**Supplementary Information**

# Nitrogen supply influences photosynthesis establishment along the sugarcane leaf

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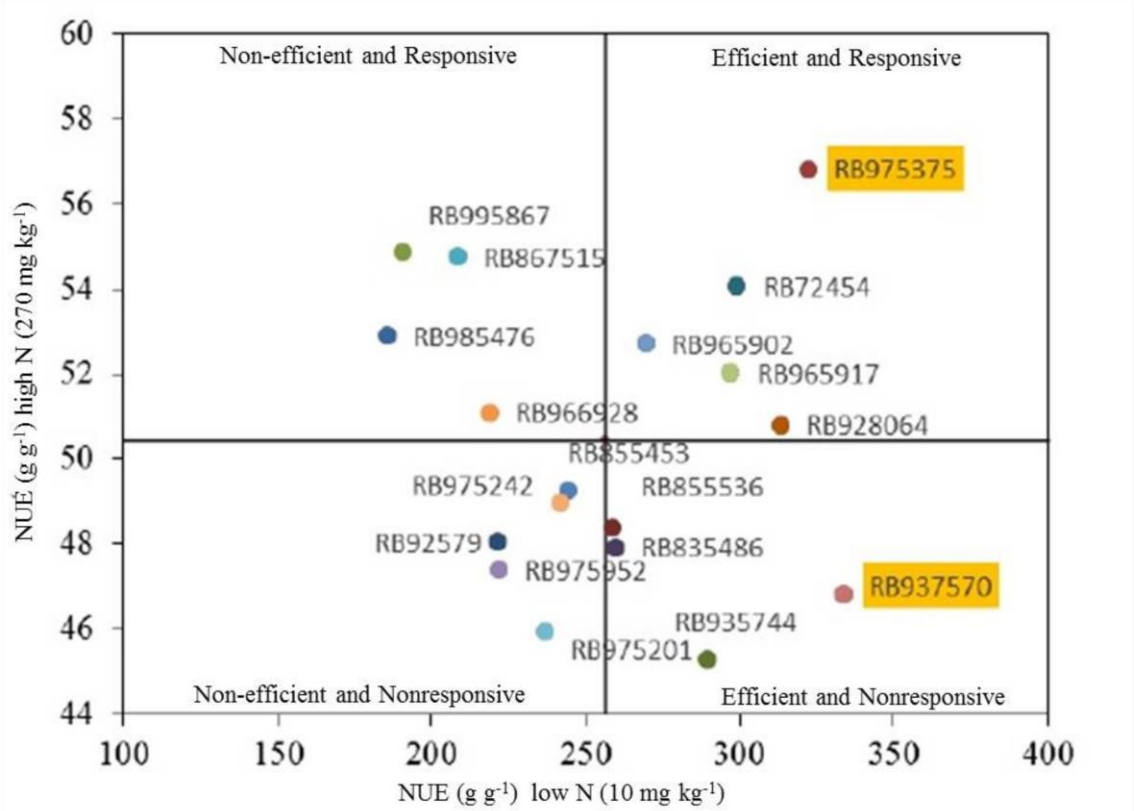
**Supplementary Methods**

Biometric measurements (number of tillers, number of leaves, number of green leaves, number of drought leaves, culm diameter, leaf +1 height, SPAD and foliar area) drought biomass (root and shoot) and physiological parameters (photosynthesis, stomatal conductance, internal CO2 concentration, electron transport rate, photochemical efficiency of PSII and transpiration), totalling 17 traits, were submitted to principal component analysis (PCA) using the mixOmics R package[1] in order to validate the contrasting response of genotypes in relation to N supply.

Leakiness was calculated according to the formula described by Farquhar[2] and was determined in the M segment. Total protein content was determined with Bradford solution (0.25%). Absorbance readings were measured in a microplate spectrophotometer (Infinite 200 PRO NanoQuant, Tecan, CH), and readings were taken at a wavelength of 595 nm.

