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## **Maize(*Zea mays*L.) interaction with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* allows mitigation of nitrogen deficiency stress: physiological and molecular characterization**

Béregère Decouard, Niaz Bahar Chowdhury, Aurélien Saou, Martine Rigault, Isabelle Quilleré, Thomas Sapir, Anne Marmagne, Christine Paysant Le Roux, Alexandra Launay-Avon, Florence Guerard, et al.

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1 [Maize \(\*Zea mays\* L.\) interaction with the arbuscular mycorrhizal fungus](#)  
2 [Rhizophagus irregularis allows mitigation of nitrogen deficiency stress:](#)  
3 [physiological and molecular characterization](#)

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36 Key words: N use efficiency; Nitrogen uptake; arbuscular mycorrhizal fungi, Maize, metabolic  
37 modelling.

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41

42 **Abstract**

43 Maize is currently the most productive cereal crop in the world ([www.faostat.org](http://www.faostat.org)). Maize can  
44 form a symbiotic relationship with the Arbuscular Mycorrhizal Fungus MF, *Rhizophagus*  
45 *irregularis*. In this relationship, the fungus provides the plant with additional water and  
46 mineral nutrients, while the plant supplies carbon compounds to the fungus. Two maize lines  
47 were studied, and they exhibited contrasting responses to AMF inoculation based on their  
48 physiological and molecular characteristics. Interestingly, the beneficial effects of the AMF  
49 were observed mainly under conditions of limited N fertilization. Under such conditions, the  
50 AMF helped maintain plant biomass production even when there was a significant reduction  
51 in N supply. The availability of nitrogen was found to be a crucial factor influencing all the  
52 traits studied. This suggests that the level of N supply plays a pivotal role in determining how  
53 the maize plants interact with the AMF. Despite the two maize lines showing different  
54 transcriptomic and metabolomic responses to *R. irregularis*, their agro-physiological traits  
55 remained similar. This indicates that while there may be genetic differences in how the plants  
56 respond at the molecular level, the overall growth and productivity outcomes are comparable.  
57 Both the plant and fungal transcriptomes were more significantly influenced by the level of N  
58 nutrition rather than the specific maize genotype. This suggests that N availability has a more  
59 profound impact on gene expression in both organisms than the genetic makeup of the  
60 maize plant. To understand the metabolic implications of this symbiotic relationship, we  
61 integrated transcriptomic data into a multi-organ Genome-scale metabolic model (GSM)  
62 called iZMA6517 based on a stoichiometric approach. This modelling approach highlighted  
63 nucleotide and ureides metabolism as previously unrecognized factors contributing to the  
64 symbiotic N nutrition facilitated by *R. irregularis*, thereby enhancing maize growth.

## 65 **Introduction**

66 Nitrogen (N) is the fourth most abundant component of plant biomass, which explains its  
67 pivotal role in agriculture (Hirel *et al.*, 2011; Hirel and Krapp, 2020). Indeed large inputs of N  
68 fertilizers are necessary to ensure optimal crop productivity (Hirel *et al.*, 2007, 2011).  
69 However, only 30 to 50% of the N available in the soil is taken up by most crops, resulting in  
70 important N losses which can cause damage to the environment such as water pollution and  
71 increased greenhouse effect (Schlesinger, 2009; Lassaletta *et al.*, 2016). It is urgent to  
72 minimize these impacts while simultaneously meeting the increasing demand for food  
73 production resulting from population growth. Ensuring crop productivity while minimizing N  
74 supply can be monitored by measuring nitrogen use efficiency (NUE). A better understanding  
75 of how to enhance NUE is a major challenge in the context of agroecology as it represents  
76 the capacity of the plant to produce grains or vegetative biomass per unit of available N (Hirel  
77 *et al.*, 2007; Lea and Mifflin, 2018; Wang *et al.*, 2018; Liu *et al.*, 2022). Three physiological  
78 processes contribute to NUE: (Masclaux-Daubresse *et al.*, 2010; Lea and Mifflin, 2018) : 1) N  
79 uptake efficiency involved in acquisition N from the soil by means of root-specific inorganic or  
80 organic N transporters (Fan *et al.*, 2017; Wang *et al.*, 2018); 2) N assimilation efficiency  
81 which is the incorporation of inorganic N into organic N-containing molecules necessary for  
82 plant growth and development (Hirel *et al.*, 2011; Hirel and Krapp, 2020; Wani *et al.*, 2021);  
83 and 3) N remobilization efficiency which represents the capacity of the plant to remobilize N  
84 and transfer it to storage organs (Masclaux-Daubresse *et al.*, 2010; Kant, 2018). Direct  
85 uptake of minerals including N and P from the soil by plants is ensured by transporters  
86 known to drive the so called “direct transport”. Interestingly, to fulfil their N-associated  
87 metabolic needs, plants can establish symbiosis with beneficial soil microbes which help to  
88 improve their N nutrition by different mechanisms, some of which are well characterized.  
89 Exploiting the capacity of a crop to benefit from beneficial microbes in terms of N nutrition in  
90 order to maintain yields while reducing chemically synthesized N input is challenging in the

91 context of a more sustainable agriculture (Gianinazzi *et al.*, 2010; Verzeaux *et al.*, 2017; Wipf  
92 *et al.*, 2019; Dellagi *et al.*, 2020; Gojon, 2022; Xie *et al.*, 2022).

93 The best characterized symbiosis which improves legume N nutrition occurs when symbiotic  
94 bacteria from the Rhizobiaceae family allows fixation of atmospheric N<sub>2</sub> and its reduction to  
95 NH<sub>4</sub><sup>+</sup> readily usable by the host plant (Udvardi and Poole, 2013; Masson-Boivin and Sachs,  
96 2018). Nitrogen can also be transferred from the soil to the roots when the plant is colonized  
97 by Arbuscular Mycorrhizal Fungi (AMFs), a symbiotic process occurring in 80% of land plants  
98 including cereals (Gianinazzi *et al.*, 2010; Chen *et al.*, 2018a; Gojon, 2022; Xie *et al.*, 2022).  
99 AMFs are obligate biotrophs, meaning that they rely on their living host plants to complete  
100 their life cycle. The early steps of AMF symbiosis involve signal exchange between the host  
101 roots of the host plant and the fungus. This signalling process leads to establishment of a  
102 physical contact between the partners during which the fungus develops structures like a  
103 hyphopodium on the root epidermal cell followed by its growth inside these cells up to the  
104 cortical cells where it differentiates into highly branched structures called “arbuscules”.  
105 Around these arbuscules, the cortical cells undergo strong reorganization leading to the  
106 formation of a structure called “periarbuscular membrane” (PAM). Dual nutritional exchange  
107 occurs at the interface of this membrane where the transfer of carbon containing molecules  
108 derived from photosynthesis such as hexoses and lipids feed the fungus, which in turn  
109 provides minerals such as N and P (Smith and Read, 2002; Gianinazzi *et al.*, 2010; MacLean  
110 *et al.*, 2017; Choi *et al.*, 2018; Dellagi *et al.*, 2020). The extra-radical mycelium which  
111 represents an extension of the arbuscular structures, allows the fungus to explore the  
112 surrounding rhizosphere and to take up minerals further translocated to the periarbuscular  
113 interface to be finally delivered to the host plant. In many case, an AMF symbiotic association  
114 is able to increase tolerance to abiotic and biotic stresses (Choi *et al.*, 2018; Berger and  
115 Gutjahr, 2021).

116 It is well established that phosphorus (P) deficiency is the main driver for the establishment  
117 of mycorrhizal symbiotic associations (Breuillin *et al.*, 2010; Smith *et al.*, 2011; Balzergue *et*

118 *al.*, 2013; Wang *et al.*, 2017; Paries and Gutjahr, 2023). Phosphorus deficiency triggers  
119 exudation from roots of signaling molecules such as strigolactones that stimulate AMF  
120 hyphal branching (Lanfranco *et al.*, 2018; Hull *et al.*, 2021). In addition, isotope tracing  
121 experiments have shown that AMF provide phosphorus to their hosts (Sawers *et al.*, 2017;  
122 Das *et al.*, 2022). In contrast, the benefit of a mycorrhizal symbiosis in terms of N uptake  
123 and utilization by the host plant, remains to be fully characterized (Chen *et al.*, 2018a; Dellagi  
124 *et al.*, 2020; Gojon, 2022; Xie *et al.*, 2022). It is known that N can be transported to the plant  
125 by AMF (Courty *et al.*, 2015; Garcia *et al.*, 2016; Chen *et al.*, 2018a). Two transporters that  
126 mediate the mycorrhizal N transport pathway have been identified so far. For example, in  
127 rice, a transporter specifically involved in the transfer of nitrate to the roots of the host plant  
128 via the AMF has been described (Wang *et al.*, 2020). In maize, an ammonium transporter  
129 enabling the uptake of inorganic N has recently been characterised (Hui *et al.*, 2022).

130 Maize is currently the most productive cereal crop in the world ([www.faostat.org](http://www.faostat.org)) with a  
131 global yield exceeding that of rice (Liu *et al.*, 2020). Maize is grown for several purposes  
132 including animal feed, food and biotechnological applications (Ranum *et al.*, 2014; Revilla *et*  
133 *al.*, 2022). Different maize genotypes are able to develop a symbiotic association with AMF  
134 (Sawers *et al.*, 2018a; Berger and Gutjahr, 2021). Several studies have shown that AMF can  
135 supply substantial amount of N to maize (Quiroga *et al.*, 2020; Hui *et al.*, 2022). However,  
136 physiological and molecular characterization of maize's interaction with AMF under different  
137 N supply levels has never been undertaken. We reasoned that utilizing AMF to reduce N  
138 fertilization requires investigating the maize physiological responses to AMF under different  
139 levels of N fertilization. In the present study, we have studied in an integrated manner the  
140 impact of N nutrition on the interaction between maize and the AMF *Rhizophagus irregularis*  
141 both at the physiological and molecular levels. Using phenotyping, transcriptomic and  
142 metabolomic approaches, we have characterized such interaction using two maize lines  
143 which benefit from symbiotic association with the fungus in terms of agronomic performance.

144 Genome-scale metabolic modeling (GSM) is a computational tool to model plant metabolism  
145 and can highlight potential key metabolic pathways in plant in response to environmental

146 cues (Morris *et al.*, 2023). Initial efforts on maize metabolic models were limited to its leaf  
147 (Simons *et al.*, 2014) and root (Chowdhury *et al.*, 2022b) GSMs, which showed excellent  
148 agreement with existing literature regarding phenotypical predictions. Most recently, a multi-  
149 organ GSM of maize line B73, *iZMA6517* (Chowdhury *et al.*, 2023), is proposed which  
150 includes full scale GSMs of root, stalk, kernel, and leaf; connected via phloem tissue.  
151 *iZMA6517* predicted organ-specific metabolic bottlenecks accurately and explored symbiotic  
152 relationship with *R. irregularis*. Thereby, apart from experimental works, we used *iZMA6517*  
153 to predict plant agronomic performance. Interestingly, *iZMA6517* identified purine and  
154 pyrimidine biosynthetic pathways as key physiological processes involved in the efficiency of  
155 N utilization in the symbiotic association.

## 156 **Material and methods**

### 157 **Plant material, growth conditions and inoculation**

158 The maize inbred lines used in this study B73 and Lo3 are described in Supplementary table  
159 1. The reference maize genotype B73, for which the genome was fully sequenced,  
160 represents the reference genome for the maize species (Schnable *et al.*, 2009). Additionally,  
161 B73 is known be responsive to AMF, resulting in increased biomass (Ramírez-Flores *et al.*,  
162 2019; Berger and Gutjahr, 2021). Lo3, was selected due to its enhanced biomass response  
163 following inoculation with *R. irregularis*. This selection was based on the outcomes of two  
164 rounds of screening aimed at identifying maize lines that exhibit increased biomass in the  
165 presence of AMF under limiting N conditions. These screening round were performed on the  
166 core collection previously described (Cañas *et al.*, 2017). The purpose was to identify maize  
167 genotypes which benefits from AMF inoculation (data not shown). The fungal strain used is  
168 the highest quality of purity strain *Rhizophagus irregularis* DAOM197198 (Agronutrition,  
169 Toulouse, France). Inoculum was prepared in distilled water and the required volume of  
170 spore suspension was added next to the germinated seed or plantlet with a pipette.

171 In all experiments, seeds were surface-sterilized as follows. Seeds were first incubated in  
172 ethanol for 5 minutes at 28°C followed by rinsing with distilled water. Then they were  
173 incubated for 45 minutes in a 15% commercial bleach solution with 0.01% Triton x100 then  
174 rinsed in distilled water. They were placed on wet sterile Whatman paper in Petri dishes  
175 closed with parafilm and incubated in the dark for 48-72h at 20°C. Germinated seeds were  
176 selected and then sown in pots of 4 litres containing a mix of sterilized unfertilized peat (40%  
177 Baltic blond peat 0-10 mm, 60% Brown peat 0-10 mm, 75 g oligoelements, pH CaCl<sub>2</sub> 5.3 –  
178 5.8, Stender, France) and dried clay beads (reference: 17831050 BILLE ARGILE LIA 8/16,  
179 Soufflet, France) 1:1/v:v. The experimental design and the different steps of growing and  
180 harvesting of the experiment in 2020 and 2021 are presented in Supplementary Figure 1 and  
181 2 respectively. Meteorological conditions within the greenhouse during 2020 are depicted in  
182 Supplementary Figure 3. For each condition 9-10 plants were used. Three inoculations with  
183 spores of *R. irregularis* were performed as follow: The first inoculation with 500 spores/plant  
184 at sowing, second inoculation 1 week after sowing with 200 spores/plant and a third  
185 inoculation 2 weeks after sowing with 200 spores/plant. Control plants were treated with  
186 distilled water. Plants were grown under three different nitrogen regimes: low N (LN, 1mM  
187 NO<sub>3</sub><sup>-</sup>), medium N (MN, 5 mM NO<sub>3</sub><sup>-</sup>) and high N (HN, 10 mM NO<sub>3</sub><sup>-</sup>). The specific composition  
188 of these regimes is provided in Supplementary Table 2. For each N regime, plants were  
189 irrigated for the first three weeks with the solution corresponding to the chosen N regime with  
190 low P (named P-) containing 10 µM phosphate in order to trigger AMF colonization. Then, at  
191 dates indicated in Supplementary Figure 1 and 2, irrigation was changed to the same N level  
192 but 300 µM P (named P+, Supplementary Table 2). Increasing P level in the irrigation  
193 solution was necessary to allow plants reach the maturity stage. To determine <sup>15</sup>N uptake,  
194 <sup>15</sup>N labelling was performed at indicated dates (Supplementary Figure 1). 100 mL of 45 mM  
195 KNO<sub>3</sub> solution enriched at 2% <sup>15</sup>N was applied to each plant in order to provide 1,25mg of  
196 <sup>15</sup>N.

## 197 **Harvesting procedures**

198 Plants grown in 2020 were used for the phenotyping, metabolomics, RNA-seq and RT-qPCR  
199 experiments. Plants grown in 2021 were to monitor fungal colonization and RT-qPCR  
200 experiments. For RNA and metabolite extractions, leaf and root samples were harvested and  
201 frozen in liquid nitrogen before RNA extraction. Depending on the experiment and the  
202 biological question asked, one, two or three developmental stages were chosen for tissue  
203 harvesting. Those stages were determined based a combination of two criteria: (i) the  
204 presence of arbuscular symbiotic structures in maize roots and (ii) plant developmental  
205 stages. The first one is the vegetative stage (VS) corresponding to the 7- to 8-leaf stage  
206 (Cañas *et al.*, 2017). This stage is an indicator of a highly metabolically and  
207 photosynthetically active leaf tissue but only few fungal structures start to be visible in roots.  
208 The second stage corresponds to 15 days after the Vessel Tasselling stage (VT+15 stage)  
209 where root colonization is clearly established and plant is in the early phase of flowering. This  
210 stage was chosen because it allows harvesting roots without imposing too great a stress on  
211 the plant in order to make sure kernels are not affected. The third stage is maturity stage  
212 (MS) and corresponds to plants displaying mature ears. Dates of harvesting for VS, VT+15  
213 stage and MS differed depending on the condition and the year of experiment  
214 (Supplementary Figure 1 and 2). For leaf tissues, harvesting consisted in cutting a piece of  
215 1 cm x 10 cm of leaf mesophyll along the midrib from the 6<sup>th</sup> fully emerged leaf for the VS.  
216 For VT+15 stage and MS, a piece of 1 cm x 10 cm leaf surface from the leaf below the ear of  
217 each individual plant was harvested. The leaf below the ear was chosen for providing a good  
218 proxy of the sink-to-source transition during grain filling (Prioul and Schwebel-Dugué, 1992).

219 Roots were harvested at VS, VT+15 stage or MS depending on the type of experiment. For  
220 each treatment/condition, roots were collected from 5 plants. Thin secondary roots were  
221 harvested from the surrounding root system, close to the pot borders. They were washed  
222 with distilled water, then frozen in liquid nitrogen or stored in distilled water. Following root  
223 harvesting, the plants grown in 2020 were left to grow until maturity. Thus, the roots used for

224 the different omics analyses corresponded to the same plants as those that produced grains  
225 and were phenotyped for physio-agronomical traits.

#### 226 **Quantification of yield, biomass, C, N, <sup>15</sup>N and P**

227 Plant organs were harvested then oven dried at 80°C for 3 to 5 days then ground to fine  
228 powder. Yield was quantified by weighing dry grains. Biomass of the aerial parts including  
229 stalks, leaves, and cobs were weighed after drying. The upper regions of the root system  
230 was harvested. This did not allow to quantify the whole root biomass since it was impossible  
231 but allowed to quantify the percentage of C, N and P. For each plant and each organ, 5,000  
232 µg of dry powder was placed in tin capsules to quantify the total N and C concentrations  
233 using the FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Villebon,  
234 France) and the <sup>15</sup>N enrichment using the Delta V Advantage isotope ratio mass  
235 spectrometer (Thermo Fisher Scientific, France). Supplementary Table 3 indicates the  
236 calculations of different traits with C and N content. A% (Atom percent) represents the <sup>15</sup>N  
237 enrichment in the sample [<sup>15</sup>N/(total N)]. Since the natural <sup>15</sup>N abundance in N unlabeled  
238 samples was 0.3663 (A% control), specific enrichments due to the <sup>15</sup>N uptake were  
239 calculated as E% = (A% - 0.3363). The P concentration of shoots, grains and roots was  
240 measured using the molybdate blue method on a Shimadzu UV-160 spectrophotometer  
241 (Shimadzu Biotech) after acid digestion (Murphy and Riley, 1962).

#### 242 **Quantification of root colonization level by AMF structures**

243 Thin secondary roots were harvested as indicated above and carefully washed in distilled  
244 water. They were incubated in 10% KOH overnight at room temperature. They were then  
245 rinsed in distilled water and incubated in 5% black ink (Sheaffer Skrip Bottled Ink, France)  
246 solution for 10 min at 70°C. Washing was performed by incubating roots in 8% acetic acid for  
247 25 min. For the quantification of % of AM colonization (total, arbuscules, vesicles) 50 pieces  
248 of 1 cm length each were mounted on a slide for observation of AM structures under light

249 microscope using the slide method (Giovannetti and Mosse, 1980). Quantifications were  
250 performed on roots collected from 5 plants for each condition.

