolarization and block the anion transport system

400(Ullrich, 1992). This trend, however, could be reversed by growing the plant in a NO_3^--rich

401medium (George, 2014). The same observation was also noted in pearl millet (Smith *et al.*,

4021990), likely due to the inhibition of NO_3^- reductase activity by NH_4^+.

403   Overall, we found that plant preference is dependent upon NH_4^+:NO_3^- ratio,

404although plants have a certain degree of preference in their ability to absorb either NH_4^+ or

405NO_3^-. In natural ecosystems, pine trees, that have generally been considered as NH_4^+

406absorber, can show high reliance on NO_3^- as NO_3^- becomes more available in the soil

407(Takebayashi *et al.*, 2010). In our experiment, stronger preference for NO_3^- was observed

408in corn (Fig. 5), consistent with the observation that most agricultural crops appeared to

409prefer NO_3^- over NH_4^+ (Britto and Kronzucker, 2013). Some exceptions were observed in

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410plants that have the ability to assimilate NH_4^+ (i.e., legumes) (Fig. 5) or adapted to live in

411waterlogged and acid condition (e.g., rice) (Zhao *et al.*, 2013). Since high NH_4^+

412concentration is toxic to plants, the amount of NH_4^+ is kept to a minimum in plant tissue by

413two mechanisms: (i) assimilation in the roots and/or (ii) enhanced efflux of NH_4^+ to the

414external medium (Britto and Kronzucker, 2013). Both processes have been suggested to

415eliminate any energetic advantages conferred by the uptake of NH_4^+ (Britto and

416Kronzucker, 2013). Ammonia assimilation in the roots reduces the amount of carbon

417available for plant growth and maintenance, and at the same time, increasing efflux of

418NH_4^+ to external medium is an 'energetically costly futile cycle' (Britto and Kronzucker,

4192013). Indeed, a reduction in the amount of soluble sugar content in corn shoot tissue was

420significantly reduced when plant was treated with NH_4^+ due to high requirement of carbon

421skeletons for NH_4^+ incorporation into amino acids (Magalhães *et al.*, 1993).

422   In our study, it was shown that irrespective of generation, both corn and soybean

423consistently exhibited a preference for the N species that dominate the mineral N pool in

424the growing medium and stronger responses were generally observed in roots compared to

425other plant tissues. While plants will try to make use of the most available form of N in its

426growing medium, plant species, physiological characteristics (i.e., maturity, association

427with mycorrhiza) and plant nutrient status also determine the N uptake preference. In our

428study, the magnitude of the response of soybean (legume) towards NH_4^+ was generally

429stronger than that of corn (non-legume). Although our study showed that soybean and corn

430did not have consistent plant preference (or 'memory' effect), there was a possibility that

431either plasticity or consistency was genetically inherited. Consistency across plant

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432generations (as observed by Wang and Macko (2011), however, are more likely observed

433in habitat specialists, such conifers that live in acid soils. For agricultural crops, plasticity,

434as shown in our study, is beneficial from the evolutionary point of view because plants can

435effectively acquire available N to alleviate their N demand according to availability of the

436dominant N forms (Cui et al., 2017) and maintain their productivity. From an agronomical

437perspective, our understanding on plant nutrient preference and a plant's ability to switch

438between different N forms is also important to adapt to other environmental changes,

439particularly changes in precipitation. Reduction or increase in the amount of rainfall may

440potentially increase NH_4^+:NO_3^- ratio by slowing down the rate of nitrification or reducing

441the concentration of NO_3^- since NO_3^- is a much more leachable N species than NH_4^+.