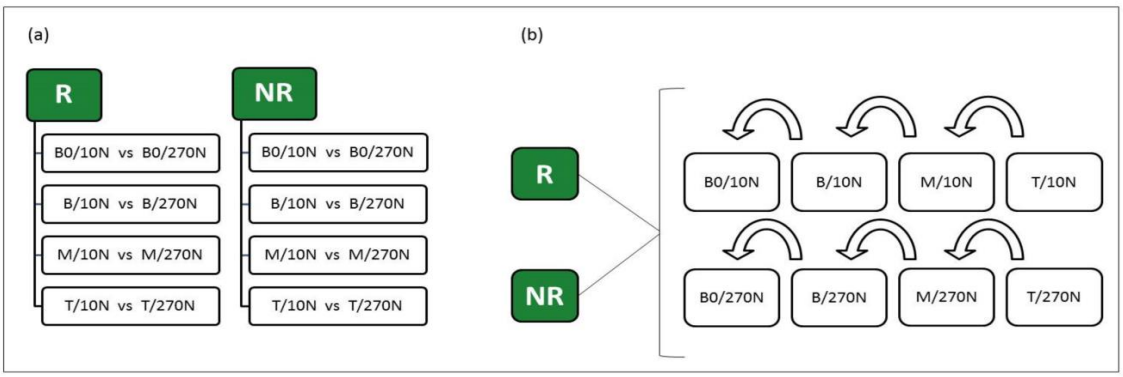



**Supplementary Figure S1. Screening of 20 sugarcane genotypes using NUE (nitrogen use efficiency) analysis based on methodology developed by Robinson[3].** NUE was determined by dividing biomass yield by crop N uptake. The responsiveness of genotypes was determined according to the threshold (NUE ≈ 50 g g-1 under high-N conditions) used in the screening analysis. The nonresponsive genotypes presented NUE < 50 and responsive genotypes NUE ≈ 50."

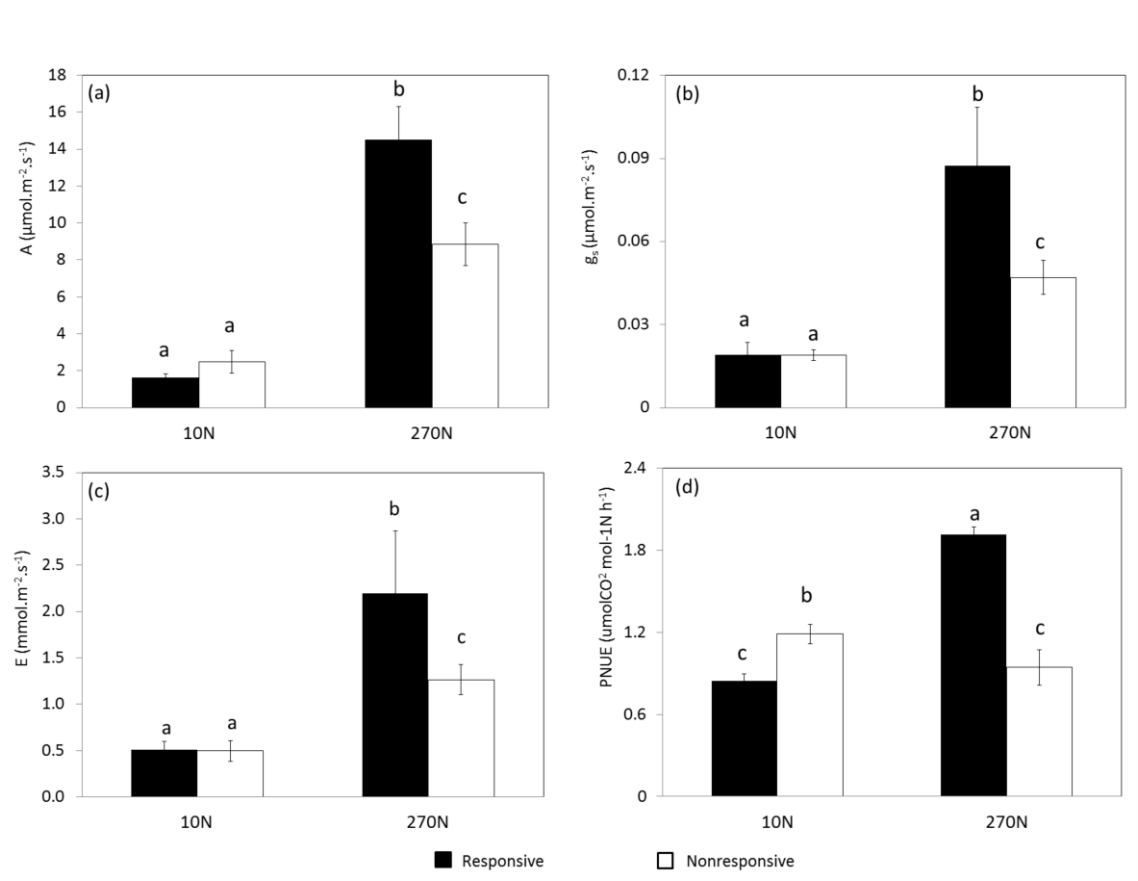
**Supplementary Table S1. Content of macronutrients and micronutrients used in the fertirrigation solution.**