### 251 **RNA extraction for RT qPCR and RNAseq**

252 RNA extraction was performed with TRIzol (Thermo Fisher Scientific, USA) protocol. Fresh  
253 tissue of leaves or roots (100 mg) were frozen in liquid nitrogen then ground to fine powder  
254 that was homogenized in 1 mL of TRIzol. Two glass beads are added to the extract in the  
255 tube which was then vortexed for 5 minutes, then the samples were incubated for 5 minutes  
256 at room temperature (RT). Two hundred microliters of chloroform were added then the  
257 sample was vortexed for 5 minutes and incubated 3 minutes at RT. Samples are centrifuged  
258 at 12,000 g for 15 minutes at 4°C. The aqueous phase (approximately 500 µL) was  
259 transferred to a new tube with an equal volume of ethanol (100%). Samples mixed with  
260 ethanol were then transferred to a column (Zymo-Spin™ IC, Germany); for purification  
261 following manufacturer's instructions.

262

### 263 **cDNA synthesis and RT-PCR**

264 1 µg of RNA was first treated with DNase (Thermo Fisher Scientific, USA) in a 10 µL total  
265 volume. 1 µg of DNase treated RNA was then used for reverse transcription using oligo-dT  
266 and the reverse transcriptase Revert aid (Thermo Fisher Scientific, USA) following the  
267 manufacturer's instructions. Quantitative PCR reactions were carried out as described (Verly  
268 *et al.*, 2020). For each sample, a cycle threshold (Ct) value was determined and converted  
269 into Starting Quantity (SQ) also named Arbitrary Units (AU) values based on a standard  
270 curve equation. The AU values obtained for each gene of interest was then divided by the  
271 mean of AU values obtained for the chosen reference gene (indicated in each dataset)  
272 leading to the normalized transcript level value. Primer sequences are described in  
273 Supplementary table 4.

### 274 **RNAseq and Bioinformatic Analysis**

275 RNA-seq libraries were constructed by the POPS platform (IPS2) using the Illumina Stranded  
276 mRNA Prep kit (Illumina®, California, U.S.A.) according to the supplier's instructions.  
277 Libraries were sequenced by the Genoscope (Évry-Courcouronnes, France) on a Novaseq  
278 6000 instrument (Illumina) with 150 base paired-end sequences.

### 279 **Raw Data cleaning**

280 The raw data were treated as follow:

281 1- Illumina filter. Performed as described with chastity cutoff of 0,6.

282 To remove the least reliable data from the analysis, the raw data were filtered to remove any  
283 clusters that have "too much" intensity corresponding to bases other than the called base.  
284 The purity of the signal from each cluster was examined over the first 25 cycles and  
285 calculated as  $\text{Chastity} = \frac{\text{Highest\_Intensity}}{\text{Highest\_Intensity} + \text{Next\_Highest\_Intensity}}$  for  
286 each cycle. The default filtering implemented at the base calling stage allows at most one  
287 cycle that is less than the Chastity threshold (0,6).

288

289 2- Genoscope filter. An in-house procedure was used in order to remove adapters and  
290 primers on the whole read and low-quality nucleotides from both ends (while quality value is  
291 lower than 20) and then continue next steps with the longest sequence without adapters and  
292 low-quality bases. Remove sequences between the second unknown nucleotide (N) and the  
293 end of the read. This step is done on trimmed (adapters + quality) reads. Discard reads  
294 shorter than 30 nucleotides after trimming. Remove read pairs that come from the low-  
295 concentration spike-in library of Illumina PhiX Control. Reads pairs corresponding to  
296 ribosomal RNA were removed using sortMeRNA (v2.1, (Kopylova *et al.*, 2012).

297 These previous two steps were performed by the sequencing facility at Genoscope. Cleaned  
298 reads from Genoscope were adapter trimmed again by the POPS platform with BBduk from  
299 the BBmap suite (v38.84, Bushnell, 2014) with the options `k=23 ktrim=r qtrim=r`

300 useshortkmers=t mink=11 trimq=20 hdist=1 tpe tbo minlen=30 using adapter sequences file  
301 included in the BBTools package.

### 302 *Mapping and Gene expression quantification*

303 Filtered reads were then mapped and counted using STAR (v2.7.3a, (Dobin *et al.*, 2013))  
304 with the following parameters --alignIntronMin 5 --alignIntronMax 60000 --  
305 outSAMprimaryFlag AllBestScore --outFilterMultimapScoreRange 0 --outFilterMultimapNmax  
306 20 on a combined reference genome made by concatenating the files of the *Zea mays* B73  
307 (V4.49) and *Rhizophagus irregularis* (V2) reference genomes and the associated GTF  
308 annotation files (also combined). Between 75.3 and 96.1 % of the reads were uniquely  
309 mapped (median = 91.9%). Between 68 and 88 % of the reads were associated to annotated  
310 genes (median = 85.3%).

### 311 Statistical Analyses of Expression Data

312 Statistical analyses were conducted on R v3.6.2 (R Core Team, 2020) using the script-based  
313 tool DiCoExpress (Lambert *et al.*, 2020; AU - Baudry *et al.*, 2022). DiCoexpress used the  
314 Bioconductor package edgeR (v 3.28.0, (Robinson *et al.*, 2010; McCarthy *et al.*, 2012).The  
315 raw count file used for the statistical analyses only contain the reads of the *Zea mays*  
316 organism. Dicoexpress DiCoExpress analyse RNAseq projects with at most two biological  
317 factors. For this reason and to answer biological questions of interest, 4 separate statistical  
318 analyses were conducted on samples from (i) B73 leaves, (ii) B73 roots, (iii) Lo3 leaves, (iv)  
319 Lo3 roots.

### 320 Gene Filtering and Normalization

321 For the four analyses, low counts genes giving low counts were filtered using the  
322 "FilterByExpression" feature available through the edgeR package with a minimum count  
323 threshold of 15. Raw counts were normalized with the trimmed mean of M values (TMM)  
324 method implemented in edgeR package.

## 325 *Differential Expression Analysis*

326 The differential analysis is based on a negative binomial generalized linear model. For the 4  
327 *Zea mays* analyses, the log<sub>2</sub> of the average normalized gene expression is an additive  
328 function of a condition effect (LN or HN), a treatment effect (Rhizo equivalent to *R. irregularis*  
329 inoculation) or Ctrl (equivalent to control treatment), an interaction between the condition and  
330 the treatment and the technical replicate factor (3 modalities). To make the processing of  
331 these multifactorial designs easier, Differential Expression (DE) analyses were performed  
332 using the `DiffAnalysis_edgeR` function of DiCoExpress to generate automatically the  
333 contrasts. In each analysis, we considered the difference between the two treatments at  
334 each condition and averaged on the two conditions, the difference between the two  
335 conditions given a treatment and averaged on the two treatments and the interaction effect  
336 defined as the difference between the two treatments at a given condition (HN) minus the  
337 difference between the two treatments at the second condition (LN). It leads to a total of 8  
338 contrasts per analysis and only biologically meaningful contrasts were interpreted. For each  
339 contrast a likelihood ratio test was applied and raw p-values were adjusted with the  
340 Benjamini–Hochberg procedure to control the false discovery rate. The distribution of the  
341 resulting p-values followed the quality criterion described by Rigaiil *et al.* (Rigaiil *et al.*, 2018).  
342 A gene was declared differentially expressed if its adjusted p-value was lower than 0.05.

343 For the fungal transcripts, expression ratios were considered relative to the N nutrition  
344 condition. Fungal transcripts displaying a FC higher or lower than 1.5 in LN compared to HN  
345 conditions were considered Each transcript ID was converted into the protein ID using the  
346 fungal genomic resource MycoCosm at the JGI website:  
347 [https://mycoCosm.jgi.doe.gov/pages/search-for-genes.jsf?organism=Rhiir2\\_1](https://mycoCosm.jgi.doe.gov/pages/search-for-genes.jsf?organism=Rhiir2_1) (Tisserant *et*  
348 *al.*, 2013; Chen *et al.*, 2018b). Based on KOG (euKaryotic Orthologous Groups), functional  
349 classes to which the genes belong were determined Over-represented functional KOG  
350 categories of these fungal genes were determined using the 'KOGMWU' 1.2 R package

351 (Date 2019-02-19. Author Mikhail V. Matz). The fold enrichment value is based on a Mann-  
352 Witney, U-test that uses for each gene, a combination of the FC and the KOG category.

### 353 **Metabolomic analyses**

354 All extraction steps were performed in 2 mL Safelock Eppendorf tubes. The ground dried  
355 samples (5 mg DW) were resuspended in 1 mL of frozen (-20°C)  
356 Water:Acetonitrile:Isopropanol (2:3:3) containing Ribitol at 4 mg/L and extracted for 10 min at  
357 4°C with shaking at 1500 rpm in an Eppendorf Thermomixer. Insoluble material was removed  
358 by centrifugation at 13500 rpm for 10 min. 700 µL were collected and 70 µL of myristic acid  
359 d27 at 0.3 g/L was added as an internal standard for retention time locking. After  
360 centrifugation, aliquots of each extract (100 µL) were dried for 4 h at 35 °C in a Speed-Vac  
361 and stored at -80°C. Two Blank tubes underwent the same steps as the samples.

362 Sample derivatization and analysis : All steps of GC-MS analyses were done as described in  
363 Fiehn et al. (Fiehn, 2006; Fiehn *et al.*, 2008). Agilent literature PUB: 5989-8310EN (Agilent  
364 G1676AA Agilent Fiehn GC/MS Metabolomics RTL Library User Guide. June 2008. Agilent  
365 P/N: G1676-90000, Agilent Fiehn GC/MS Metabolomics RTL Library Product Note. May  
366 2008). Samples were taken out of -80°C, warmed 15 min before opening and speed-vac  
367 dried again for 1 hour at 35 °C before adding 10 µL of 20 mg/mL methoxyamine in pyridine  
368 to the samples and the reaction was performed for 90 min at 30°C under continuous shaking  
369 in an Eppendorf thermomixer. 90 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA)  
370 (REGIS TECHNOLOGIES 1-270590-200 -10x1g) were then added and the reaction  
371 continued for 30 min at 37°C. After cooling, 90 µL were transferred to an Agilent vial for  
372 injection.

373 4 hours after derivatization 1 µL of sample was injected in splitless mode on an Agilent  
374 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer. The column was  
375 an ZORBAX DB5-MS from Agilent (30 m with 10 m integraguard column - ref 122-5532G).  
376 An injection in split mode with a ratio of 1:30 was systematically performed for saturated  
377 quantification compounds. Oven temperature ramp was 60 °C for 1 min then 10 °C/min to

378 325 °C for 10 min. Helium constant flow was 1.1 mL/min. Temperatures were as follows:  
379 injector: 250°C, transfer line: 290°C, source: 230 °C and quadripole 150 °C. The quadrupole  
380 mass spectrometer was switched on after a 5.90 min solvent delay time, scanning from 50-  
381 600  $\mu$ . Absolute retention times were locked to the internal standard d27-myristic acid using  
382 the RTL system provided in Agilent's Masshunter software. Retention time locking reduces  
383 run-to-run retention time variation. Samples were randomized. A fatty acid methyl esters mix  
384 (C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, C30) was injected in the  
385 middle of the queue for external RI calibration.

386 Data processing and quantification: Raw Agilent datafiles were analyzed with AMDIS  
387 <http://chemdata.nist.gov/mass-spc/amdis/>. The Agilent Fiehn GC/MS Metabolomics RTL  
388 Library (version June 2008) was employed for metabolite identifications. This library is the  
389 most comprehensive library of metabolite GC/MS spectra that is commercially available. It  
390 contains searchable GC/MS EI spectra and retention time indexes from approximately 700  
391 common metabolites. Peak areas determined with the Masshunter Quantitative Analysis  
392 (Agilent) in splitless and split 30 modes. Because automated peak integration was  
393 occasionally erroneous, integration was verified manually for each compound in all analyses.  
394 Resulting areas were compiled in one single Excel file for comparison. Peak areas were  
395 normalized to Ribitol and Dry Weight. Metabolite contents are expressed in arbitrary units  
396 (semi-quantitative determination).

### 397 **Amino acid quantification by OPA-HPLC**

398 Extraction procedure was identical that one described for metabolomics. Here 200 $\mu$ L of the  
399 extraction solution was taken and dried in a Speed-Vac evaporator for 2 h at 30°C before  
400 adding 1,3 mL of H<sub>2</sub>O milliQ. The o-phthalaldéhyde (OPA) reagent was made 48 h before  
401 first use by dissolving OPA at 12mg/mL in 200  $\mu$ L of methanol and adding 1.8 mL of sodium  
402 borate 0.5M (pH 9.5) and 40  $\mu$ L of 2-mercaptoethanol. The reagent was filtered into an  
403 autosampler vial and used for up to 3 days. Precolumn derivatization was performed in the  
404 injection loop by automated mixing of 10  $\mu$ L sample and 10  $\mu$ L OPA reagent, followed by a

405 delay of 2 min prior to injection. The HPLC (Alliance Waters 2695, Waters Corporation,  
406 Massachusetts, USA) fitted with a fluo detector (Multi  $\lambda$  Fluorescence Detector 2475) was  
407 used. Compounds were measured at  $\lambda$  excitation of 340 nm and  $\lambda$  emission of 455 nm. The  
408 chromatographic separation was performed with Symmetry C18 column (3.5 $\mu$ m, 4.6\*150  
409 mm) by gradient elution at 40 °C using buffer A (20% methanol, 80% sodium acetate, 1%  
410 tetrahydrofuran, pH 5.9) and buffer B (80% methanol, 20% sodium acetate, pH 5.9). Buffer  
411 flow rate was 0.8 mL/min throughout and total run time per injection was 42 min. The  
412 chromatography data were analysed by Empower software. Peak identity was confirmed by  
413 co-elution with authentic standards. Concentration was calculated with calibration curve  
414 using peak area of compound of interest.

#### 415 **Statistical analyses**

416 All statistical analyses were performed with RStudio 2023.06.0+421 "Mountain Hydrangea"  
417 under the free software environment R version 4.3.1 (Beagle Scouts). All Anova were  
418 implemented with the "aov" R package. All heatmaps were generated with the estimated  
419 marginal means that were calculated using the R package "emmeans". Heatmap for physio-  
420 agronomical traits was generated with 'pheatmap' 1.0.12 package (Date 2019-01-04. Author  
421 Raivo Kolde) by implementing the ward.D2 hierarchical clustering method (Murtagh and  
422 Legendre, 2014). Heatmap for physio-agronomical traits was generated with the 'pheatmap'  
423 R package 1.0.12 Date 2019-01-04 (Author Raivo Kolde). Heatmaps for metabolomics,  
424 including the interactive ones, were generated with "heatmaply" R package (Galili *et al.*,  
425 2018)

426 Metabolomics data were analyzed with the R package MultiVarSel (Perrot-Dockès *et al.*,  
427 2018) which is the implementation of the variable selection approach in the multivariate linear  
428 model proposed in (Perrot-Dockès *et al.*, 2018a), theoretically analysed in (Perrot-Dockès *et*  
429 *al.*, 2018b) and applied in several contexts of omics data analyses such as (Grandclaudon *et*  
430 *al.*, 2019; Perrot-Dockès *et al.*, 2022). The list of R packages used in this study: Chiquet J,  
431 Donnet S, Barbillon P (2023). *sbm: Stochastic Blockmodels*. R package version 0.4.5-9000,

432 <<https://grosssbm.github.io/sbm/>>. Kolde R (2019). `_pheatmap`: Pretty Heatmaps\_. R  
433 package version 1.0.12, <<https://CRAN.R-project.org/package=pheatmap>>. Lenth R (2023).  
434 `_emmeans`: Estimated Marginal Means, aka Least-Squares Means\_. R package version  
435 1.8.6, <<https://CRAN.R-project.org/package=emmeans>>. Perrot-Dockès M, Lévy-Leduc C,  
436 Chiquet J. R package `MultiVarSel` available from the Comprehensive R Archive Network,  
437 2017. <<https://CRAN.R-project.org/package=MultiVarSel>>.

### 438 **Genome-Scale Metabolic Modelling (GSM) and Transcriptomics Data Integration**

439 To investigate the metabolism of maize from systems level, we used *iZMA6517* as the  
440 template model (Chowdhury *et al.*, 2023). The model is specific to B73 genotype and consists  
441 of organ-specific GSMs of root, stalk, leaf, and kernel. All these individual organ GSMs are  
442 connected through a common phloem pool. The model has been described (Chowdhury *et al.*  
443 *et al.*, 2023). The model was contextualized with HN Control, HN Rhizo, LN Control, and LN  
444 Rhizo using EXTREAM algorithm (Chowdhury *et al.*, 2023). The upper and lower bounds for  
445 the mentioned conditions are presented in the Supplementary Table 5. The General  
446 Algebraic Modeling System (GAMS) version 24.7.4 with IBM CPLEX solver was used to run  
447 FBA and FVA, E-Flux, and the FSA algorithm on the model. Each of the algorithms was  
448 scripted in GAMS and then run on a Linux-based high-performance cluster computing  
449 system at the University of Nebraska-Lincoln. Instructions for running files in GAMS through  
450 NEOS server have been published (Chowdhury *et al.*, 2022a). All the necessary files for  
451 COBRApy users can also be accessed through the *iZMA6517* publication (Chowdhury *et al.*,  
452 2023).

### 453 **Metabolic Bottleneck Analysis (MBA)**

454 To determine the metabolic bottlenecks in each of HN Control, HN Rhizo, LN Control, and  
455 LN Rhizo conditions, we used Metabolic Bottleneck Analysis (MBA) (Chowdhury *et al.*,  
456 2023). MBA expands the flux space of each reaction separately and assesses its impact on  
457 the whole plant biomass growth rate. Further details on MBA can be accessed in the original

458 publications. MBA pinpoints plant-wide metabolic bottlenecks, which were further analyzed  
459 for biological insights.

## 460 **Results**

### 461 **The impact of *Rhizophagus irregularis* on maize growth and yield is strongly** 462 **dependent on the level of N nutrition**

463 To investigate the molecular and physiological mechanisms involved in maize interaction  
464 with AMF in terms of N nutrition and have an overview of the diversity of these mechanisms  
465 we used two AMF-responsive maize lines B73 and Lo3. In order to find at which level of N  
466 fertilization the two lines benefit most from the fungal inoculation, these were grown in a  
467 greenhouse under low N (LN), medium N (MN) or high N (HN) corresponding to 1 mM NO<sub>3</sub><sup>-</sup>,  
468 5 mM NO<sub>3</sub><sup>-</sup> and 10 mM NO<sub>3</sub><sup>-</sup> in the nutrient solution respectively. The impact of *R. irregularis*  
469 inoculation on maize physio-agronomic traits was monitored by measuring shoot biomass,  
470 grain yield, total N content and <sup>15</sup>N enrichment allowing the calculation of other traits such as  
471 NUE as described in Supplementary table 3. In inoculated plants, an increase in the shoot  
472 biomass production was only observed under LN in both lines, whereas an increase in grain  
473 yield only occurred in B73 (Figure 1A, 1B). A slight negative impact of *R. irregularis*  
474 inoculation was observed on B73 grain yield when plants were grown under HN.  
475 Interestingly, *R. irregularis* allowed inoculated plants grown under LN to produce a similar  
476 vegetative biomass compared to non-inoculated plants fed with 5 times more NO<sub>3</sub><sup>-</sup>. However,  
477 AMF inoculation did not influence shoot biomass of either maize genotypes under MN or HN  
478 (Figure 1B).