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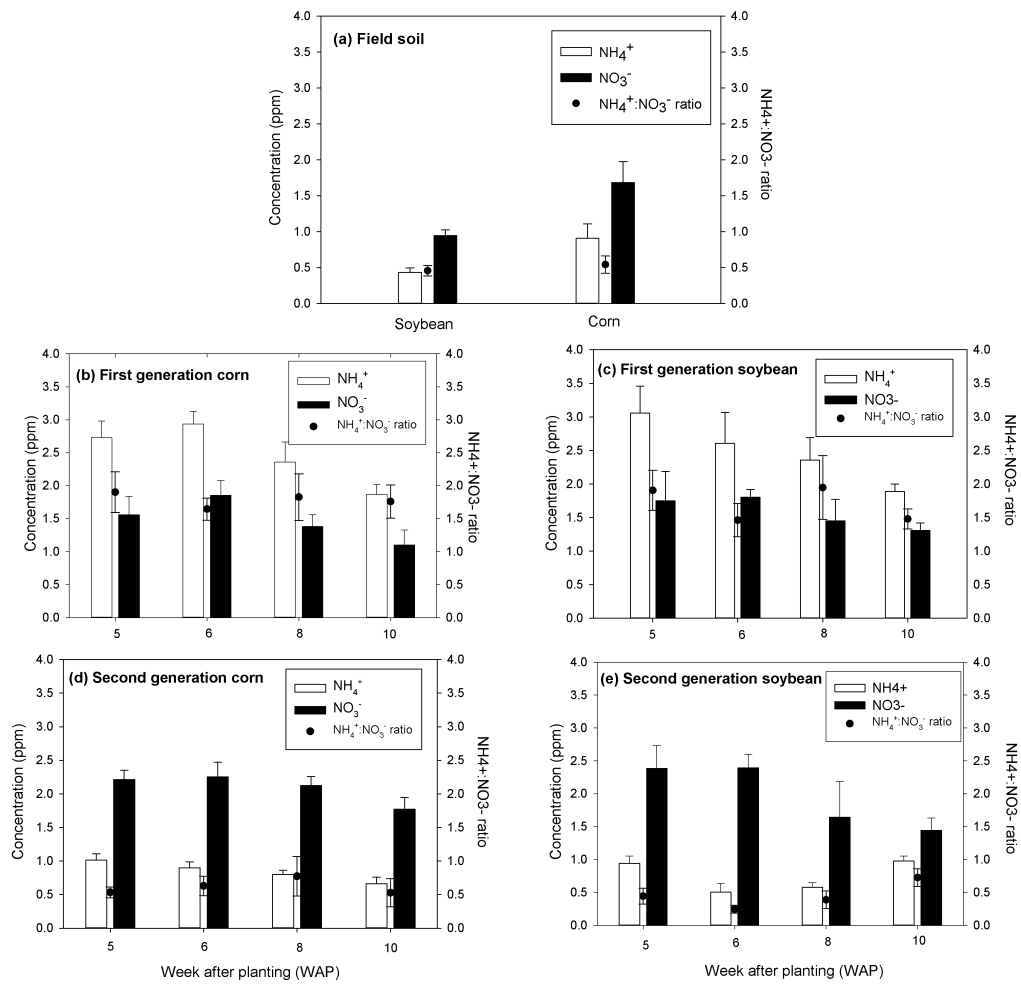557 soybean and sunflower. *Plant Physiol* **49**:138-41.

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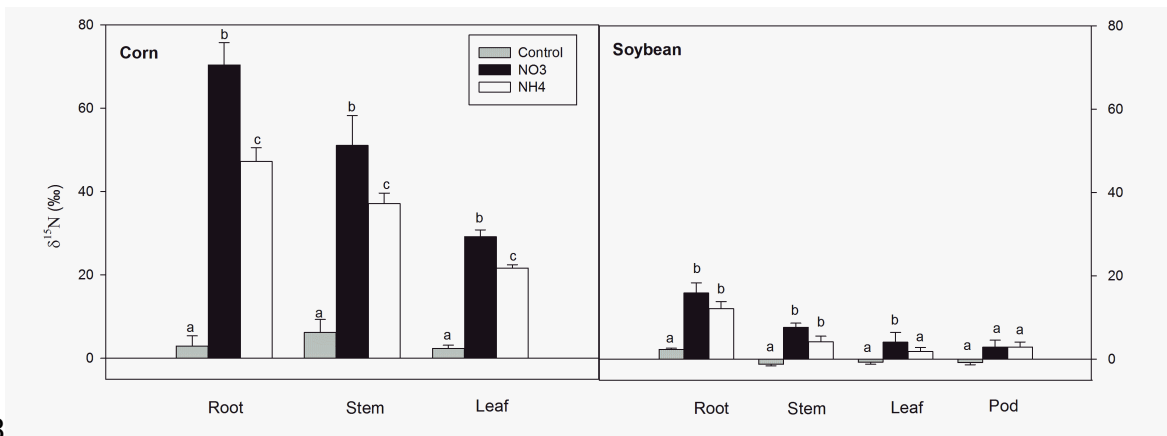
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Figure captions

565Fig. 1. Ammonium and nitrate concentration (mean ± SD) in plant growing medium across

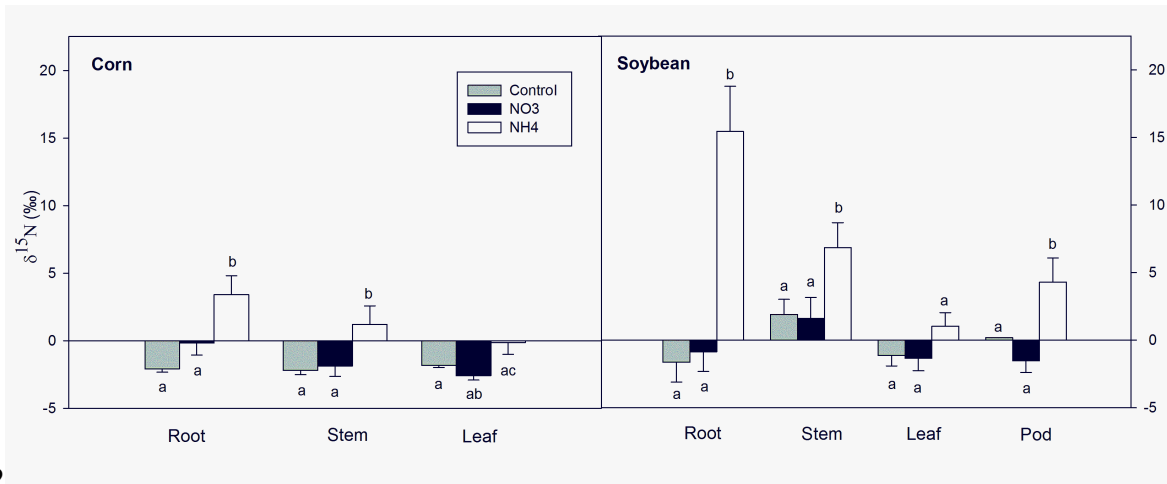566several generations of soybean and corn.