| | Macronutrients | | | | | Micronutrients | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $H_2PO_4^-$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | $SO_4^-$ | $H_3BO_3$ | $Cu^{2+}$ | $Fe^{2+}$ | $Zn^{2+}$ | $Mn^{2+}$ | $MoO_4^{2-}$ |
| Content (mg per kg of sand) | 100 | 100 | 20 | 25 | 100 | 1 | 0.5 | 5 | 3 | 4 | 0.3 |

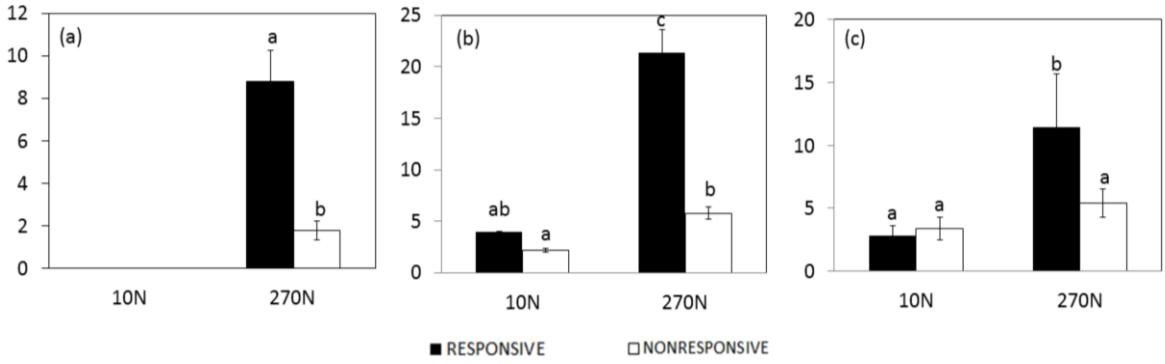

**Supplementary Figure S2**. **Three-month-old sugarcane plants.** R corresponds to the responsive genotype and NR to the nonresponsive genotype; 10N and 270N indicate 10 and 270 mg of N per kg of sand treatments, respectively.