479 In inoculated plants, an increase in NUE for grain was observed under LN and MN  
480 fertilization conditions only in B73 (Figure 1D). Aerial parts NUE increased only in LN  
481 conditions for both B73 and Lo3 (Figure 1C). Together, these data indicate that benefit from  
482 AMF in terms of maize yield and NUE is higher under limiting N supply than under ample N  
483 supply and depends on the genotype. When we examined the impact of the AMF on N

484 resource allocation under the three levels of N, a significant increase of biomass allocation to  
485 the grain was observed in both B73 and Lo3 only in LN (Figure 1E). Chlorophyll quantities  
486 were monitored during the vegetative phase and were also increased by AMF inoculation  
487 under LN only (Figure 1F).

488 The grain N content, considered as a proportion relative to dry mass, remains consistent  
489 across genotype and the N feeding conditions. However, an exception to this trend is  
490 observed in the B73 genotype, where the N content is notably lower in inoculated LN and MN  
491 plants (Figure 2A). Both the shoot and the root N contents were roughly proportional to the  
492 amount of N provided to the plant. Fungal inoculation did not change N content under LN.  
493 However, under higher N supply levels, a significant increase in the N content of aerial parts  
494 was observed in response to *R. irregularis* under MN for B73 and under HN for Lo3  
495 indicating that these lines are able to store higher levels of N in the vegetative parts upon  
496 association with the AMF even under ample N (Figure 2C). The C/N ratio exhibited minimal  
497 change, as a slight decrease in B73 inoculated LN plants aerial parts and increase in grains  
498 in response to *R. irregularis* under MN for B73 was observed (Figure 2G-H). Changes in  
499 carbon (C) content were not statistically significant.

500 To determine whether N uptake is modified during the symbiotic interaction, plants were  
501 labelled with a pulse of  $^{15}\text{N}$  provided at the vegetative stage. The total quantity of  $^{15}\text{N}$  in aerial  
502 parts and grains measured at maturity is unchanged whatever the treatment (Supplementary  
503 Figure 4). These data indicate that the fungus does not affect N uptake at the vegetative  
504 stage. However, under LN conditions,  $^{15}\text{N}$ -enrichment in the different organs of the inoculated  
505 plants was much lower than in control plants for both B73 and Lo3 in line with a biomass  
506 increase resulting in  $^{15}\text{N}$  dilution in AMF inoculated plants (Supplementary Figure 4). These  
507 data indicate that the higher uptake of N triggered by *R. irregularis* is more likely to occur  
508 after the vegetative stage.

509 It is well established that mycorrhizae play a pivotal role in the improvement of plant P  
510 nutrition and uptake (Choi *et al.*, 2018; Plassard *et al.*, 2019; Paries and Gutjahr, 2023). To

511 determine if the amount of N supplied to the plant and the presence of *R. irregularis* modify  
512 B73 and Lo3 phosphate content, total P content (TPC) was measured by the molybdate blue  
513 method. In inoculated plants, an increase in the TPC content was observed under HN  
514 conditions both in the grains and in the roots of Lo3, whereas for B73 it was in the shoots  
515 and in the roots under HN (Figure 2B-D-F). Together, these data indicate that N supply has a  
516 strong impact on *R. irregularis* mediated P nutrition and that this benefit depends on maize  
517 genotype.

### 518 **Inoculation by *R. irregularis* mitigate the physiological impact of N-deficiency**

519 Physio-agronomic phenotyping prompted us to determine if there were traits that could  
520 discriminate inoculated and non-inoculated plants. For this purpose, hierarchical clustering  
521 analysis (HCA) was performed using the physio-agronomic traits listed in Supplementary  
522 Table 3. The HCA presented in Figure 3 shows that the N supply, the genotype, and the  
523 symbiotic association had an impact on the monitored traits. All the LN plants were grouped  
524 in cluster A. Within cluster A, values for the traits related to N and P uptake including root P  
525 content and <sup>15</sup>N enrichment (R\_Ppercent and R\_15NEpercent respectively), aerial part P  
526 content, NUE and <sup>15</sup>N enrichment (AP\_Ppercent, AP\_NUE, AP\_15NEpercent respectively),  
527 were higher. LN plants in cluster A were distant from the HN MN plants clustered in group B.  
528 In cluster B, genotypes were distributed into two subgroups, B1 and B2. Traits related to  
529 yield and biomass were grouped in cluster B irrespective of the inoculation status.  
530 Interestingly, in cluster A, traits related to N deficiency exhibited higher values in the control  
531 compared to the inoculated plants indicating that *R. irregularis* allowed plants to behave  
532 more similarly to group B plants, albeit still under N stress deficiency. These data show that  
533 *R. irregularis* inoculation can mitigate the impact of N limitation on physio-agronomic traits.

### 534 **Marker genes for N uptake and assimilation are differentially regulated in response to** 535 **AMF inoculation and N supply**

536 The finding that several NUE traits, notably those related to  $\text{NO}_3^-$  uptake were modified by  
537 AMF treatment prompted us to investigate further which molecular components were  
538 involved. Before investigating in detail, the molecular mechanisms taking place in regard to  
539 on N supply we had to identify the time point of the interaction process at which symbiotic  
540 markers were expressed at their maximum. For this purpose, the expression levels of  
541 symbiotic marker genes were monitored at three stages (see materials and methods). Then,  
542 the level of transcripts for the plant genes *ZmAMT3.1*, *ZmPHT6.1* and *ZmNPF4.5* markers of  
543 the symbiotic association and representative of  $\text{NH}_4^+$ , phosphorus and  $\text{NO}_3^-$  transport  
544 respectively was quantified (Sawers *et al.*, 2017; Wang *et al.*, 2020; Xie *et al.*, 2022; Hui *et*  
545 *al.*, 2022). For the three genes, the levels of root transcripts were higher at VT+15 stage in  
546 both HN and LN conditions (Supplementary Figure 5). Fungal colonization was greater under  
547 HN compared to LN both in B73 and Lo3 (Supplementary Figure 5A). Hence, the VT+15  
548 stage was selected for all subsequent experiments.

549 The expression of selected marker genes involved in direct N transport, P and N transport  
550 mediated by AMF was then monitored in roots following inoculation by the AMF and  
551 according to the level of N supply. By monitoring the level of *R. irregularis* constitutive  
552 transcript *RiLSU* we confirmed that high N input led to increased fungal biomass in roots  
553 (Figure 4B). The expression level of the four well known symbiotic markers mentioned  
554 previously, *ZmPHT1.6*, *ZmNPF4.5*, *ZmHA1*, a proton pump, and *ZmAMT3.1* (Wang *et al.*,  
555 2014, 2020; Sawers *et al.*, 2017; Xie *et al.*, 2022; Hui *et al.*, 2022), is up-regulated in  
556 response to the fungus in both genotypes and under all N supply conditions. It is interesting  
557 to notice that the higher the N nutrition level, the higher the expression of these markers  
558 (Figure 4A). To know more about direct N transport and N assimilation genes in those  
559 conditions, we investigated expression levels of two maker genes of N starvation stress  
560 (*ZmNRT2.1a*, and *ZmNRT2.2*) encoding high affinity transporters, known to be upregulated  
561 under N deficiency (Krapp *et al.*, 2014; Wang *et al.*, 2018; Dechorgnat *et al.*, 2019; Hirel and  
562 Krapp, 2020) (Figure 4A). As expected, the level of N supply had a drastic effect in both

563 maize genotypes on transcripts levels for the nitrate transporters *ZmNRT2.1a*, *ZmNRT2.2*  
564 which were more highly expressed in LN plants compared to MN and HN plants. These data  
565 indicate that the presence of the fungus mimics an additional N supply.

566 **Leaf and root transcriptome reprogramming in response to *R. irregularis* colonization**  
567 **integrates a genotype and N-availability effects**

568 To further characterize the biological processes involved in maize response to *R. irregularis*  
569 colonization as a function of N fertilization, a whole genome transcriptomic approach was  
570 conducted in leaves and roots of plants grown under HN and LN conditions. First, we defined  
571 the differentially expressed genes (DEGs) as those exhibiting a fold change of the transcript  
572 levels in inoculated plants compared to non-inoculated plants that was higher than 1.5 or  
573 lower than -1.5 ( $p < 0.05$ ). It is important to note that RNAs used for the transcriptomic  
574 approach were obtained from the plants that were phenotyped at the end of their life cycle for  
575 physio-agronomical traits. Indeed, root harvesting for molecular studies was not destructive,  
576 allowing plants to complete their life cycle.

577 Details for the numbers of DEGs depending on organ, N nutrition level and genotype are  
578 shown in Supplementary Figure 6. First, we focused on the DEGs in roots and in leaves  
579 irrespective of the genotype and irrespective of the level of N fertilization. In leaves, only 2  
580 genes were up-regulated and only 2 genes were down regulated, whereas in roots many  
581 more genes were differentially expressed in all conditions in response to the fungus. The two  
582 genes up-regulated in all conditions in leaves encode a H(+)-exporting diphosphatase  
583 (Zm00001d015569) involved in vacuolar ion transport and a histidine-containing  
584 phosphotransferase protein 4 (Zm00001d043505) involved in signalling. The two down-  
585 regulated genes encode a Mannan endo-1,4-beta-mannosidase (Zm00001d010039)  
586 involved in cell wall remodelling and a Nuclear transcription factor Y subunit (NF-Y)  
587 (Zm00001d006835).

588 When considering root DEG genes irrespective of the genotype or N supply, there was an  
589 increase in the expression level of 345 genes whereas a decrease was observed for 59  
590 genes (Supplementary Figure 6). GO enrichment analysis revealed that for those common  
591 up-regulated genes in roots, the biological processes overrepresented were those related to  
592 proteolysis inhibition, lipid metabolism, ethylene response and membrane transport. The  
593 common down regulated genes were represented by five main functional categories  
594 including cell wall biogenesis, metal homeostasis, phosphate transport, stress responses and  
595 lipid metabolism (Figure 5).

596 To gain insight into the specific mechanisms taking place in each line (B73 and Lo3) upon  
597 symbiotic association as a function of N nutrition, enriched GO functions in DEG under LN or  
598 HN in each organ were determined. In B73 roots under LN, main overrepresented categories  
599 of up-regulated transcripts are related to plant immunity represented by hormone signaling  
600 and phenylpropanoid pathway, cell wall remodeling and nucleotide metabolism, while the  
601 main down-regulated categories were enriched in post translational modifications such as  
602 histone de-ubiquitination. In B73 roots under HN, the main overrepresented categories in up-  
603 regulated genes are related to defense, signaling, glycolysis, lipid metabolism and cellular  
604 trafficking while the overrepresented categories in the down-regulated genes are related to  
605 sugar and steroid metabolism and cell division.

606 In inoculated Lo3 roots under LN, stress responses, amino acid biosynthesis and autophagy  
607 are overrepresented in the up-regulated genes, while in the down-regulated genes, DNA and  
608 amino acid metabolism as well as cell wall remodeling are overrepresented. Under HN, *R.*  
609 *irregularis* inoculation of Lo3 triggers the up-regulation of root transcripts mainly related to  
610 photosynthesis, stress responses, DNA metabolism, water transport, amino-acid metabolism,  
611 root development and terpenoid biosynthesis. In roots of HN grown Lo3 plants, the main  
612 overrepresented categories of down-regulated genes belong to nitrate assimilation,  
613 oligopeptide transport and stress responses categories.

614 We performed the same data analysis in leaves (Figure 5). In response to *R. irregularis*, the  
615 B73 leaves main overrepresented categories of up-regulated transcripts under LN conditions  
616 are related to translation and auxin signaling, while the main down-regulated categories were  
617 enriched in symbiotic interactions, signaling. Under HN, in B73 leaves of inoculated plants,  
618 the main overrepresented categories in up-regulated genes are related to redox reactions,  
619 carbohydrate transport and defense while the main over-represented categories in the down-  
620 regulated genes are related to protein folding and response to inorganic substances.

621 In leaves of inoculated Lo3 plants, under LN, the main overrepresented categories in the up-  
622 regulated genes are photosynthesis, cell wall remodeling, cytoskeleton remodeling, stress  
623 response, signaling, while in the down-regulated genes, gamma-amino butyric acid (GABA)  
624 metabolism and pyrimidine biosynthesis are overrepresented. Under HN, *R. irregularis*  
625 inoculation of Lo3 plants triggers the up-regulation of leaf transcripts mainly related to amino  
626 acid catabolism, flavin-containing compounds related metabolism and organic acid transport  
627 as well as iron homeostasis, while down-regulated genes belong to lipid metabolism,  
628 pyrimidine metabolism, and stress response. Taken together, these data show that leaf and  
629 root transcriptomic responses to the AMF differ depending on maize genotype and N supply.

### 630 **Leaf and root metabolomic profiles in response to *R. irregularis* are shaped by N** 631 **nutrition**

632 To further characterize the metabolic responses of the two maize lines to *R. irregularis*  
633 inoculation, a primary metabolome profiling was performed on the same leaf and root  
634 samples as those studied in the previous section. Metabolic profiles showed that plant organ  
635 and N supply had the strongest impact on leaf and root metabolite composition  
636 (Supplementary Figures 7A and B). AMF inoculation and maize genotype had globally  
637 weaker impacts on metabolite profiles (Figures 6 A and B and supplementary Figure 7C and  
638 D). In leaf samples 132 water soluble metabolites were detected of which 103 were  
639 significantly different in at least on condition after ANOVA statistical analysis ( $P < 0.05$ )  
640 followed by a Bonferroni post-hoc test and correction. In roots, there were 120 water-soluble

641 metabolites of which 117 were found to be significantly different in at least one condition. The  
642 identity of metabolites can be found on the interactive heatmap Supplementary interactive  
643 File 1.

644 In roots, fungal inoculation triggered accumulation of lipids such as trans-13-octadecenoic  
645 acid and palmitoleic in both lines Supplementary Figure 8. The accumulation of these two  
646 lipids was much higher under HN than under LN. We observed that in inoculated roots, there  
647 was a global decrease in sucrose, glucose and fructose levels in roots of Lo3 under LN.  
648 Several metabolites linked to N metabolism were differentially accumulated in inoculated  
649 roots. For example, Glu, Arg, citrulline, Asn and  $\beta$ -Ala levels were increased in response to  
650 *R. irregularis* in roots under HN. Glutamine levels remained unchanged in inoculated roots.  
651 Some metabolites related to immunity were accumulated differentially in response to the  
652 fungus. For instance, shikimic acid was accumulated in LN roots of inoculated B73 plants  
653 and trehalose was accumulated in HN inoculated roots of both lines compared to controls.

654 In leaves, lipids and soluble sugars (glucose and fructose) did not show differential  
655 accumulation in response to the fungus. Sucrose, however, showed a significant decrease  
656 only for HN and this in both lines (Supplementary Figure 9). Gln and Asn accumulated in Lo3  
657 leaves under HN in response to *R. irregularis*. Xylulose accumulated in Lo3 R LN leaves.

658 To identify metabolites representative of the plant response to *R. irregularis* under each  
659 growth condition, metabolites for which abundance is significantly correlated with specific  
660 treatment conditions were identified by using a variable selection approach in a multivariate  
661 linear model conducted with the MultiVarSel approach (see materials and methods, Figure 6  
662 C and D). For example, we found that under LN, xylulose was accumulated in leaves of Lo3  
663 inoculated plants (Figure 6C). In roots, benzoic acid was accumulated in inoculated B73  
664 roots of plants grown under LN (Figure 6D). Palmitoleic acid is accumulated to a lower extent  
665 in Lo3 HN control conditions compared to all the other conditions. These data indicate that  
666 specific metabolites are accumulated depending on the plant genotype and the N supply in  
667 response to *R. irregularis*.

668 **Multi-organ metabolic modelling reveals key metabolic processes triggered by *R.***  
669 ***irregularis* allowing maize to mitigate N limitation stress**

670 To identify key metabolic reactions involved in yield and biomass reshaping in response to *R.*  
671 *irregularis* symbiosis, we integrated the transcriptomic data in a recently reconstructed multi-  
672 organ genome scale modelling (GSM) named *iZMA6517* (Chowdhury *et al.*, 2023), This  
673 model was built for the B73 maize genotype using the transcriptomic data integration  
674 algorithm, EXTREAM (Chowdhury *et al.*, 2023). This was followed by a metabolic bottleneck  
675 analysis (MBA) to pinpoint metabolic bottlenecks for biomass production. The B73 leaf and  
676 root transcriptomic data were used as input to EXTREAM to contextualized *iZMA6517*. The  
677 modelling predicted that growth rates were higher under LN in response to *R. irregularis*  
678 inoculation compared to non-inoculated plants, which resulted in grain yield and biomass  
679 increase while under HN, a negative impact on maize growth rate was observed (Figure 7A).  
680 Strikingly, these data are in agreement with the observed yield and biomass experimental  
681 data in Figure 1, confirming the accurate phenotypic prediction of the *iZMA6517*. This result  
682 prompted us to determine the metabolic processes that could explain the improved yield  
683 under LN B73 plants in response to *R. irregularis* inoculation by identifying the potential  
684 metabolic bottlenecks relieved by the AMF under limiting N. For this purpose, we compared  
685 the bottlenecks identified in LN control plants to the bottlenecks identified in inoculated LN  
686 plants (Figure 7B; Table 1). Four metabolic bottlenecks relieved by *R. irregularis* inoculation  
687 were identified : photosynthesis, purine and pyrimidine metabolism, aromatic acid  
688 metabolism; and folate metabolism (Figure 7B, Table 1). Interestingly 13 new bottlenecks  
689 were emerged following *R. irregularis* inoculation. These new bottlenecks are involved in the  
690 metabolism of nicotinate, nicotinamide, purine, aromatic amino acids, Calvin cycle and  
691 glycolysis, On the other hand, we found that in HN plants, four metabolic bottlenecks that  
692 were present in LN control plants were relieved. Three of them were common with those  
693 relieved by *R. irregularis* (photosynthesis, purine and pyrimidine metabolism, aromatic acid  
694 metabolism), the fourth one is involved in ascorbate and aldarate metabolism. The HN

695 treatment created 14 new metabolic bottlenecks compared to LN control conditions. These  
696 new bottlenecks are involved in glycolysis, TCA cycle, Calvin cycle, metabolism of alanine,  
697 aspartate, glutamate, pyrimidine, purine, fructose, mannose, aromatic amino acids. These  
698 data indicate that different metabolic processes are modulated by N supply and *R. irregularis*  
699 inoculation. The most interesting result is the fact that photosynthesis, pyrimidine  
700 metabolism, aromatic amino acid biosynthesis are 3 processes likely to play key roles on *R.*  
701 *irregularis* mediated N nutrition in maize.