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569 Fig. 2. Differences of N uptake in different plant parts for corn and soybean in the field (P

570 generation; mean ± SD). Letters 'a', 'b' and 'c' indicate significant differences at $P<0.05$.
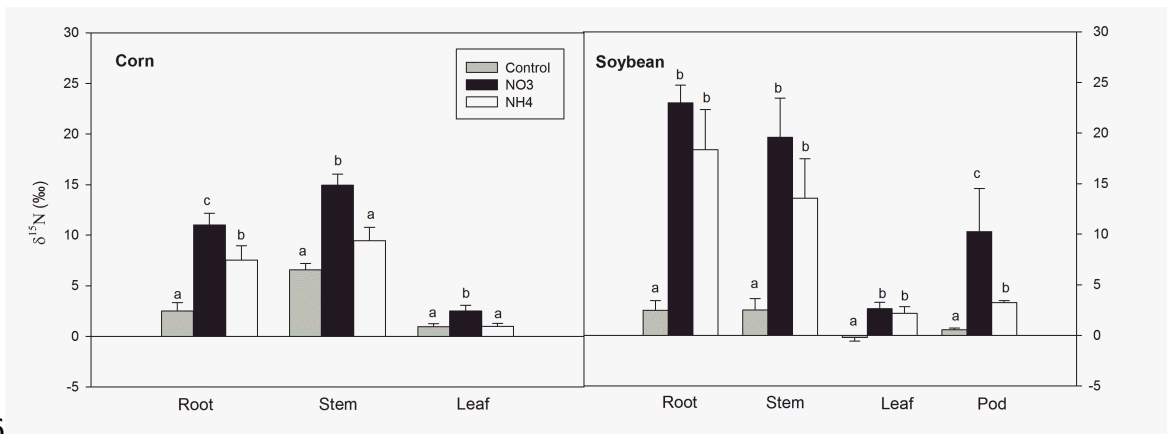
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573 Fig. 3. Differences of N uptake in different plant parts for corn and soybean during F1

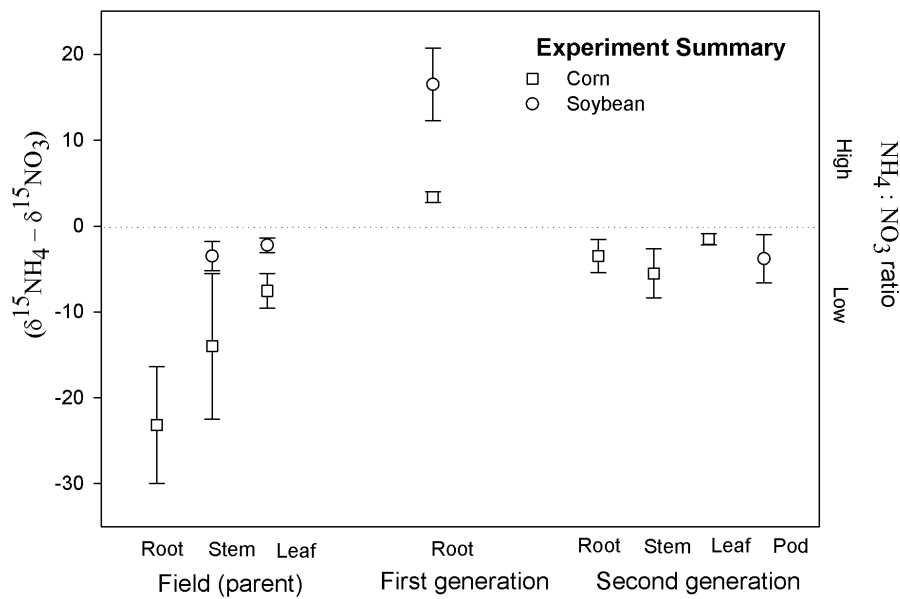574 generation (mean ± SD). Letters 'a', 'b' and 'c' indicate significant differences at $P<0.05$.

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577 Fig. 4. Differences of N uptake in different plant parts for corn and soybean during F2
578 generation (mean ± SD). Letters 'a', 'b' and 'c' indicate significant differences at $P<0.05$.

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581 Fig. 5. Summary of N preference for soybean and corn (mean ± SD) under a changing
582 NH_4^+:NO_3^- ratio.