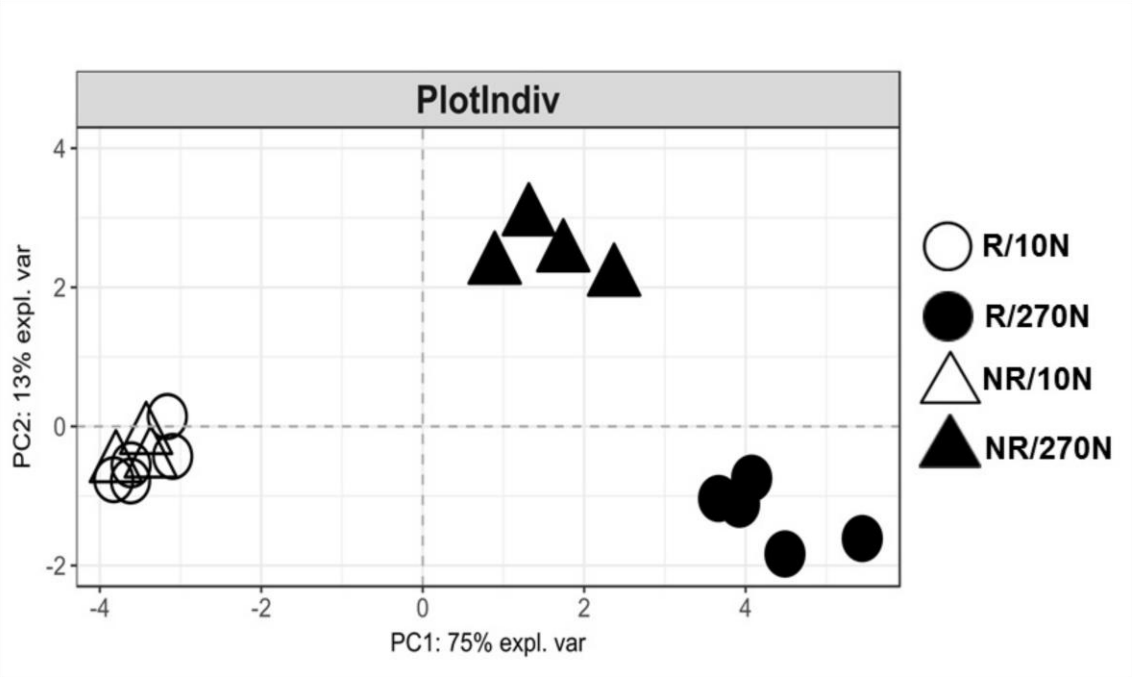

**Supplementary Figure S3. Schematic of the strategies used for t-test analysis applied to metabolomics data.** (a) Comparisons between treatments for each segment within each genotype; (b) Comparisons among distal segment to the previous segment within each leaf and each treatment for each genotype; R corresponds to the responsive genotype and NR to the nonresponsive genotype; 10N and 270N correspond to 10 and 270 mg of N per kg of sand treatments, respectively; B0, Base "zero"; B, Base; M, Middle; T, Tip.



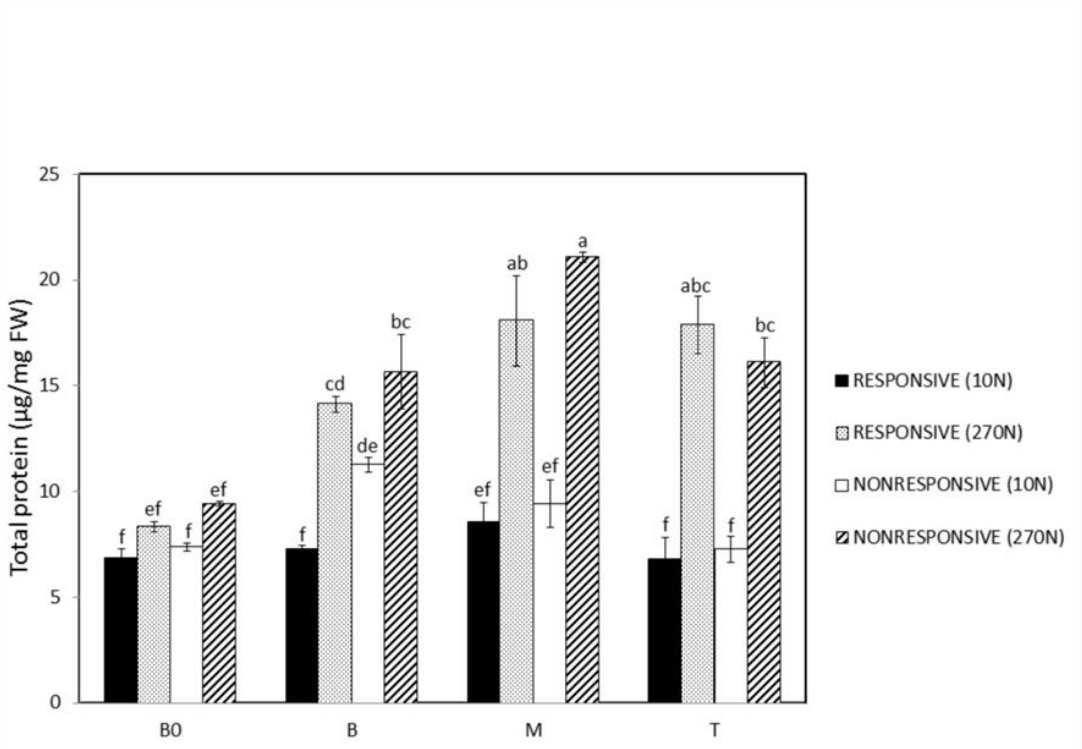

**Supplementary Figure S4: Effects of different N treatments on physiological parameters of two contrasting genotypes.** Physiological data were collected at middle leaf region of leaf +1 from three-month old plants. (a) Leaf photosynthetic rate; (b) Stomatal conductance rate; (c) Transpiration rate; (d) PNUE, photosynthetic nitrogen-use efficiency; 10N and 270N correspond to 10 and 270 mg of N per kg of sand, respectively. Data are presented as the mean ± SE. Letters indicate statistical significance using ANOVA followed by Fisher test ($n = 3$; $p \leq 0.05$).