### 702 **Transcriptome of *R. irregularis* in inoculated maize roots is driven by N availability**

703 To gain insight into the metabolic cross talk between *R. irregularis* and maize, we  
704 investigated the biological processes taking place in the fungal partner according to the N  
705 supply conditions. For this purpose, we determined the fungal genes displaying a higher  
706 expression in LN roots compared to HN roots and vice versa. This selection was performed  
707 for B73 and Lo3 separately. The Venn diagrams in Figure 8A and 8B show that under LN,  
708 1251 fungal genes were up-regulated in Lo3 while they were 1247 in B73, including 548  
709 transcripts up-regulated in both maize lines. In HN roots, 1596 fungal transcripts were up-  
710 regulated in Lo3 and 1359 were up-regulated in B73 roots including 609 genes up-regulated  
711 in both maize lines. In both maize lines, the functional categories overrepresented in *R.*  
712 *irregularis* DEG were similar in each N nutrition condition Figure 8C. However, an important  
713 remodelling in *R. irregularis* overrepresented DEG functional categories in response to N  
714 supply was observed. For example, fungal genes up-regulated under LN were involved in  
715 nuclear structures, vesicular trafficking, translation and transcription, whereas those up-  
716 regulated under HN had a role in metabolic activity of primary and secondary metabolism, in  
717 particular nucleotide and lipid metabolism. Only few maize line-specific functional categories  
718 could be observed. For instance, defence mechanisms was a functional category of *R.*  
719 *irregularis* DEGs specifically expressed in Lo3. These data show that the fungal  
720 transcriptome is strongly affected by the nutritional N status of the host and the soil. Among  
721 the DEG functional categories, several genes were involved in amino acid and nucleotide

722 metabolism and transport as well as in inorganic transport. We focused on the expression  
723 profiles of nucleotide and amino acid metabolism genes and transporter genes as well as  
724 phosphate and ammonium acid transport genes. Supplementary Table 7 shows the  
725 expression pattern of these fungal genes. Interestingly, fungal ammonia transporters are  
726 more highly expressed under LN than under HN. We did not find any fungal nitrate  
727 transporter genes in the differentially expressed AMF genes. The fungal amino acid  
728 biosynthetic genes up-regulated are mainly involved in asparagine, arginine, glutamine,  
729 glutamate and serine pathways. Several fungal amino acid transporter genes were found to  
730 be up-regulated either in LN or HN roots. The fungal nucleotide biosynthesis genes up-  
731 regulated in HN roots are related to pyrimidine de novo biosynthesis or salvage pathways.

## 732 **Discussion**

733 To better understand the biological and genetic basis of how AMF improve maize N nutrition,  
734 we investigated the physiological and molecular responses of different maize genotypes  
735 when they are inoculated with the AMF *R. irregularis* under different N supply conditions. In  
736 our study, the first interesting result was to find an increase in kernel yield in the two maize  
737 lines B73 and Lo3 inoculated with *R. irregularis* when N nutrition is reduced. We showed that  
738 a symbiotic association with *R. irregularis* was able to compensate for decreased maize plant  
739 performance when N fertilization is reduced. In the literature, depending on both the N supply  
740 level and the form of N supplied, different effects on yield and/or biomass production have  
741 been reported, including an increase, no effect, or a reduction in productivity when the plant  
742 develops a symbiotic association with an AMF (Ingraffia *et al.*, 2020; Quiroga *et al.*, 2020;  
743 Yooyongwech *et al.*, 2022). In the present investigation, we estimated that kernel yield can  
744 be maintained with a 2- to 5-fold reduction in N input when two maize lines were inoculated  
745 with *R. irregularis*. Such a beneficial impact was previously observed for maize plant biomass  
746 production under low N fertilizer inputs (Quiroga *et al.*, 2020). One can, therefore, conclude  
747 that the positive impact of AMF on plant performance can be variable depending on the AMF  
748 and the host genotype as well as the level and type of available N source (Verzeaux *et al.*,  
749 2017; Ingraffia *et al.*, 2020; Xie *et al.*, 2022).

750 Our transcriptome study allowed us to provide an overview of biological processes putatively  
751 involved during the establishment of a symbiotic association between the AMF *R. irregularis*  
752 and maize. In the leaves of inoculated plants, four maize genes were found to be  
753 differentially expressed irrespective of the N-feeding conditions and the genotype  
754 (Zm00001d015569, Zm00001d043505, Zm00001d010039, Zm00001d006835). Although  
755 these four genes did not allow us to identify a core biological process occurring in inoculated  
756 plants, their function was representative and biologically relevant to an interaction with an  
757 AMF such as biomass production (Zm00001d015569) (Menadue *et al.*, 2021), cytokinin  
758 signaling (Zm00001d043505) (Yonekura-Sakakibara *et al.*, 2004), cell wall remodeling

759 (Zm00001d010039), nitrate transport and flowering (Zm00001d006835) (Su *et al.*, 2018;  
760 Cheng *et al.*, 2021).

761 Whatever the level of N fertilization or the maize lines, the core DEGs that were either up or  
762 down-regulated in roots were enriched in transcripts encoding proteins involved in a variety  
763 of biological functions : defense, hormone signaling (especially jasmonate and cytokinin), N  
764 and C metabolism, cell wall and chromatin remodeling, proteolysis inhibition, metal  
765 homeostasis, phenylpropanoid and lipid metabolism. Similar results were previously obtained  
766 in several other model and crop species, indicating that most of these biological functions are  
767 shared when plant develop symbiotic associations with AMFs (Fiorilli *et al.*, 2009; Gallou *et*  
768 *al.*, 2012; Schaarschmidt *et al.*, 2013; Meng *et al.*, 2015; Handa *et al.*, 2015; Vangelisti *et al.*,  
769 2018; Kaur and Suseela, 2020; Wang *et al.*, 2020; Hui *et al.*, 2022). In particular, the most  
770 highly up-regulated genes in roots of B73 and Lo3 under both N nutrition conditions in  
771 response to *R. irregularis* inoculation are encoding peptidases, peptidase inhibitors,  
772 chitinases and senescence genes (Supplementary Table 10a and b). Interestingly,  
773 autophagy genes are overrepresented in Lo3 genes up-regulated in roots under LN following  
774 inoculation with *R. irregularis* (Supplementary Table 9, Figure 5). These data suggest that  
775 important proteolysis processes are taking place in inoculated roots that may involve  
776 autophagy especially in the Lo3 maize line. Autophagy is a process known to recycle N  
777 during senescence, and is known to provide N assimilates necessary for maize kernel filling  
778 (Li *et al.*, 2015; Masclaux-Daubresse *et al.*, 2017).

779 Changes in the level of expression of genes involved in sugar, amino acid, and lipid  
780 metabolism were confirmed by metabolomics analyses (Supplementary Figure 8 and 9).  
781 Under HN, the amounts of root soluble carbohydrates sucrose, fructose, and glucose were  
782 lower in inoculated plants compared to controls, which is consistent with the export of C-  
783 containing molecules to the fungus. As this did not occur under LN, one can hypothesize that  
784 the roots and/or the fungus do not constitute an important sink for C or that the plant limits  
785 fungal proliferation under N-limiting conditions. Under LN, genes involved in the control of

786 plant immunity were up-regulated in roots of inoculated plants, especially in line B73, which  
787 is consistent with the accumulation of shikimic acid (Supplementary Figure 8). Both  
788 transcriptomic data and metabolomics data show modifications in lipid metabolism and the  
789 accumulation of lipids in roots. In roots, when N fertilization was not limiting, we observed an  
790 accumulation of two lipids: trans-13-octadecenoic acid and palmitoleic acid (Supplementary  
791 Figure 8). Lipid accumulation in roots colonized by AMFs was already observed (Keymer *et al.*,  
792 2017; MacLean *et al.*, 2017), notably in *R. irregularis*, to which their reduction and  
793 transfer from the host to the fungus is considered to be a way of fueling the symbiotic fungus  
794 with a carbon source other than sugars (Keymer *et al.*, 2017; MacLean *et al.*, 2017). In the  
795 present study, lipid accumulation occurred only under HN, which is consistent with the fact  
796 that due to higher photosynthetic activity, as revealed by the accumulation of soluble  
797 carbohydrates, the plant is able to synthesize more fatty acids to feed the fungus. In  
798 inoculated plants, arginine (Arg), known to be a key molecule involved in the transfer of N  
799 from the fungus to the plant (Chen *et al.*, 2018a; Kaur and Suseela, 2020; Dellagi *et al.*,  
800 2020; Xie *et al.*, 2022), accumulates under HN conditions not only in the roots of line B73 HN  
801 but also in the leaves.

802 Unexpectedly, another process that appears to be playing a crucial role in AMF symbiosis is  
803 pyrimidine biosynthesis. Our transcriptomic analysis demonstrates that, regardless of  
804 nitrogen feeding conditions, pyrimidine biosynthesis processes are consistently  
805 downregulated in leaves of inoculated plants (Figure 5). Furthermore, under low nitrogen  
806 (LN) conditions, several key genes encoding enzymes involved in nucleotide metabolism,  
807 including carbamoyl-phosphate synthase (Witte and Herde, 2020b), are downregulated in  
808 both leaves and roots (Supplementary Table 8). For example, maize carbamoyl-phosphate  
809 synthase genes *Zm00001d044250* *Zm00001d011192* are downregulated in B73 roots when  
810 inoculated with *R. irregularis* under LN conditions (Supplementary Table 8). A critical gene in  
811 this context is *Zm00001d023833*, encoding a putative nucleoside diphosphate kinase  
812 (NDPK), which participates in pyrimidine and purine nucleotide salvage (Witte and Herde,

813 2020a). This enzyme was identified as a metabolic bottleneck relieved by AMF inoculation  
814 (Figure 7). More precisely, the reactions relieved by *R. irregularis* inoculation corresponds to  
815 those indicated in Figure 7. In non-inoculated maize plants under LN, this maize *NDPK* gene  
816 is up-regulated in leaves compared to HN suggesting that pyrimidine salvage is activated  
817 under LN to help plants cope with N Limitation (Supplementary Table 8). On the contrary, in  
818 the symbiotic association, *NDPK* gene expression remains unaltered compared to  
819 uninoculated LN, suggesting the absence of constraints on this enzymatic reaction in  
820 symbiotic plants (Supplementary Table 8). The level of  $\beta$ -Ala, a known product of purine  
821 degradation drops in inoculated LN and HN roots suggesting that *R. irregularis* inoculation  
822 represses the degradation of nucleotides.

823 In line with these data, fungal transcriptomic analysis by RNAseq shows that pyrimidine and  
824 ureide metabolism play a crucial role in the symbiosis with regard to N nutrition. The fungal  
825 metabolism appears to differ depending on N supply. The integration of maize and fungal  
826 RNAseq and metabolome analyses has allowed us to construct a simplified model depicting  
827 the various biological events occurring in the symbiotic association (Figure 9). Under LN,  
828 fungal argininosuccinate lyase, carbamoyl-phosphate synthase and arginase genes are  
829 upregulated, potentially leading to urea accumulation. However, urea level drops in LN  
830 inoculated roots a fact that is consistent with higher expression of *R. irregularis* urease under  
831 LN. It is thus likely that under LN, the major way the fungus provides plant with N is based on  
832 its urea cycle followed by urea conversion into ammonium which is then transferred to the  
833 plant.

834 Under HN, several amino acids accumulate in response to fungal inoculation in roots (Gln,  
835 Glu, Asn, Arg, Citr, Orn). Fungal pyrimidine de novo biosynthetic genes and the pyrimidine  
836 salvage pathway are upregulated in HN roots. This suggests that under HN, the fungus  
837 activates the metabolism of nucleotide monophosphate biosynthesis that may then be  
838 transferred to the plant. The transfer of nucleotides from the fungus to the plant could also

839 take place under LN but to a lower extent because metabolic modeling identified the NDPK  
840 enzyme as being the metabolic bottleneck relived by *R. irregularis* inoculation under LN.

841 On the other hand, allantoin accumulates exclusively in HN roots and its level remains  
842 unaffected by inoculation. This metabolite can be converted into allantoic acid, which may be  
843 released into the apoplast (Tegeder, 2014). A fungal allantoicase is upregulated in HN roots,  
844 indicating possible conversion of allantoic acid into urea under HN conditions. This transfer of  
845 ureides is akin to the process observed in symbiotic associations between tropical legumes  
846 and Rhizobia species (Tegeder, 2014). Previous studies have also identified upregulation of  
847 genes involved in allantoin synthesis in roots of AMF-inoculated plants (Nanjareddy *et al.*,  
848 2017). Consequently, it is plausible that ureide transfer mirrors that occurring in legume  
849 nodules.

850 Fungal arbuscules are known to undergo controlled senescence (Choi *et al.*, 2018; Paries  
851 and Gutjahr, 2023), our data suggest that autophagy is upregulated in maize roots, with  
852 higher expression in Lo3 LN roots (Supplementary Table 9). Autophagy likely contributes to  
853 the degradation of fungal cellular products, potentially releasing nucleosides. The fungal  
854 nucleotide salvage process can then recycle nucleosides that may repress de novo plant  
855 pyrimidine biosynthesis pathways (Tegeder, 2014; Witte and Herde, 2020*b*). This implies  
856 that AMF likely supplies the host plant with nucleotides and nucleosides, potentially  
857 pyrimidines, to circumvent the deficiency in NDPK activity, thereby alleviating the associated  
858 metabolic bottleneck. This observation is consistent with the known role of NDPK in plant  
859 survival with respect to pyrimidine salvage (Tegeder, 2014; Witte and Herde, 2020*a*). It is  
860 noteworthy that pyrimidine biosynthesis can be an essential process for plant N management  
861 (Zrenner *et al.*, 2006; Fernie *et al.*, 2020).

862 Pyrimidine metabolism holds significance not only in crop performance but also in nitrogen  
863 use efficiency (NUE) (Tegeder, 2014; Lee *et al.*, 2018; Melino *et al.*, 2022). Recent findings  
864 demonstrate a correlation between maize leaf nitrogen accumulation and NDPK protein  
865 levels (Urrutia *et al.*, 2023), further supporting the hypothesis that *R. irregularis* stimulates the

866 reprogramming of plant pyrimidine metabolism to enhance maize performance under  
867 nitrogen-limiting conditions.

868 In conclusion, we propose that inoculating maize with *R. irregularis* offers a promising  
869 strategy to significantly reduce nitrogen fertilizer usage while maintaining maize kernel yield.  
870 Our findings highlight a metabolic interplay between the host and AMF, with pyrimidine and  
871 ureide metabolism serving as key players in this interaction (BARROSO *et al.*, 1988; Thakur  
872 and Garg, 2023). Furthermore, the observed differences in how our two studied maize lines  
873 embrace different strategies to benefit from the fungus illustrate the importance of leveraging  
874 maize's extensive genetic diversity for a comprehensive understanding of this symbiotic  
875 interaction (Schmidt *et al.*, 2016; Sawers *et al.*, 2018*b*; Liu *et al.*, 2020).

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877 **Data Availability**

878 RNAseq project is deposited (Gene Expression Omnibus,Edgard R. et al. 2002):  
879 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE235654> ; accession no.  
880 GSE235654. All steps of the experiment, from growth conditions to bioinformatic analyses,  
881 were detailed in CATdb (Gagnot *et al.*, 2008): <http://tools.ips2.u-psud.fr.fr/CATdb/>; Project:  
882 NGS2021\_19\_Rhizophagus according to the MINSEQE 'minimum information about a high  
883 throughput sequencing experiment'.

