SHORT COMMUNICATION

Plant Signaling & Behavior 10:3, e991596; March 2015; © 2015 Taylor & Francis Group, LLC

Mild ammonium stress increases chlorophyll content in *Arabidopsis thaliana*

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Keywords: ammonium, *Arabidopsis thaliana*, chlorophyll, natural variation, nitrogen nutrition, SPAD

Abbreviations: Chl, chlorophyll; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate.

Nitrate (NO_3^-) and ammonium (NH_4^+) are the main forms of nitrogen available in the soil for plants. Excessive NH_4^+ accumulation in tissues is toxic for plants and exclusive NH_4^+-based nutrition enhances this effect. Ammonium toxicity syndrome commonly includes growth impairment, ion imbalance and chlorosis among others. In this work, we observed high intraspecific variability in chlorophyll content in 47 *Arabidopsis thaliana* natural accessions grown under 1 mM NH_4^+ or 1 mM NO_3^- as N-source. Interestingly, chlorophyll content increased in every accession upon ammonium nutrition. Moreover, this increase was independent of ammonium tolerance capacity. Thus, chlorosis seems to be an exclusive effect of severe ammonium toxicity while mild ammonium stress induces chlorophyll accumulation.

Nitrogen (N), constituent of essential molecules like amino acids and nucleic acids, is a crucial element for plant growth. Most non-legume plants require 20–50 g of N taken up by their roots to produce 1 kg of dry biomass. Thus, natural supply of soil N usually limits crops productivity and requires the supplementation of N compounds.[1] Nitrate (NO_3^-) and ammonium (NH_4^+) are the main forms of N available for plants. Amino acids may also represent an important N source under particular conditions of soil decomposition. The preferred form in which N is taken up depends on plant adaptation to soil environment. Generally, plants adapted to alkaline and oxygenated soils prefer NO_3^- while plants adapted to acidic and reducing conditions preferentially uptake NH_4^+.[2] The intensive use of fertilizers has become agriculture a major contributor to global environmental threats.[3,4] For instance, excessive NO_3^- addition provokes its leaking leading to water eutrophication. Interestingly, the use of ammonium-based fertilizers together with nitrification inhibitors has proven useful to both reduce nitrate leaching and the emission of greenhouse gases (N_2O, NO_x) associated to N-fertilization.[5] Nevertheless, NH_4^+ accumulation is toxic to most plants, although toxicity thresholds greatly depend on the ammonium dose in function of the species.[6] In fact, NH_4^+ in high concentrations is also toxic to fungi and animals.[7] Even when some plants grow almost equally well on NO_3^- and NH_4^+, they may differ in many aspects from their metabolic activities and ion composition to morphological aspects.[8] In general, high intraspecific variation exists in ammonium tolerance in different species including Arabidopsis.[9,10]

Ammonium accumulation in soils might come not only from its direct application as fertilizer, but from NH_4^+/NH_3 deposition from the atmosphere and this accumulation is especially relevant in soils with low nitrification rates.[7] Common ammonium toxicity symptoms include biomass reduction, reactive oxygen species overproduction, pH deregulation or ion imbalance. However the concept of "ammonium toxicity" is not completely clear, since ammonium nutrition can lead to biomass reduction, but to an increase in the quality of the final product.[11]

Chlorosis is also a well-established symptom of ammonium toxicity.[12] Indeed, leaf chlorosis has been used for the screening of Arabidopsis mutants hypersensitive to ammonium.[13] In this sense, the aim of our work was to determine chlorophyll (Chl) content in a collection of *A. thaliana* accessions with contrasted tolerance toward ammonium nutrition[10] to check the possible relationship between Chl content and Arabidopsis intra-specific variability in ammonium tolerance. Thus, we evaluated chlorophyll (Chl) content, using a SPAD-502 meter, in a collection of 47 Arabidopsis world natural accessions (http://publiclines.versailles.inra.fr/naturalAccession/index) grown under limiting N-supply conditions (1 mM) with NH_4^+ or NO_3^- as N source (specific growth conditions are described in Sarasketa et al.[10]).

SPAD-502 is a handy apparatus that measures the transmittance of red and infrared radiation and calculates a value that corresponds to the amount of Chl present in the sample leaf. For Arabidopsis the calibration equations to convert SPAD values to total Chl content have been calculated per

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Submitted: 10/13/2014; Revised: 11/17/2014; Accepted: 11/18/2014
http://dx.doi.org/10.4161/15592324.2014.991596

Original article: Sarasketa A, González-Moro MB, González-Murua C, Marino D. Exploring ammonium tolerance in a large panel of Arabidopsis thaliana natural accessions. J Exp Bot. In press doi: 10.1093jxberu342.