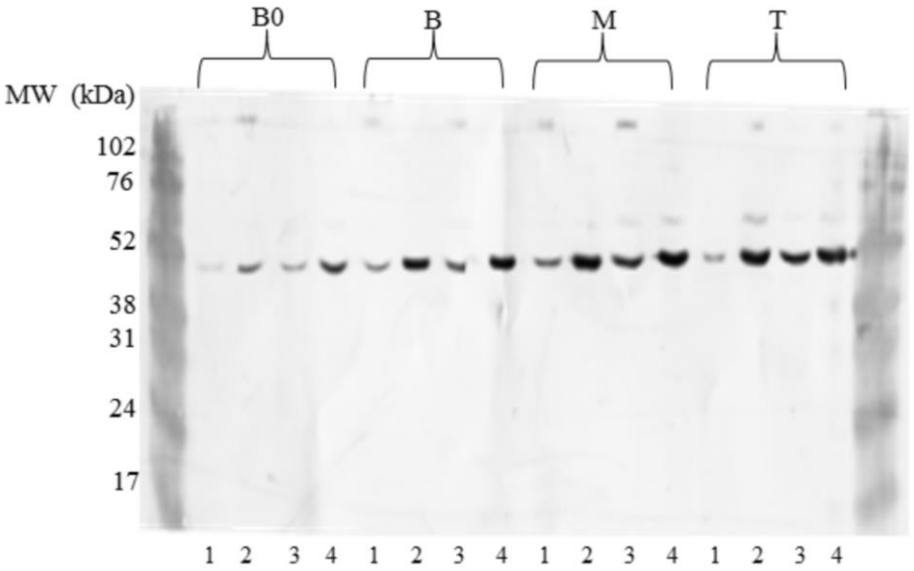

**Supplementary Figure S5: Effects of different N treatments on biometric traits.** (a) tillering; (b) number of green leaves; (c) number of dry leaves. 10N and 270N correspond to 10 and 270 mg of N per kg of sand, respectively. Data are presented as the mean ± SE. Letters indicate statistical significance using ANOVA followed by Fisher test ($n = 3$; $p \leq 0.05$).



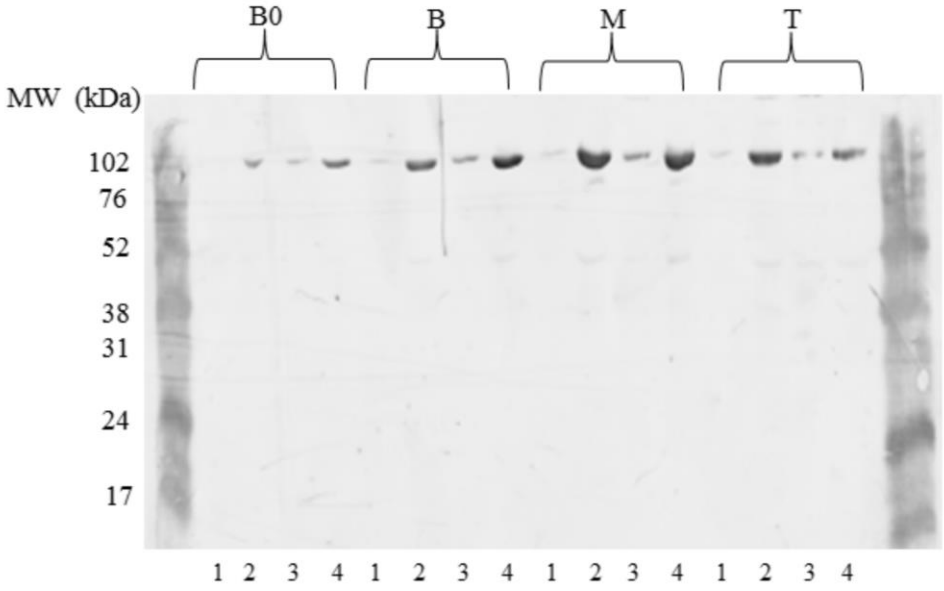

**Supplementary Figure S6: Multivariate PCA analysis using two principal components**. A total of 17 traits were used: tillering, total number of leaves, number of green leaves, number of dry leaves, culm diameter, height of leaf +1, SPAD index, leaf area, dry leaf biomass, dry culm biomass, dry root biomass, photosynthesis (A), stomatal conductance, internal carbon concentration (Ci), electron transport rate (ETR), potential quantum efficiency of photosystem II (Fv/Fm) and transpiration rate. 10N and 270N correspond to 10 and 270 mg of N per kg of sand, respectively. R, responsive (RB975375). NR, nonresponsive (937570).