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885 **Acknowledgments:**

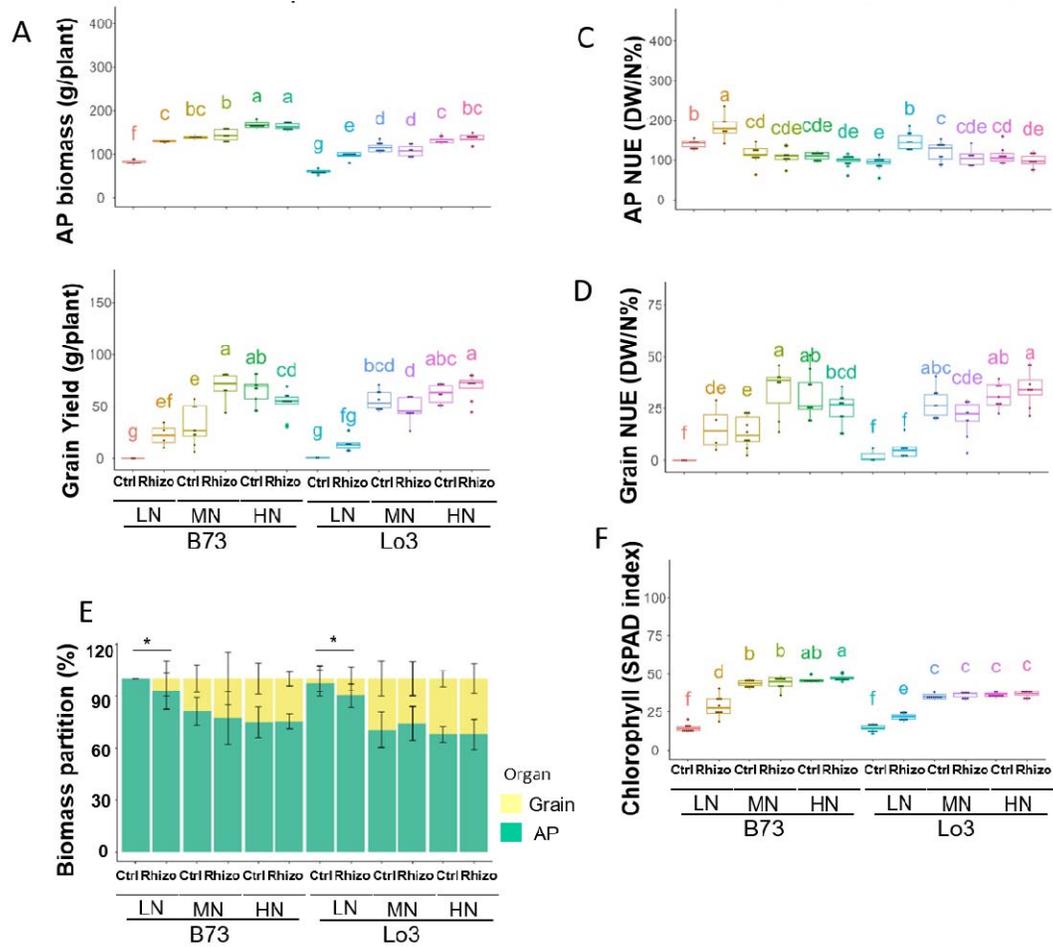
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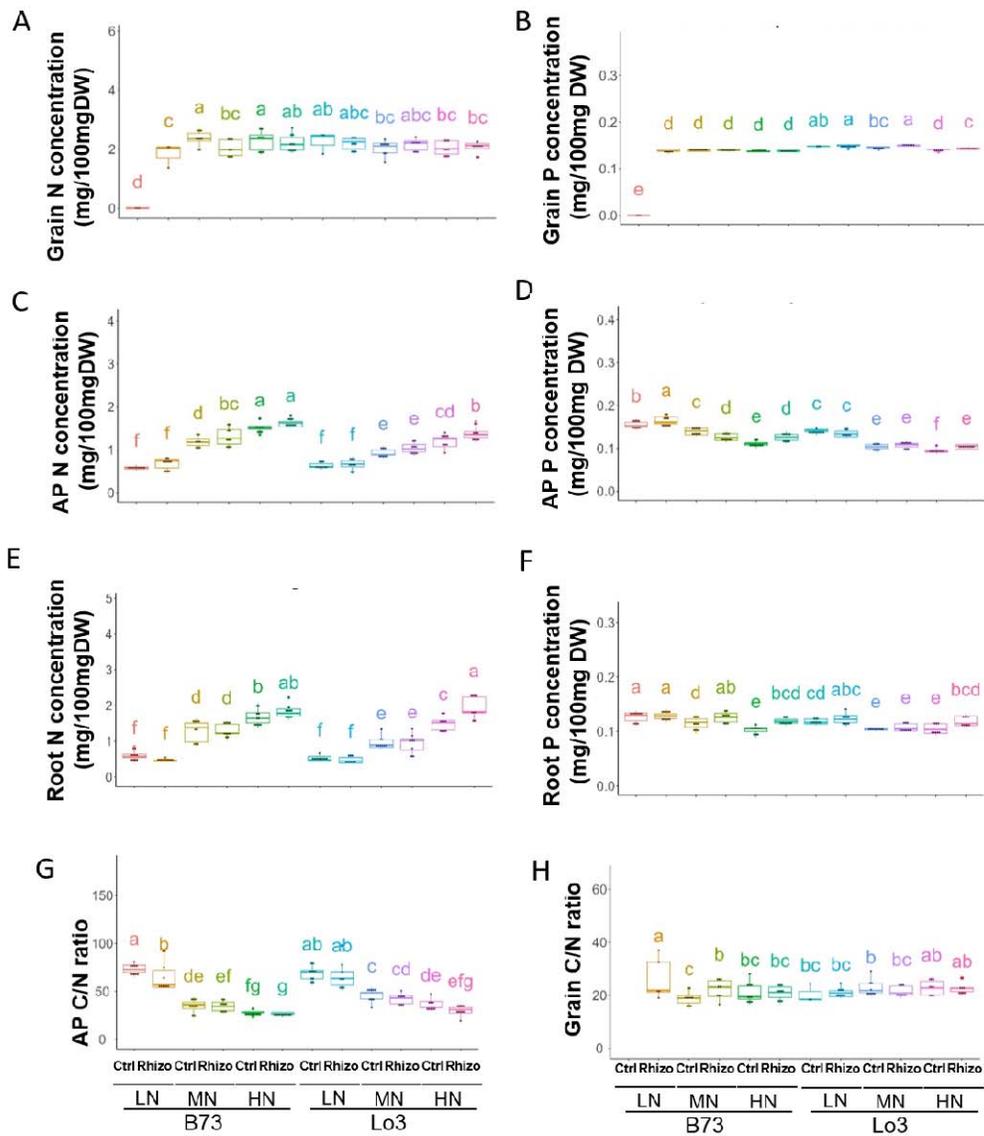
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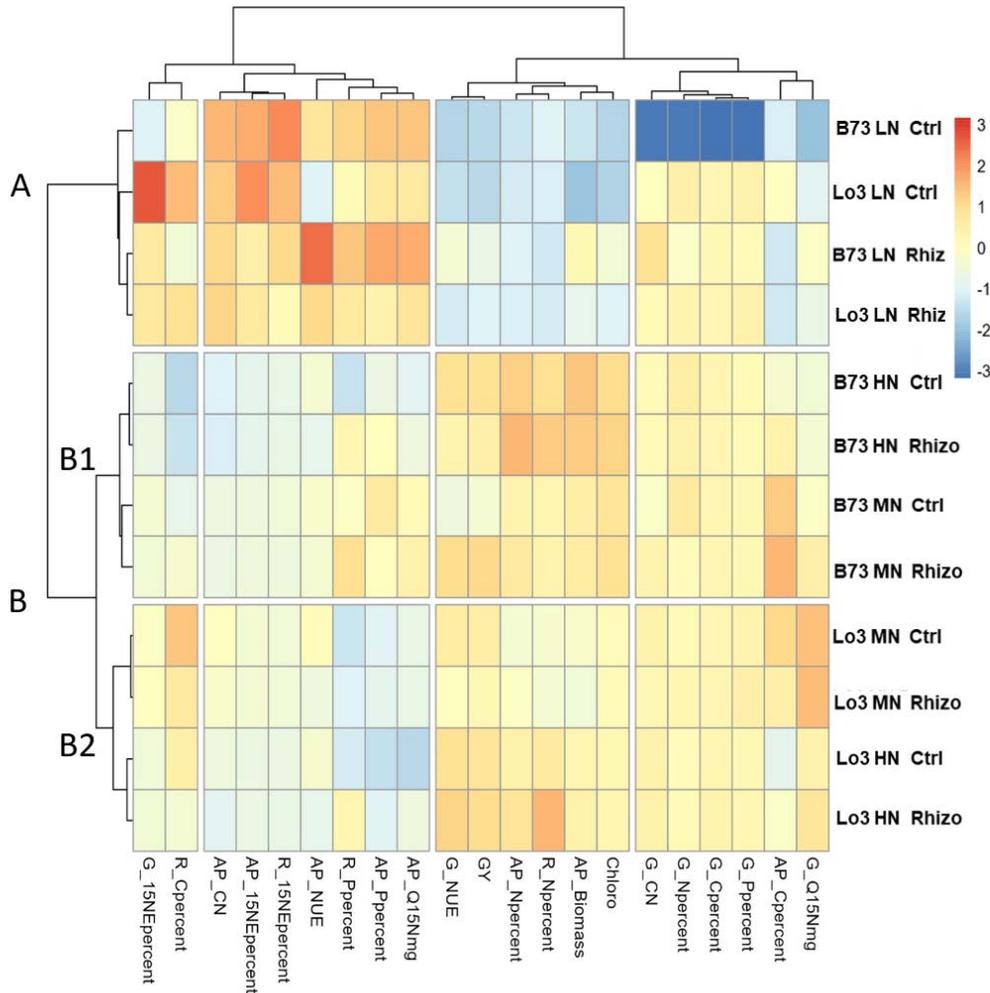
904 **Figure 1: Impact of N supply on maize responses to *R. irregularis* for biomass and N content shows more**  
 905 **pronounced beneficial AMF effects under LN.** Plants were grown and harvested as indicated in materials and  
 906 methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>), moderate nitrate (MN, 5mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). **A**  
 907 and **B** : grain yield and aerial part (AP) biomass of indicated samples. **C,D** : Nitrogen Use Efficiency (NUE) in  
 908 grains and in AP. **E**: Biomass partitioning. **F**: Average chlorophyll content measured as SPAD index on the leaves  
 909 number 4,5 and 6 over 5 weeks after sowing. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R.*  
 910 *irregularis*, AP: Biomass of Aerial Parts. N=7 to 10 plants. Different letters indicate significantly different averages  
 911 for the considered trait by ANOVA. Asterisk indicates significant difference between control and inoculated plants  
 912 t-test ( $p < 0.05$ ).  
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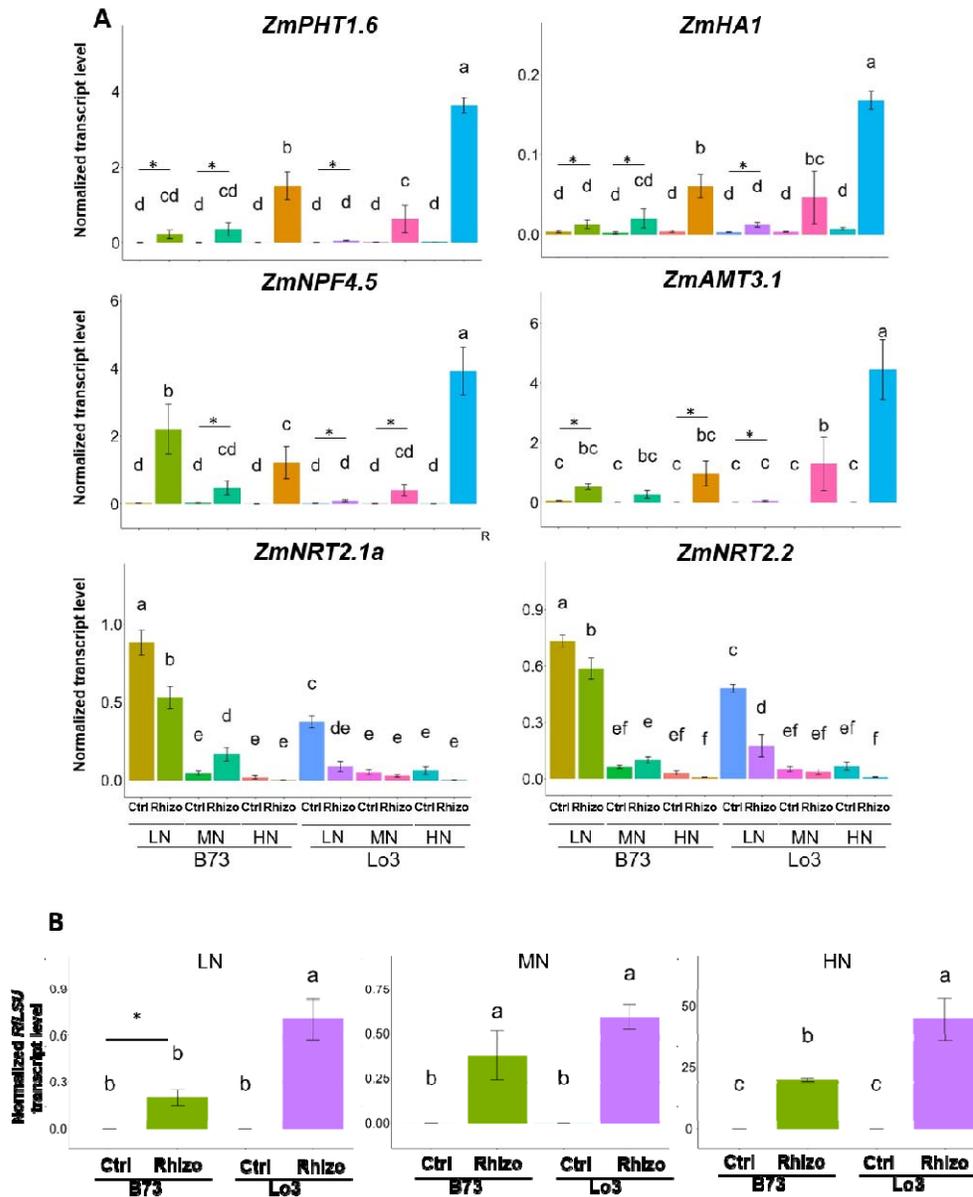


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**Figure 2: Impact of N supply on maize responsiveness to *R. irregularis* for P and N content and C/N ratio highlights poor impact of AM symbiosis on these traits.** Plants were grown and harvested in 2020 as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>), moderate nitrate (MN, 5mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). **A** to **F** : N and P content of grains, aerial parts (AP) and roots. **G** and **H** : C/N ratios in AP and grains. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*. N=7-10 plants for grains and AP. N=5 for roots. Different letters indicate significantly different averages for the considered trait by ANOVA.

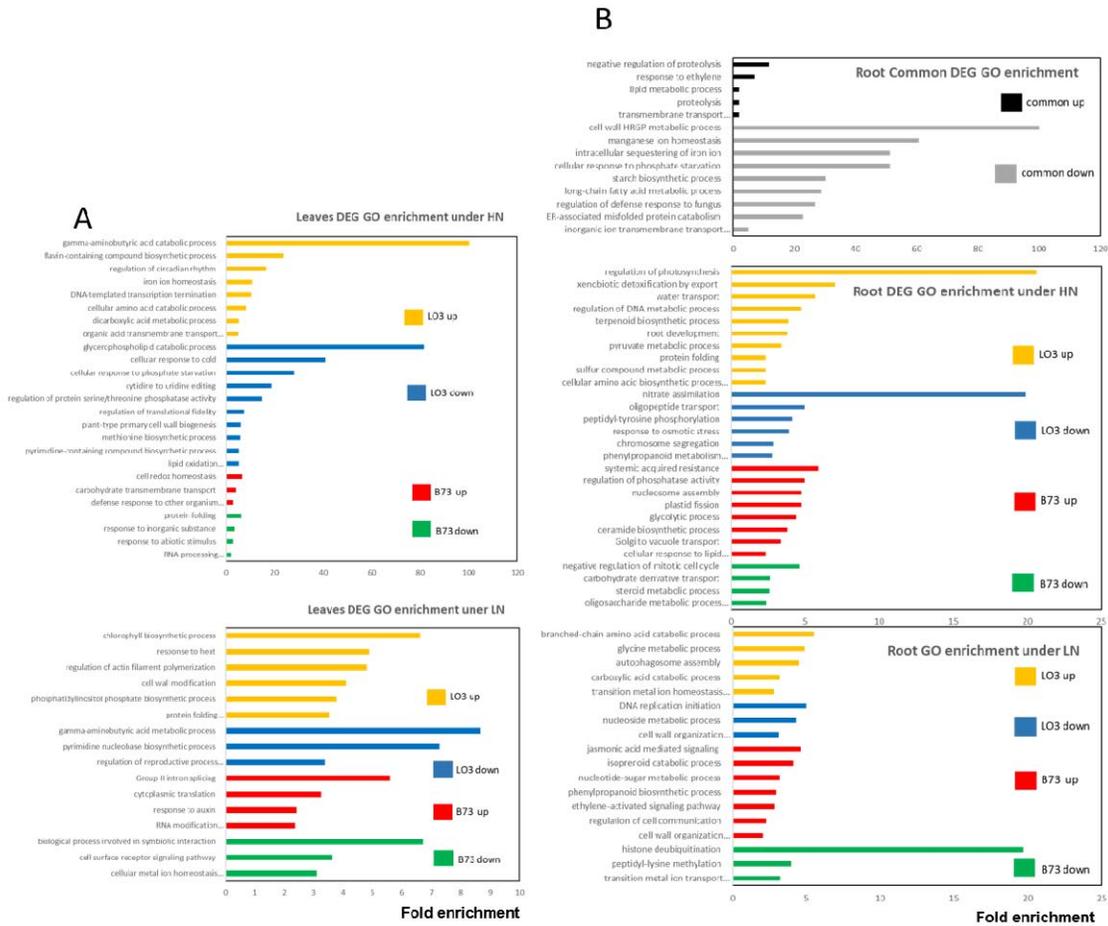


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 924 **Figure 3: Heatmap of physio-agronomical traits in different organs shows that *R. irregularis***  
 925 **inoculation of LN plants improves their performances.** Plants were grown and harvested in 2020  
 926 as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>), moderate nitrate (MN, 5mM  
 927 NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). Standardized levels of quantified physio-agronomic traits of  
 928 B73 and Lo3 lines under LN, MN or HN inoculated or not with *R. irregularis* are shown. The two HCA  
 929 were performed to group the samples and the physio-agronomic traits according to their average  
 930 values following Ward method of clustering. At the left of the HCA, the vertical bar represents the  
 931 groups of samples. Indicated traits are measured in Aerial parts (AP), roots (R) or grains (G). Group A  
 932 corresponded to plants grown under LN, Group B corresponded to plants grown under MN or HN. The  
 933 B group was subdivided into two subgroups: B1 and B2 corresponded respectively to B73 and Lo3  
 934 plants. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*. Three organs were  
 935 considered: Aerial Parts (AP), Grains (G) and Roots (G). Chloro: chlorophyll monitored by SPAD  
 936 index. Cpercent: Percentage of C. Npercent: percentage of N. CN: C/N ratio. 15NEpercent:  
 937 percentage of <sup>15</sup>N. P Percent: percentage of P. Q15Nmg: quantity of 15N in organ per plant. NUE:  
 938 Nitrogen Use Efficiency. GY: Grain Yield per plant. The color scale indicates if the trait is higher  
 939 (orange) or lower (blue) in the indicated conditions with respect to the others. Details for the formulas  
 940 used to calculate these traits are indicated in Supplementary Table 3.



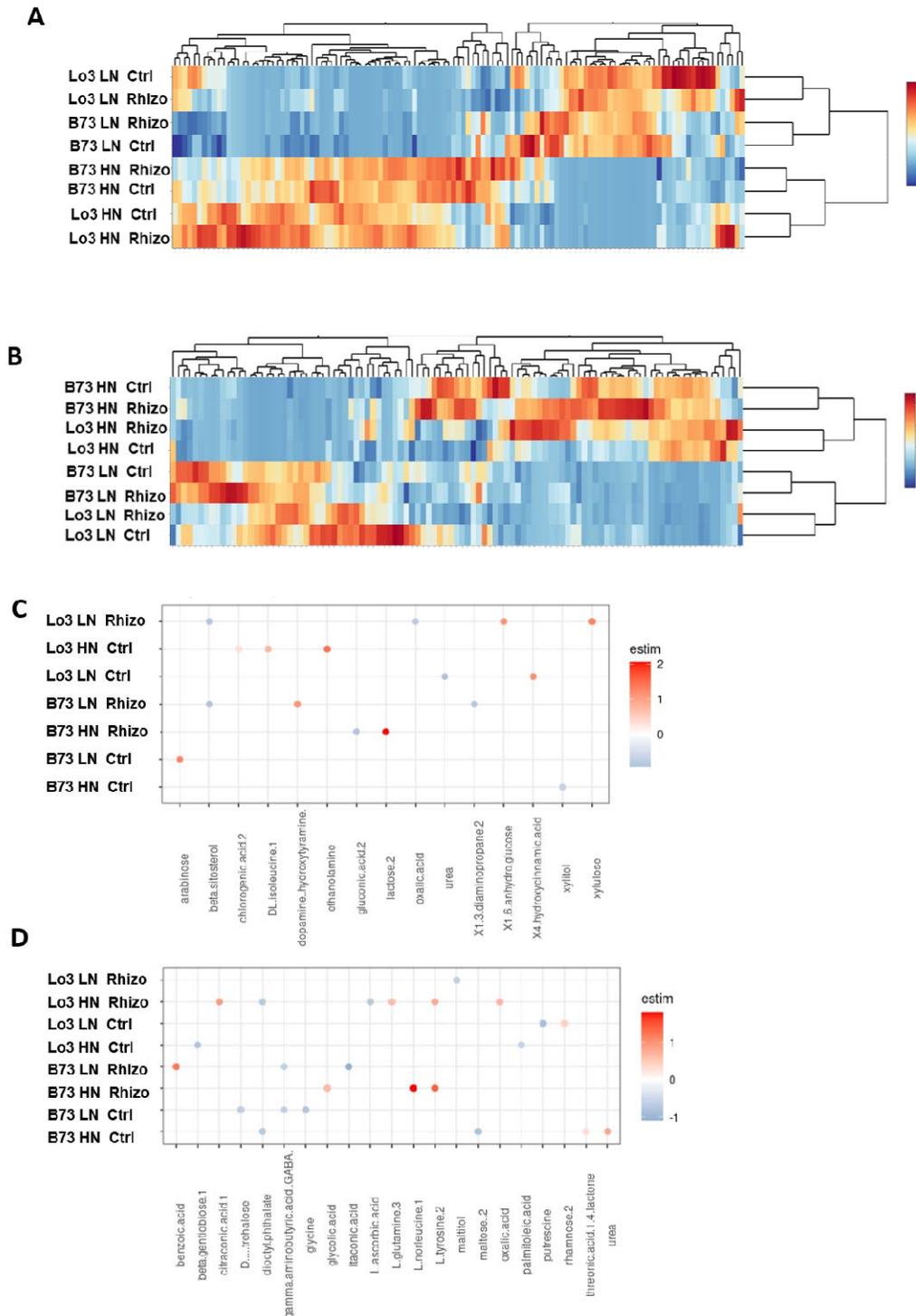
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**Figure 4: Impact of N supply on expression of genes involved in N and P transport in roots suggests that AM symbiosis alleviates N deficiency stress.** Plants were grown and harvested in 2020 as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>), moderate nitrate (MN, 5mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). Roots were harvested at VT+15 stage. **A:** Normalized transcript levels of indicated genes monitored by qRT-PCR. **B:** Quantification of *R. irregularis* in roots by qRT-PCR amplification of the *RiLSU* constitutive fungal transcript. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*, N=5. Different letters indicate significantly different gene expression levels as analyzed by ANOVA. Bars are standard errors. Asterisk indicates significant difference between mock and *R. irregularis*-inoculated plants by t-test ( $p < 0.05$ ).