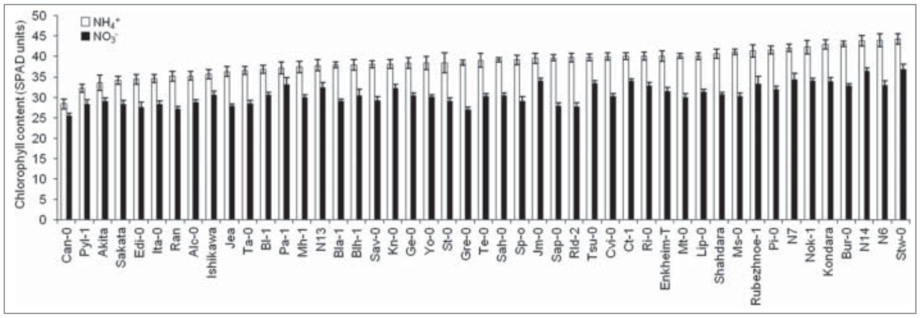

Figure 1. Natural variation in 47 *Arabidopsis thaliana* accessions chlorophyll content (SPAD units) under nitrate or ammonium as N-source. Values represent (mean ± SE for n = 8-12). Chlorophyll content for each individual plant was obtained by calculating the mean of 6 independent measurements with SPAD-502 device. Significant differences were found for every accession under ammonium compared with nitrate ($P < 0.05$).

unit of leaf area and per fresh weight with R^2 values of 0.99 and 0.98 respectively, thus validating the use of SPAD-502 for Chl measurement in Arabidopsis with a *ca*. 6% deviation on average.[14]

In a previous study, we showed that 1 mM NH_4^+ nutrition provoked a significant reduction in shoot biomass in 24 out of the 47 Arabidopsis natural accessions analyzed.[10] However, despite growth inhibition, we observed Chl content increase in every accession (**Fig. 1**). Chl content might increase in response to several environmental changes. For instance, low irradiance is commonly accepted to enhance Chl content thus resulting in foliar nitrogen investment for light harvesting.[15] However, plant growth stage, leaf age, soil water and nutrients deficiency other than N can affect these measurements.[16]

In this study, we observed high intraspecific variation in Chl content in Arabidopsis (**Fig. 1 and 2**). However, there was no correlation between Chl content and shoot biomass both under ammonium ($r^2 = 0.011$, $P = 0.942$) and nitrate nutrition ($r^2 = 0.061$, $P = 0.685$). Thus, in our conditions of growth under low-NH_4^+ supply, Chl content does not seem to be related with Arabidopsis ammonium tolerance capacity. Similarly, we found no correlation between Chl content and leaves ammonium concentration ($r^2 = 0.154$, $P = 0.301$ under ammonium nutrition and $r^2 = 0.084$, $P = 0.573$ under nitrate nutrition).

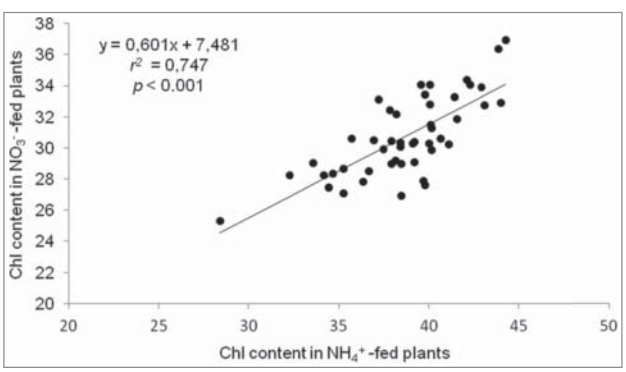

Figure 2. Scatter plot of chlorophyll content (SPAD units) of NO_3^--fed plants (vertical axis) versus NH_4^+-fed plants (horizontal axis). Linear regression and Pearson correlation coefficient (r^2) are given.

Studies based in ammonium stress in Arabidopsis have generally described Chl content reduction as a toxicity symptom.[17,18] However, Chl content increase has been also reported.[19] Interestingly, Li et al.[20] showed that under mixed nutrition (5 mM NO_3^- supplemented with increasing concentrations of NH_4^+) Arabidopsis cv. Col-0 plants exposed to low ammonium stress experienced a *ca*. 25% increase in Chl content while under high ammonium stress experienced severe chlorosis. Generally, Chl content increase under mild-ammonium toxicity could be simply due to a greater effect of the stress in leaf expansion than in Chl biosynthesis or to a side-effect due to overall accumulation of N-compounds commonly observed under ammonium nutrition.[7,10,13,21] Besides, it has been shown that enhanced photosynthetic rates induced by high irradiance favor ammonium tolerance by providing more carbon skeletons for NH_4^+ assimilation.[21,22] Thus, Chl content increase could be a strategy to somehow try to increase CO_2 assimilation and thus mitigate NH_4^+ accumulation.

In summary, it is clear that severe ammonium toxicity is associated with chlorosis and with net photosynthesis decline.[7] However, this appears to be specific of a severe stress as Chl content does not seem to be a direct effect of ammonium toxicity; since, as shown for instance in this work, inhibition of growth[10] is not accompanied by chlorosis. Similarly, we observed no correlation between Chl content variation and Arabidopsis intraspecific ammonium tolerance. Future works focused in NH_4^+ nutrition and its relationship with leaves pigment composition and biosynthesis will be helpful to understand how and why Chl content increases in relation to the N-source provided.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Anabel Robredo for technical assistance.

Funding

The research leading to these results has received funding from the Basque Government (IT526-10) and the People Program (Marie Curie Actions) of the European Union'sSeventh Framework Program (FP7/2007–2013) under REA grant agreement number 334019.

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