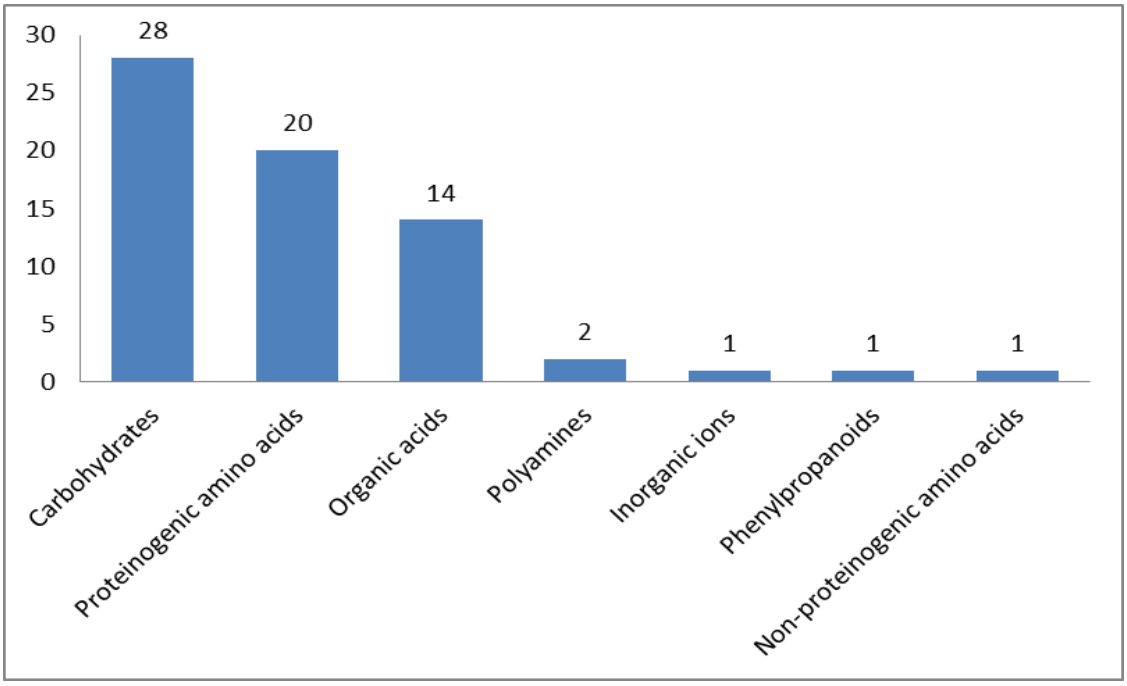

**Supplementary Figure S7. Protein content along sugarcane leaf blade.** 10N and 270N correspond to 10 and 270 mg of N per kg of sand treatments, respectively. B0, Base "zero"; B, Base; M, Middle; T, Tip. FW, fresh weight. Data are presented as the mean ± SE. Letters indicate statistical significance using ANOVA followed by Fisher test ($n = 3$; $p \leq 0.05$).