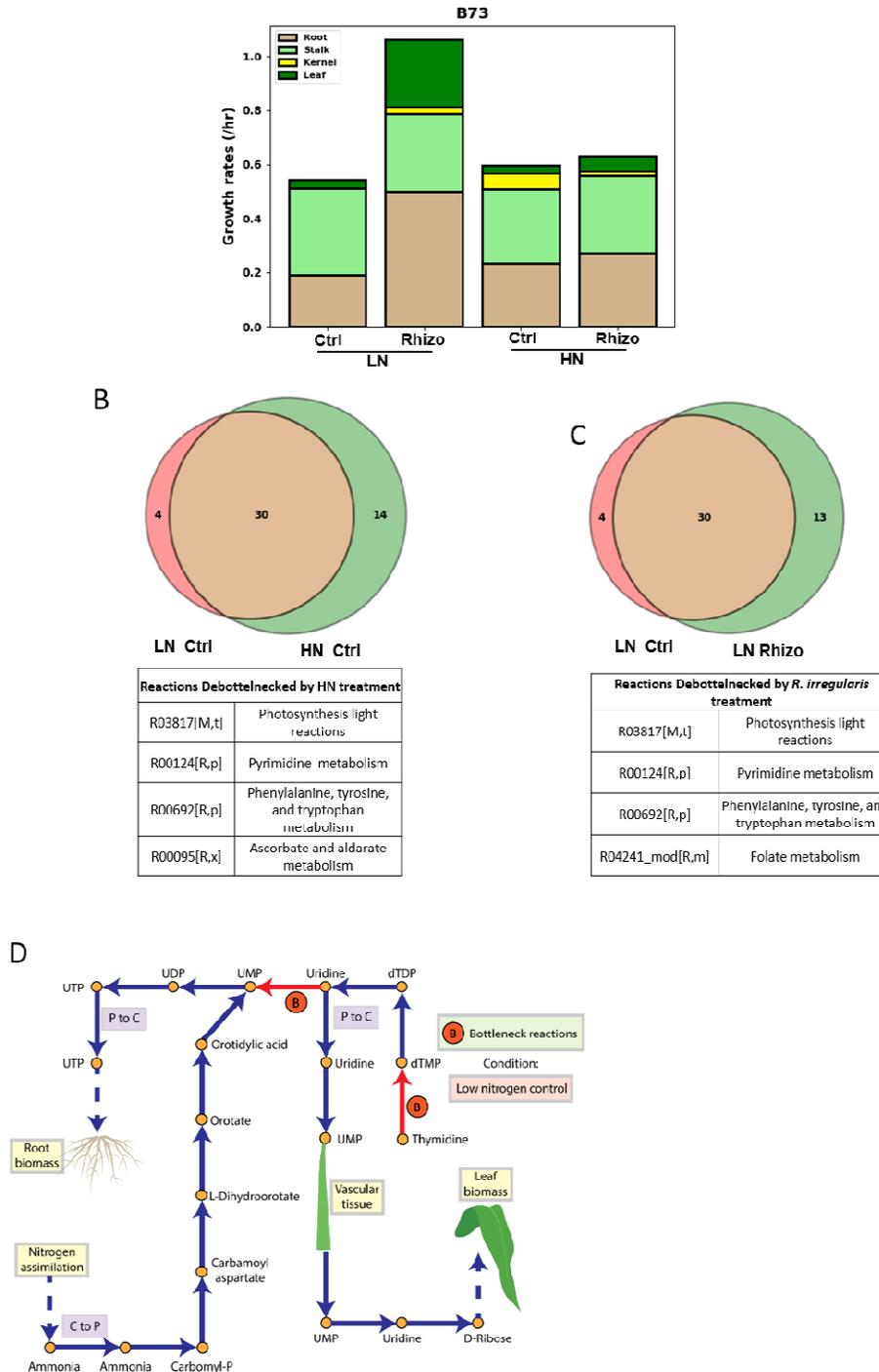
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**Figure 5 : Gene ontology of the enriched functional categories in the common DEGs. A:** Leaf genes. **B:** Root genes. Common DEGs were selected so that they have the same pattern of regulation (up or down) in the different conditions with a fold change (FC) >1.5. FC represents the ration of expression of each gene in *R. Irregularis* inoculated tissues versus the mock control tissues. There were only 2 genes in the common DEGs in leaves thus, they were not included in the GO analyses. Each bar represents the fold enrichment in the indicated category obtained by <https://pantherdb.org/>. Only categories enriched more than 2 fold and displaying more than 2 genes are represented with  $p$ -value of enrichment < 0.05.



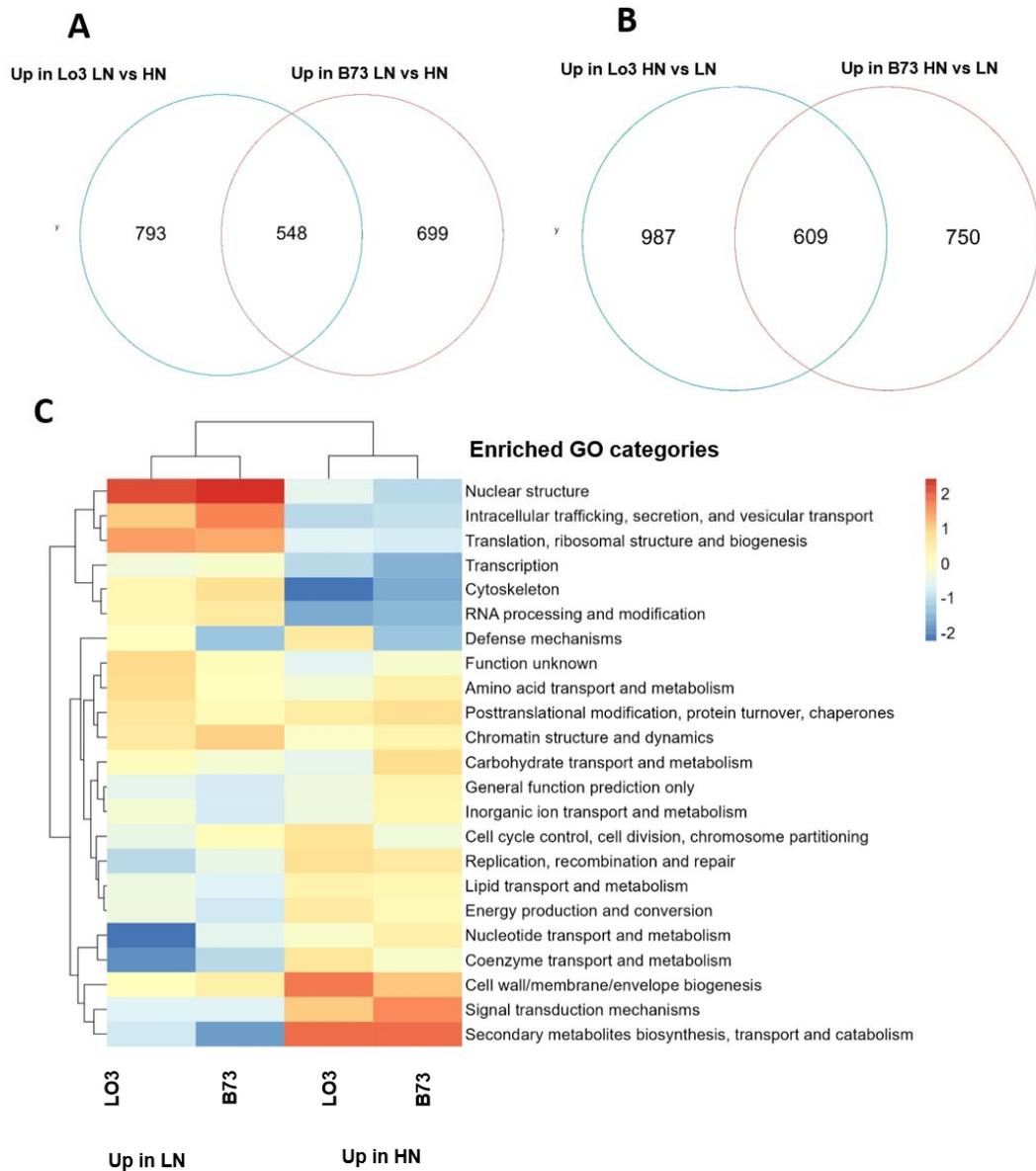
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**Figure 6: Metabolomic analyses of leaves and roots reveals key metabolites specific to symbiosis under LN or HN nutrition.** Plants were grown and harvested in 2020 as indicated in materials and methods under low nitrate (LN, 1mM  $\text{NO}_3^-$ ) or high nitrate (HN, 10mM  $\text{NO}_3^-$ ). Leaves and roots were harvested at VT+15 stage. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*, Metabolites were monitored by GS/MS. **A** and **B** : HCA heatmap showing metabolomic profiles of leaves and roots of inoculated or control plants under LN or HN in leaves and roots respectively. **C** and **D**: Analyses of metabolomics data conducted with the R package MultiVarSel with a threshold of 0.884 showing the metabolites on which the genotype, the N nutrition of the *R. irregularis* inoculation have an impact. Leaves, **C**; roots, **D**. The color scale indicates if the metabolites are accumulated (red) or down accumulated (blue) in the indicated conditions with respect to the others.

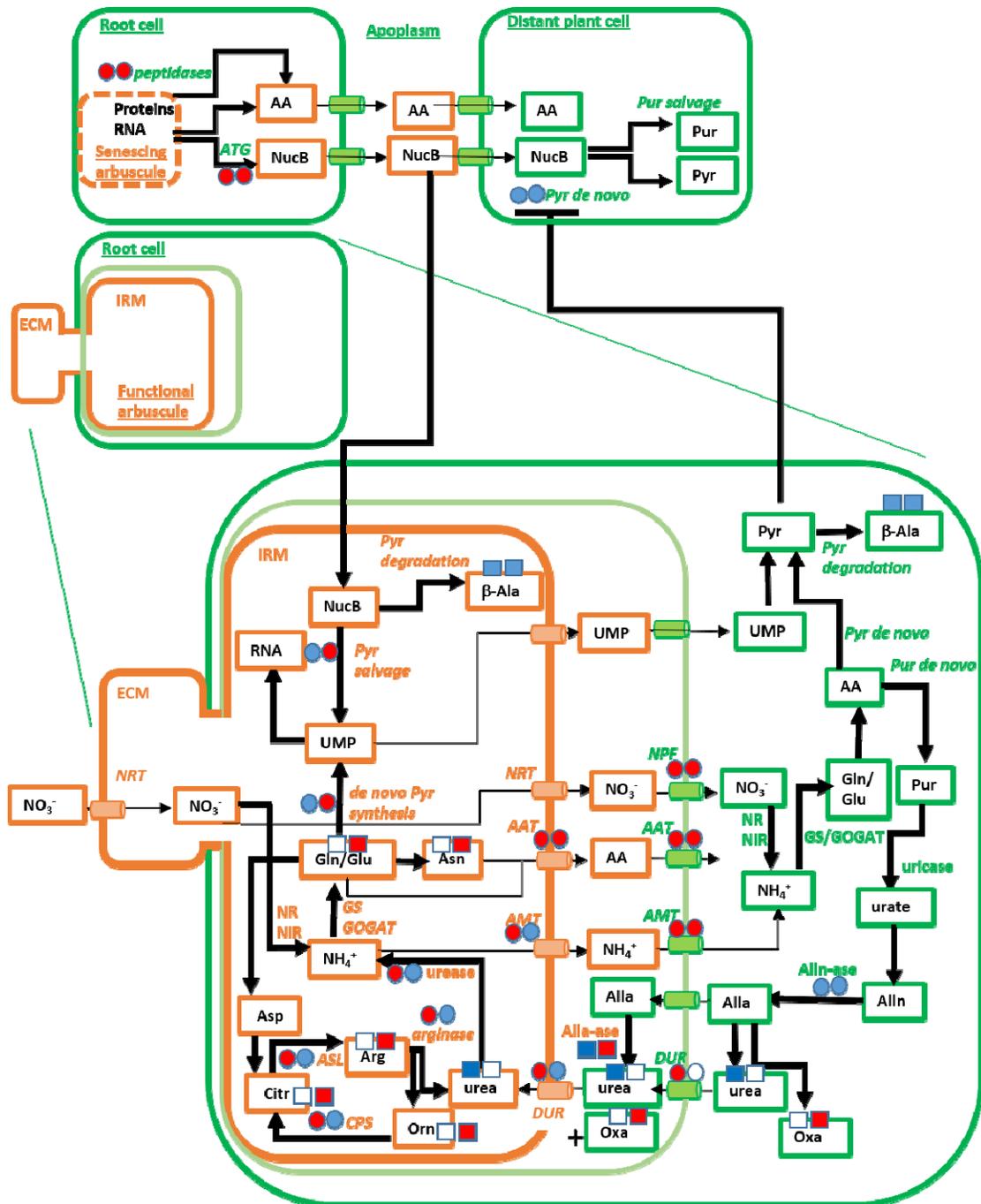


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**Figure 7: Genome based metabolic modeling reveals essential impact of AMF on pyrimidine metabolism.** **A:** RNAseq data were used to predict different organ growth rates in the B73 maize line. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*, EXTREAM algorithm predicted higher yield under LN in B73. **B** and **C:** Venn diagrams showing the metabolic bottlenecks under indicated conditions highlighting the debottlenecked reactions by uninoculated HN versus uninoculated LN (B) and by *R. irregularis* inoculation under LN vs uninoculated LN (C). **D:** Metabolic reactions in the pyrimidine pathway de-bottlenecked by *R. irregularis* inoculation and by HN treatment shown in **B** and **C**. P: Plastid compartment. C: Cytosolic compartment.



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980 **Figure 8 : *Rhizophagus irregularis* transcriptome in maize roots is shaped by N supply.** Plants were grown  
981 and harvested as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM  
982 NO<sub>3</sub><sup>-</sup>). Roots inoculated with *R. irregularis* were harvested at VT+15 as indicated in materials and methods. DEGs  
983 were selected so that they have a fold change (FC >1.5). **A** : Up in LN indicates *R. irregularis* genes that are more  
984 highly expressed in inoculated LN roots compared to inoculated HN roots. **B**: Up in HN indicates *R. irregularis*  
985 genes that are more highly expressed in HN compared to LN. **C**: Gene ontology of functional categories  
986 enrichment was based on KOG annotations of encoded *R. irregularis* proteins (see materials and methods). Each  
987 bar represents the functional category enriched in the given condition. p-value of enrichment was < 0.05; only  
988 categories enriched more than 2 fold and displaying more than 2 genes are represented and used to build the  
989 heatmap. The color scale indicates the over-representation or the down-representation in red and blue  
990 respectively.



**Figure 9: Hypothetical working model for maize and *R. irregularis* N nutrition under LN and HN.**

To build the model, the metabolomic data from B73 and Lo3 maize lines were pooled for simplification (Supplementary Figure 10) and we used genes in Supplementary Tables 7 and 8. In some cases genes with multigene families such as Glutamine Synthetase encoding genes have different expression profiles. For simplification purposes, these cases were not considered in the hypothetical mechanistic model below.

- Under LN fungal RNAseq data indicate up regulation of amino acid transporters (AAT) and ammonium transporters (AMT). Fungal argininosuccinate lyase (ASL) and arginase genes are up regulated under LN, which could lead to urea accumulation. A fungal urea transporter (*DUR*) is upregulated under LN, it is likely to be expressed in the IRM but could

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1004 also be expressed in the ECM. Usually DUR is an exporter of urea, it remains to be  
1005 determined how this *R. irregularis* DUR transporters acts during AM symbiosis. However,  
1006 urea is probably further degraded into ammonium by urease since the fungal urease  
1007 coding gene is up regulated under LN. This could explain the fact that under LN, *R.*  
1008 *irregularis* inoculated roots accumulate less urea. Globally lower amounts of amino acids  
1009 are found in LN roots which suggests less assimilation via GS-GOGAT in the fungal and  
1010 inoculated plant root cells. The fungal RNAseq data indicate that pyrimidine de novo  
1011 biosynthetic genes are down regulated under LN compared to HN but still expressed (for  
1012 example, UMP synthase, orototate phosphorybolsyl transferase) as well as the pyrimidine  
1013 salvage pathway (for example, dUTPase, allantoicase, deoxycitydylate deaminase,  
1014 cytosine deaminase). These data suggest that under LN, the fungus redirects its N  
1015 metabolism in order to provide  $\text{NH}_4^+$  to the plant cell, but may also provide nucleosides to  
1016 plant cells.

1017 -Under HN, high amounts of  $\text{NO}_3^-$  are available to the fungus and to the plant. Fungal RNAseq  
1018 data indicate up-regulation of some amino acid transporters that are different from those  
1019 up-regulated in LN, down regulation of ammonium permeases. Fungal argininosuccinate  
1020 lyase and arginase genes are down-regulated under HN, which could lead to urea  
1021 accumulation. A fungal urea transporter is down-regulated under HN. We observe a global  
1022 increase in the amounts several amino acids including Citr, which is a precursor of Arg. Arg  
1023 and Asn accumulate in inoculated roots indicating that fungal and/or plant assimilation  
1024 via GS-GOGAT are probably upregulated under HN in response to the fungus. The fungal  
1025 RNAseq data indicate that in HN roots, pyrimidine de novo biosynthetic genes are up-  
1026 regulated (for example, UMP synthase, orototate phosphorybolsyl transferase) as well as  
1027 the pyrimidine salvage pathway (for example, dUTPase, allantoicase, deoxycitydylate  
1028 deaminase, cytosine deaminase). Allantoin accumulates only in HN roots and not in LN  
1029 roots, and its amount is not affected by inoculation in HN roots. Allantoin can be converted  
1030 into allantoic acid which can be released in the apoplast. In HN roots a fungal allantoicase  
1031 is up-regulated suggesting that urea is produced by this pathway under HN.

1032 -In italics, genes. In boxes: metabolites. In orange : fungal genes or metabolites. In green :  
1033 plant genes or metabolites. In front of genes and metabolites there are two squares. The  
1034 square at the left indicates the status of the item under LN. The square at the right  
1035 indicates the status of the item under HN. The circles at the left indicates the status of the  
1036 gene expression under LN. The circle at the right indicates the status of the gene  
1037 expression under HN. AA: Amino acids. NucB: Nucleobases. Pur: Purine. Pyr: Pyrimidine.  
1038 IRM: Intraradical mycelium. ECM: Extraradical mycelium. .Alla: Allantoic Acid. Alln :  
1039 Allantoin. Alln-ase: Allantoinase. Arg: arginine. Asn: Asparagine. Asp : Aspartate. b-Ala :b-  
1040 alanine. Citr : Citrulline. Glu: Glutamate. Gln: Glutamine. Orn : Ornithine. GOGAT:  
1041 Glutamine Oxo-Glutarate Aminotransferase. Oxa : Oxalic Acid. NR : Nitrate reductase. NiR  
1042 : Nitrite reductase. AAT : Amino acid transporter. AMT : Ammonium transporter. DUR :  
1043 Urea transporter. NPF : plant Nitrate transporter. NRT : *R. irregularis* nitrate transporter.  
1044 CPS : Carbamoyl-phosphate synthase. ASL : Argininosuccinate lyase. Pyr de novo:  
1045 Pyrimidine de novo biosynthetic pathway. Pur de novo: Pur de novo biosynthetic pathway.

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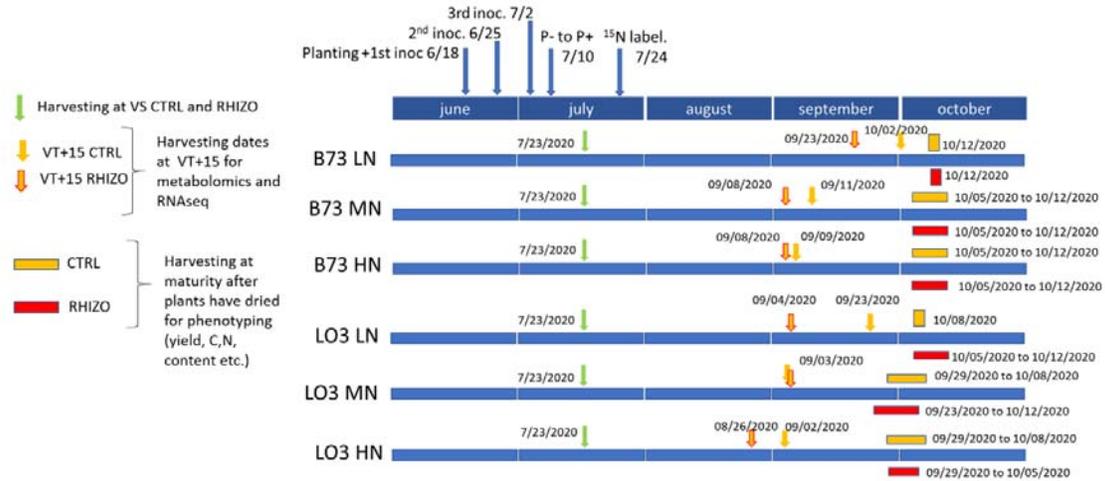
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1052 **Table 1 : List of metabolic bottlenecks relieved and created by *R. irregularis* or N**  
 1053 **supply.** Metabolic bottlenecks were identified by MBA that are relieved by either *R.*  
 1054 *irregularis* treatment or HN treatment compared to LN control conditions are indicated as  
 1055 “debottlenecked”. Those that were newly created following *R. irregularis* or HN treatment were  
 1056 compared to LN control conditions are indicated as “new metabolic” bottlenecks.

Reactions Debottlenecked by <i>R. irregularis</i> treatment	
R03817[M,t]	Photosynthesis light reactions
R00124[R,p]	Purine and Pyrimidine metabolism
R00692[R,p]	Phenylalanine, tyrosine, and tryptophan metabolism
R04241_mod[R,m]	Folate metabolism
Reactions Debottlenecked by HN treatment	
R03817[M,t]	Photosynthesis light reactions
R00124[R,p]	Purine Pyrimidine metabolism
R00692[R,p]	Phenylalanine, tyrosine, and tryptophan metabolism
R00095[R,x]	Ascorbate and aldarate metabolism
New metabolic bottlenecks with LN RHIZO	
R03346[M,c]	Nicotinate and nicotinamide metabolism
R03346[B,c]	Nicotinate and nicotinamide metabolism
R00086[M,pm]	Purine metabolism
R00986[R,p]	Purine metabolism
MR00968[B,c]	Benzoate metabolism
R00086[B,pm]	Purine metabolism
R00341[R,c]	Pyruvate metabolism
R00216[B,p]	Pyruvate metabolism
R01058[B,c]	Glycolysis
R02678[R,m]	Phenylalanine, tyrosine, and tryptophan metabolism
R00710[R,m]	Pyruvate metabolism
R01067[R,c]	Calvin-Benson-Bassham cycle
R01067[R,p]	Calvin-Benson-Bassham cycle
New bottlenecks in HN	
R00341[R,c]	Pyruvate metabolism
R00709_mod[R,m]	TCA cycle
R00713[R,m]	Alanine, aspartate, and glutamate metabolism
R01067[R,c]	Calvin-Benson-Bassham cycle
R01067[R,p]	Calvin-Benson-Bassham cycle
R01667[R,p]	Pyrimidine metabolism
R00235[B,x]	Pyruvate metabolism
R00885[B,c]	Fructose and mannose metabolism
R01058[B,c]	Glycolysis
R02080[M,c]	Phenylalanine, tyrosine, and tryptophan metabolism
R00214[M,m]	Pyruvate metabolism
R00124[B,p]	Purine metabolism
R00267_mod[M,c]	Alanine, aspartate, and glutamate metabolism

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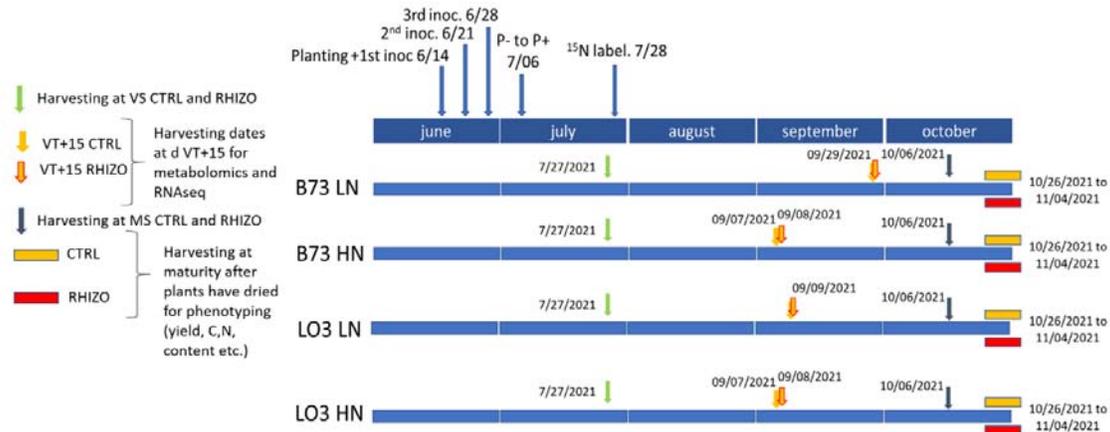
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1061 **Supplementary Figure 1: Experimental design with dates of each treatment and**  
 1062 **harvesting.** Experiments were performed at indicated dates during the summer of 2020 in  
 1063 the greenhouse. Each date is represented on the boxes below for each maize line/condition.  
 1064 For each maize line/condition, the harvesting dates for the vegetative stage (VS) and 15  
 1065 days after vessel tasseling (VT+15) stage are indicated. The horizontal red and yellow boxes  
 1066 indicate the periods of harvesting dry mature plants for physio-agronomical trait  
 1067 phenotyping. Ctrl: Mock non inoculated control. RHIZO: inoculated with *R. irregularis*.

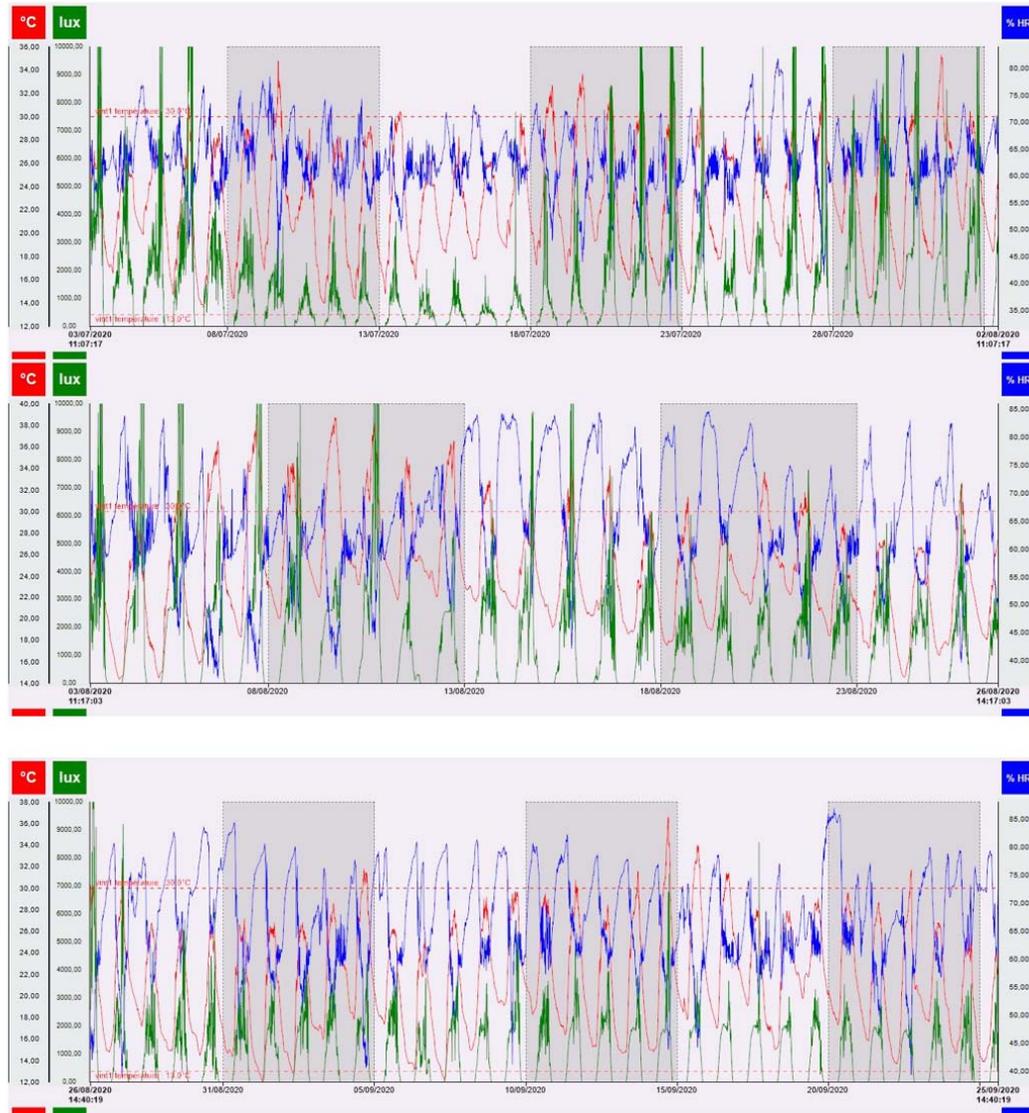
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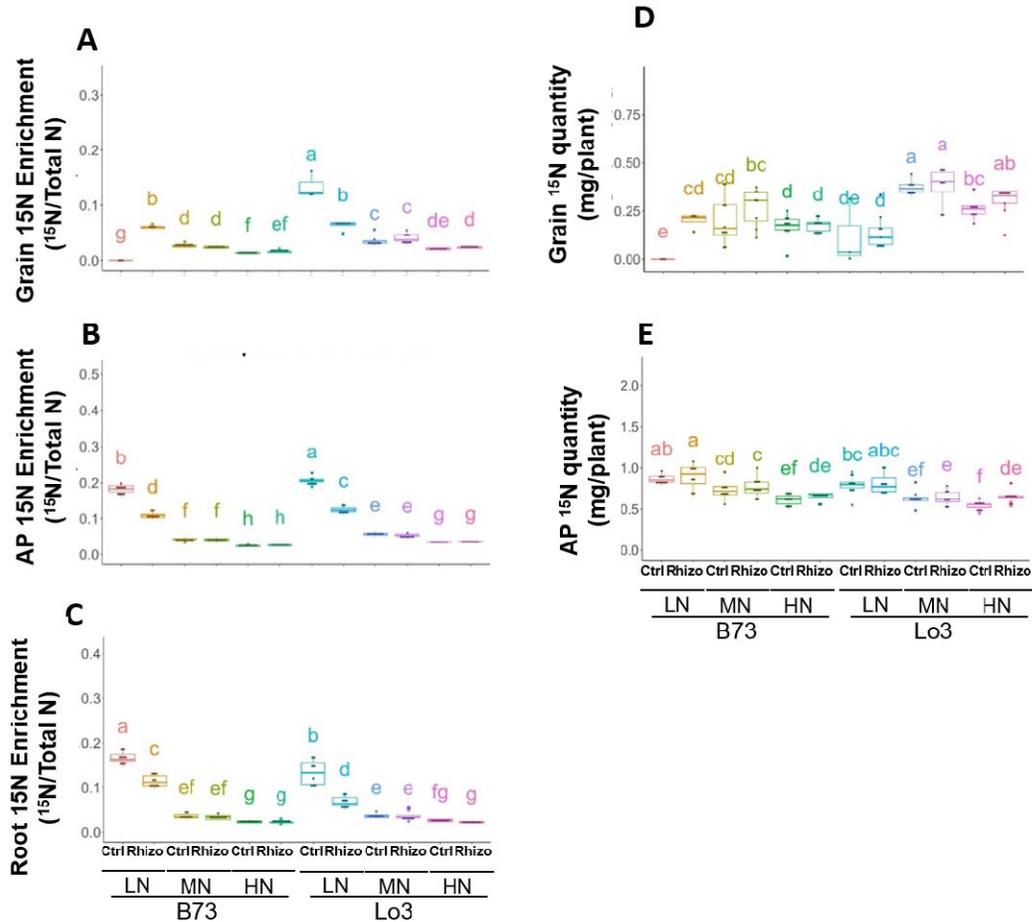
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1071 **Supplementary Figure 2: Experimental design with details for dates of each treatment**  
 1072 **and harvesting dates.** Experiments were performed at indicated dates during the summer  
 1073 of 2021 in the greenhouse. Each date is represented on the boxes below for each maize  
 1074 line/condition. For each maize line/condition, the harvesting dates for the vegetative stage  
 1075 (VS) and 15 days after vessel tasseling (VT+15) stage are indicated. The horizontal red and  
 1076 yellow boxes indicate the periods of harvesting dry mature plants for physio-agronomical  
 1077 trait phenotyping. Ctrl: Mock non inoculated control. RHIZO: inoculated with *R. irregularis*.



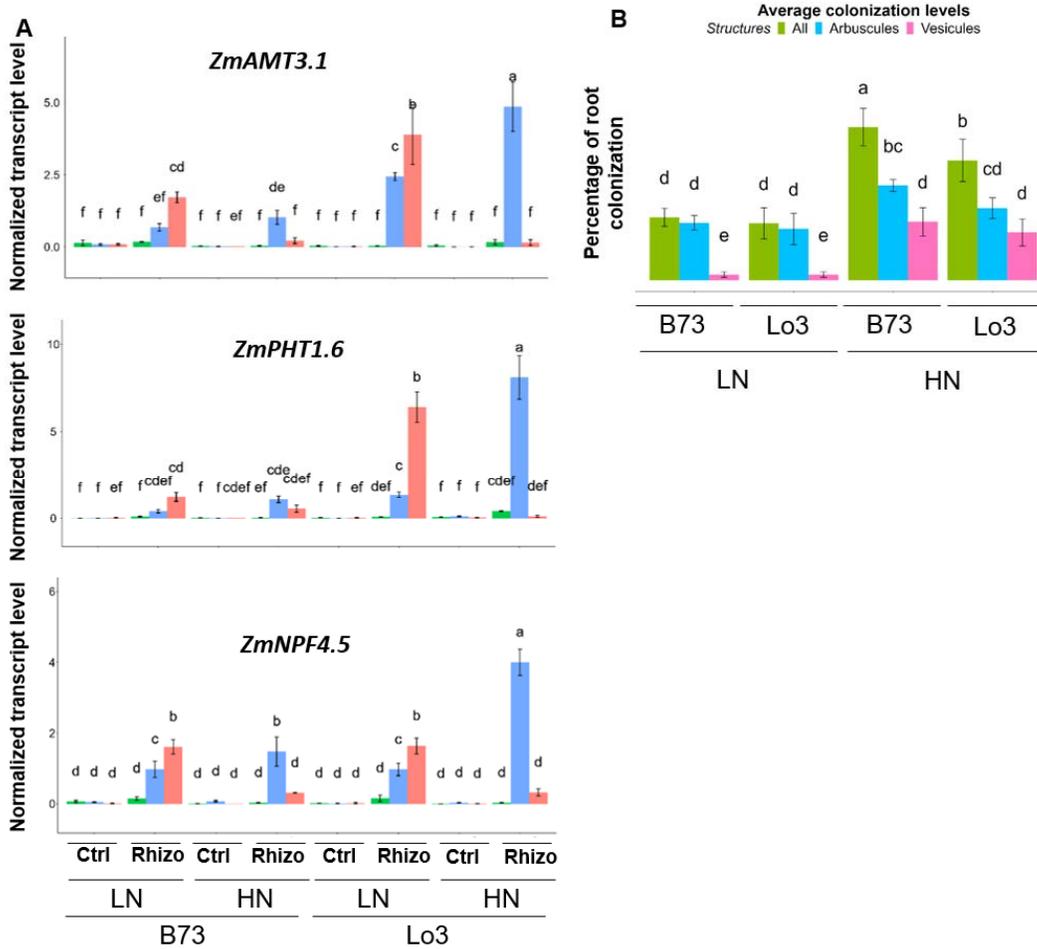
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**Supplementary Figure 3: Meteorological data collected in the greenhouse for the 2020 experiment (Supplementary Figure 1).** In red the temperature in °C. In green the light intensity in Lux. In blue, the % of humidity. Data from July 3rd to September 26th 2020 are shown.



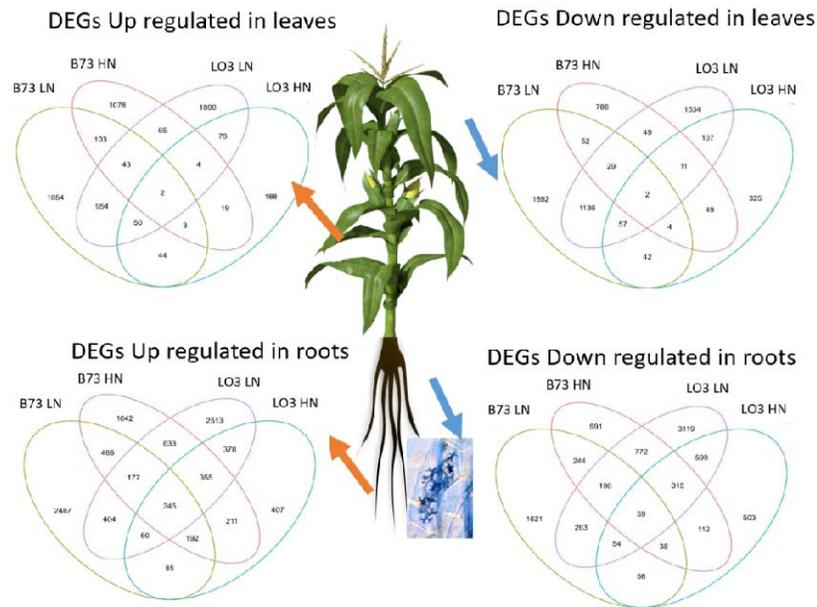
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**Supplementary Figure 4: Impact of N supply on N isotope fractionation and  $^{15}\text{N}$  uptake in response to *R. irregularis* shows that AM symbiosis and N supply have a strong effect on N uptake.** Plants were grown and harvested in 2020 as indicated in materials and methods under low nitrate (LN, 1mM  $\text{NO}_3^-$ ), moderate nitrate (MN, 5mM  $\text{NO}_3^-$ ) or high nitrate (HN, 10mM  $\text{NO}_3^-$ ). Plants were subjected to a short pulse with a  $^{15}\text{N}$  labelled nutrient solution (See Materials and Methods). **A, B** and **C** :  $^{15}\text{N}$  enrichment in grains, aerial parts (AP) and roots respectively. **D** and **E** :  $^{15}\text{N}$  quantity in mg/plant in grains and AP respectively. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*, AP: Biomass of Aerial Parts. N=7 to 10 plants. Different letters indicate significantly different averages for the considered trait by ANOVA N=7-10 plants for grains and aerial parts. N=5 for roots.