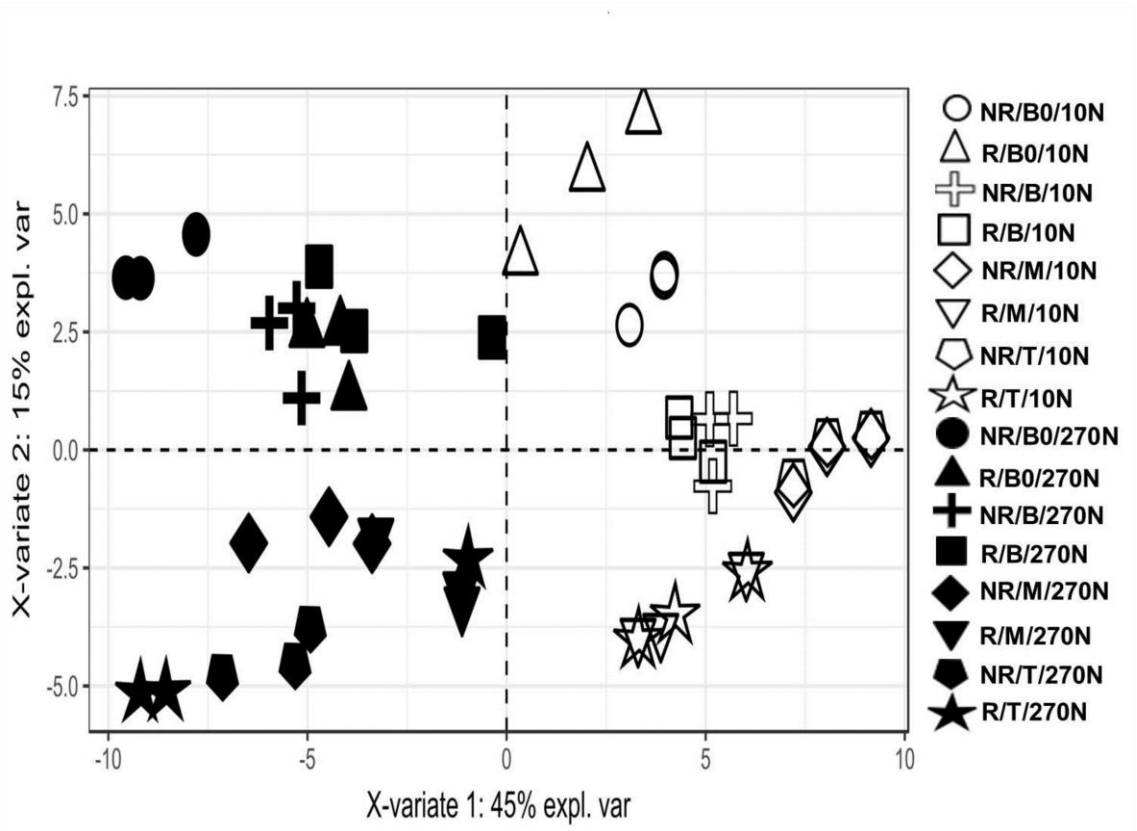

**Supplementary Figure S8: Western blot membrane of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)**. The commercial peptide sequences of RubisCO large subunit form I (Agrisera, SWE) were used. B0, Base "zero"; B, Base; M, Middle; T, Tip. 1, Responsive with 10 mg of N; 2, Responsive with 270 mg of N; 3, Nonresponsive with 10 mg of N; 4, Nonresponsive with 270 mg of N. MW, Molecular weight.




**Supplementary Figure S9: Western blot membrane of phosphoenolpyruvate carboxylase (PEPcase)**. The commercial peptide sequences of PEPC1 (Agrisera, SWE) were used. B0, Base "zero"; B, Base; M, Middle; T, Tip. 1, Responsive with 10 mg of N; 2, Responsive with 270 mg of N; 3, Nonresponsive with 10 mg of N; 4, Nonresponsive with 270 mg of N. MW, Molecular weight.




**Supplementary Figure S10. Distribution and chemical classification of the metabolites identified by gas chromatography mass spectrometry (GC-MS).**




**Supplementary Figure S11. Results of partial least square discriminant analysis (PLS-DA) in relation to metabolites detected by GC-MS.** R, responsive. NR, nonresponsive. B0, Base "zero"; B, Base; M, Middle; T, Tip. 10N and 270N correspond to 10 and 270 mg of N per kg of sand treatments, respectively.

**Supplementary Table S2. Result from over representation analysis (ORA).** The fold enrichment was calculated among all metabolites detected (67) and the metabolites present in each pathway of the customized library. Total; metabolite number of each reference pathway. Hits; metabolite number in relation to reference pathway.

| Metabolic pathway | Total | Expected | Hits | Raw p | Holm p | FDR |
|---|---|---|---|---|---|---|
| Protein biosynthesis | 20 | 1.24 | 17 | 3.58E-19 | 1.43E-17 | 1.43E-17 |
| ABC transporters | 96 | 5.96 | 30 | 2.35E-16 | 9.18E-15 | 4.71E-15 |
| Biosynthesis of plant secondary metabolites | 127 | 7.89 | 32 | 1.83E-14 | 6.97E-13 | 2.45E-13 |
| Proteinogenic amino acids degradation | 94 | 5.84 | 24 | 9.76E-11 | 3.61E-09 | 9.76E-10 |
| Sugar metabolism | 41 | 2.55 | 16 | 2.44E-10 | 8.77E-09 | 1.95E-09 |
| Urea cycle | 19 | 1.18 | 9 | 4.49E-07 | 1.57E-05 | 3.00E-06 |
| Plant hormones biosynthesis | 71 | 4.41 | 16 | 1.83E-06 | 6.22E-05 | 9.51E-06 |
| Phenylpropanoids biosynthesis | 55 | 3.41 | 14 | 1.90E-06 | 6.27E-05 | 9.51E-06 |
| Carbon metabolism | 95 | 5.9 | 18 | 5.49E-06 | 0.000176 | 2.44E-05 |
| 2-oxocarboxylic acid metabolism | 97 | 6.02 | 17 | 3.26E-05 | 0.00101 | 0.00013 |
| TCA cycle | 19 | 1.18 | 7 | 7.21E-05 | 0.00216 | 0.000262 |
| Fructose and mannose metabolism | 54 | 3.35 | 11 | 0.000261 | 0.00758 | 0.000871 |
| Photosynthesis | 32 | 1.99 | 8 | 0.00046 | 0.0129 | 0.00142 |
| Amino acids metabolism | 49 | 3.04 | 10 | 0.000508 | 0.0137 | 0.00145 |
| Starch and sucrose metabolism | 51 | 3.17 | 10 | 0.000715 | 0.0186 | 0.00191 |
| Photorespiration | 16 | 0.993 | 5 | 0.00203 | 0.0508 | 0.00508 |
| Glucose-alanine cycle | 11 | 0.683 | 4 | 0.00322 | 0.0772 | 0.00757 |
| Malate-aspartate shuttle | 8 | 0.497 | 3 | 0.0102 | 0.235 | 0.0227 |
| Glutathione metabolism | 45 | 2.79 | 7 | 0.0177 | 0.39 | 0.0373 |
| Phenylalanine and tyrosine metabolism | 13 | 0.807 | 3 | 0.0418 | 0.877 | 0.0835 |
| Ascorbate and alderate metabolism | 24 | 1.49 | 4 | 0.0562 | 1 | 0.102 |
| Gluconeogenesis | 24 | 1.49 | 4 | 0.0562 | 1 | 0.102 |
| Amino sugar and nucleotide sugars metabolism | 82 | 5.09 | 8 | 0.128 | 1 | 0.222 |
| Mitochondrial electron transport chain | 14 | 0.869 | 2 | 0.214 | 1 | 0.357 |
| Glycolysis | 18 | 1.12 | 2 | 0.309 | 1 | 0.494 |
| Propanoate metabolism | 48 | 2.98 | 4 | 0.348 | 1 | 0.535 |
| Valine leucine and isoleucine degradation | 35 | 2.17 | 3 | 0.372 | 1 | 0.551 |
| Nucleotide sugars metabolism | 9 | 0.559 | 1 | 0.44 | 1 | 0.628 |
| Aromatic compounds degradation | 31 | 1.92 | 2 | 0.585 | 1 | 0.807 |
| Pentose phosphate pathway | 17 | 1.06 | 1 | 0.667 | 1 | 0.832 |
| Porphyrin and chlorophyll metabolism | 90 | 5.59 | 5 | 0.674 | 1 | 0.832 |
| Tyrosine metabolism | 38 | 2.36 | 2 | 0.698 | 1 | 0.832 |
| Nitrogen metabolism | 19 | 1.18 | 1 | 0.707 | 1 | 0.832 |
| Pyruvate metabolism | 19 | 1.18 | 1 | 0.707 | 1 | 0.832 |
| Glycerolipid metabolism | 42 | 2.61 | 2 | 0.75 | 1 | 0.857 |
| Tryptophan metabolism | 34 | 2.11 | 1 | 0.891 | 1 | 0.99 |
| Carotenoid biosynthesis | 73 | 4.53 | 1 | 0.992 | 1 | 1 |
| Plastoquinone biosynthesis | 74 | 4.59 | 1 | 0.993 | 1 | 1 |
| Fatty acid metabolism | 105 | 6.52 | 2 | 0.993 | 1 | 1 |

Green cells represent the significant pathway at $P < 0.05$ level and FDR $\leq 0.1$; FDR; False discovery rate.

## References

1. Lê Cao, K. A., González, I. & Déjean, S. IntegrOmics: An R package to unravel relationships between two omics datasets. Bioinformatics 25, 2855–2856 (2009).

2. Farquhar, G. D. On the Nature of Carbon Isotope Discrimination in C 4Species. Aust. J. Plant Physiol. 10, 205–226 (1983).

3. Robinson, N. et al. Sugarcane genotypes differ in internal nitrogen use efficiency. Functional Plant Biol. 34(12):1122–1129 , (2007).