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**Supplementary Figure 5 : Impact of N supply on *R. irregularis* colonization and kinetics of symbiosis marker gene expression.** Plants were grown and harvested in 2021 as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). **A:** AM Symbiotic gene expression levels quantified by qRT-PCR. **B:** Quantification of root colonization level by microscopic observation of different fungal structures : arbuscules, vesicles and the global colonization level (All) in roots harvested at VT+15 stage. Ctrl: Mock non inoculated control, Rhizo: inoculated with *R. irregularis*. N=8. Bars indicate SD. Different letters indicate significantly different averages for the considered gene transcript level or trait by ANOVA.



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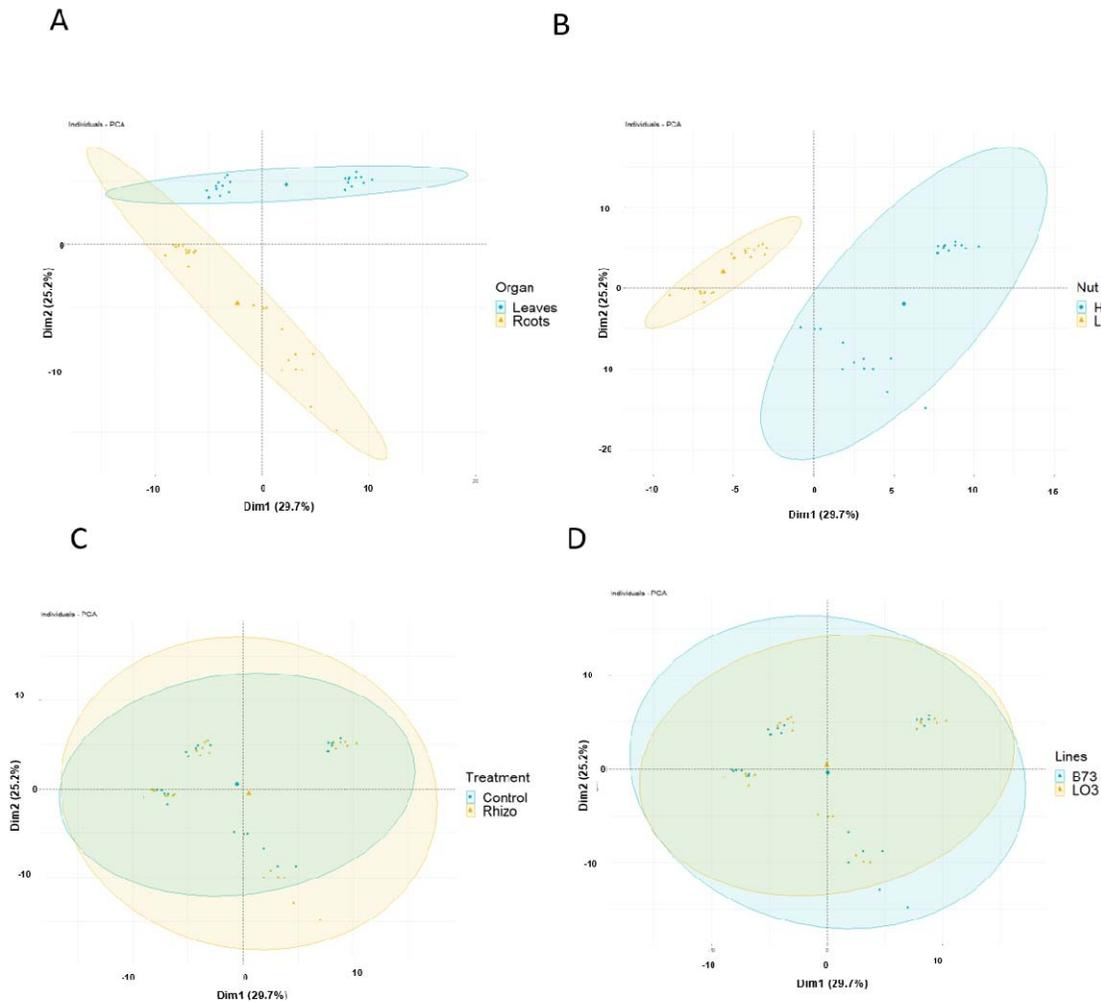
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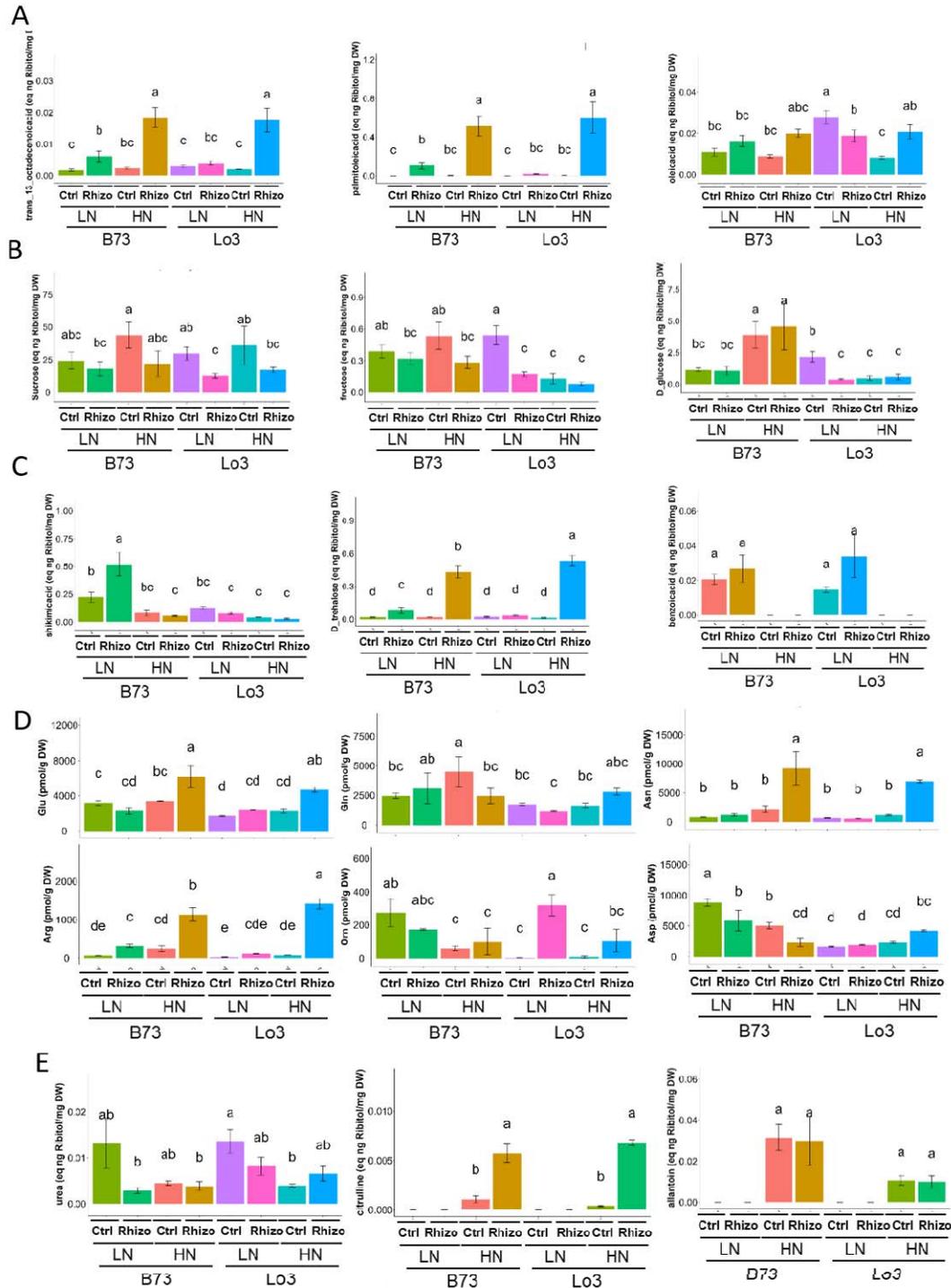
**Supplementary Figure 6 : Venn diagram of RNAseq data showing the overlap between maize DEG under LN or HN in line B73 and LO3.** Genes were selected so that their fold change (inoculated versus control) was > to 1.5 and statistically significant in the 3 replicates. B73 LN and B73 HN represent DEG genes up or down regulated in inoculated plants compared to control plants in LN or HN respectively; LO3 LN and LO3 HN represent DEG genes up or down regulated in inoculated plants compared to control plants in LN or HN respectively.

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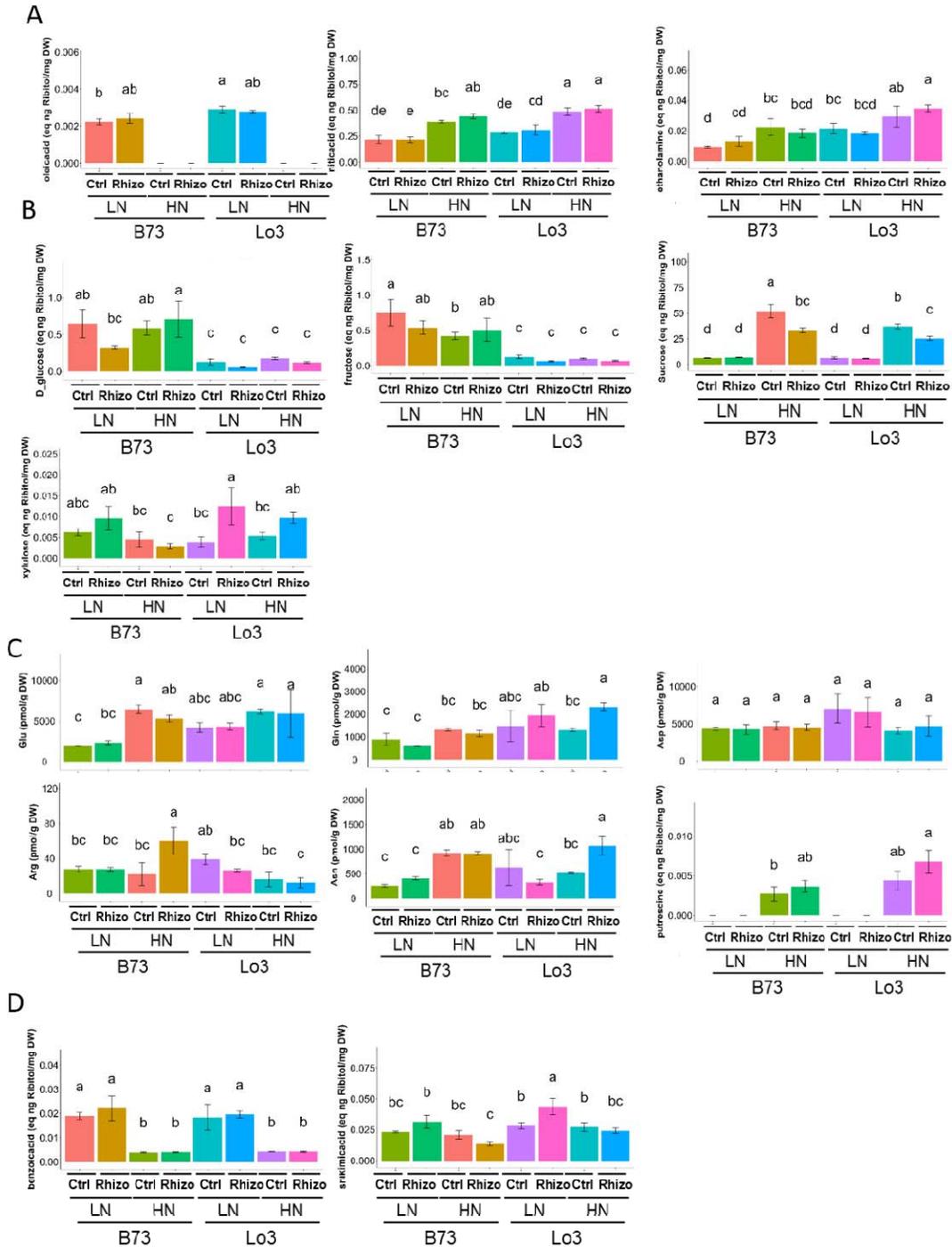
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1114 **Supplementary Figure 7: PCA on metabolomics data showing different impacts of N**  
1115 **nutrition, organ, maize genotype or *R. irregularis* inoculation on metabolome.** Plants  
1116 were grown and harvested in 2020 as indicated in materials and methods under low nitrate  
1117 (LN, 1mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>) at VT+15 stage. Primary metabolite profiles  
1118 obtained by GC/MS were used to build these PCAs from leaves and roots of inoculated  
1119 (Rhizo) or mock non-inoculated control (Ctrl) plants under LN or HN from B73 and Lo3  
1120 genotypes.



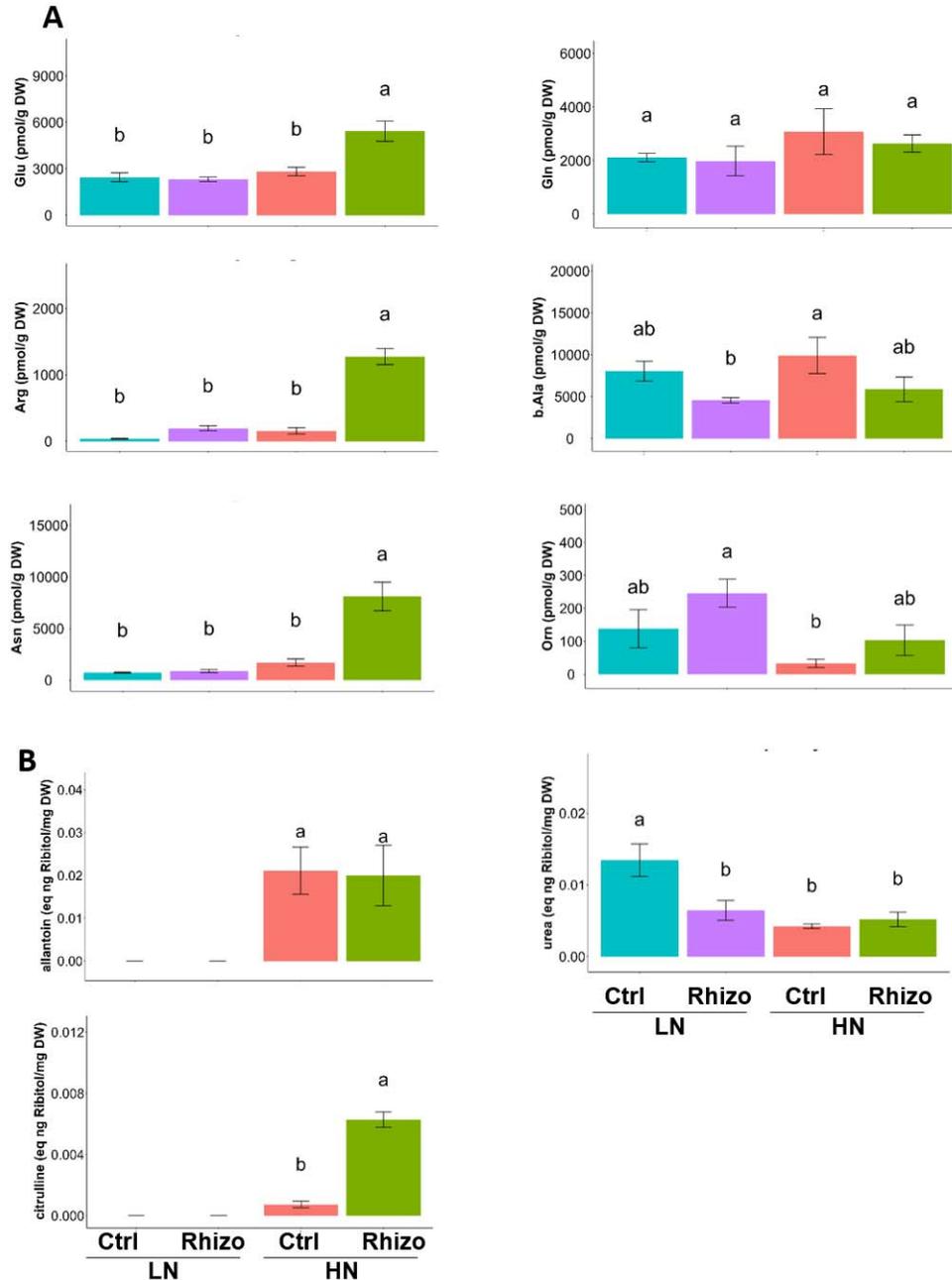
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**Supplementary Figure 8 : Examples of metabolites accumulating in roots in response to *R. irregularis*.** Plants were grown and harvested in 2020 as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). Roots were harvested at VT+15 stage. Ctrl: Mock non inoculated control, Rhizo: *R. irregularis*, N=3. Different letters indicate significantly different gene expression levels as analyzed by ANOVA. Bars are standard errors. Metabolites were quantified in identified samples by GS/MS in A, B,C and E or HPLC in D. **A**: examples of lipids. **B**: examples of soluble sugars. **C**: Examples of defense related metabolites. **D and E**: examples of N related metabolites.



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**Supplementary Figure 9 : Examples of metabolites accumulating in leaves in response to *R. irregularis*.** Plants were grown and harvested in 2020 as indicated in materials and methods under low nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). Roots were harvested at VT+15 stage. Ctrl: Mock non inoculated control, R: inoculated with *R. irregularis*, N=3. Different letters indicate significantly different gene expression levels as analyzed by ANOVA. Bars are standard errors. Metabolites were quantified in indicated samples by GC/MS or HPLC. Metabolites monitored by GC/MS are indicated by relative abundance to ribitol. Metabolites quantified by HPLC are indicated by absolute quantities (pmol/g DW). **A**: examples of lipid related metabolites. **B**: examples of sugars. **C**: Examples of N related metabolites. **D** : Examples of defense related metabolites.



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1142 **Supplementary Figure 10 : Metabolite profiles supporting hypothetical model in roots.**

1143 Plants were grown and harvested in 2020 as indicated in materials and methods under low  
 1144 nitrate (LN, 1mM NO<sub>3</sub><sup>-</sup>) or high nitrate (HN, 10mM NO<sub>3</sub><sup>-</sup>). Roots were harvested at VT+15  
 1145 stage. Metabolites related to the mecanistic hypothetical model in Figure 9 from both B73  
 1146 and Lo3 maize lines were pooled. **A**: metabolites quantified by HPLC. In **B** metabolites  
 1147 monitored by GC/MS. Ctrl: Mock non inoculated plants. Rhizo: inoculated with *R. irregularis*.

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