

Contents lists available at [ScienceDirect](http://ScienceDirect.com)

## Journal of Proteomics

journal homepage: [www.elsevier.com/locate/jprot](http://www.elsevier.com/locate/jprot)

## Evidence for a rhizobia-induced drought stress response strategy in *Medicago truncatula*



Christiana Staudinger<sup>a,\*</sup>, Vlora Mehmeti-Tershani<sup>a</sup>, Erena Gil-Quintana<sup>b</sup>, Esther M. Gonzalez<sup>b</sup>, Florian Hofhansl<sup>c</sup>, Gert Bachmann<sup>a</sup>, Stefanie Wienkoop<sup>a,\*</sup>

<sup>a</sup> Department of Ecogenomics and Systems Biology, University of Vienna, Althanstraße 14, 1090 Vienna, Austria

<sup>b</sup> Department Environmental Science, Public University of Navarra Campus Arrosadía, 31006 Pamplona, Spain

<sup>c</sup> Department of Microbiology and Ecosystem Science, University of Vienna, Althanstraße 14, 1090 Vienna, Austria

## ARTICLE INFO

## Article history:

Received 21 October 2015

Received in revised form 16 December 2015

Accepted 11 January 2016

Available online 23 January 2016

## Keywords:

Drought stress

Induced tolerance

Leaf senescence

Priming

Symbiosis

Stay-green

## ABSTRACT

Drought stress hampers plant energy and biomass production; however it is still unknown how internal C:N balance and rhizobial symbiosis impact on plant response to water limitation. Here, the effect of differential optimal nitrogen nutrition and root nodule symbiosis on drought stress and rehydration responses of *Medicago truncatula* was assessed. Two groups of plants were nodulated with *Sinorhizobium medicae* or *Sinorhizobium meliloti* – differing in the performance of N fixation; the third group grew in a rhizobia-free medium and received mineral nitrogen fertilizer. In addition to growth analyses, physiological and molecular responses of the two systems were studied using ionomic, metabolomic and proteomic techniques.

We found a significant delay in drought-induced leaf senescence in nodulated relative to non-nodulated plants, independent of rhizobial strain and uncoupled from initial leaf N content. The major mechanisms involved are increased concentrations of potassium and shifts in the carbon partitioning between starch and sugars under well-watered conditions, as well as the enhanced allocation of reserves to osmolytes during drought. Consequently, nodulated plants recovered more effectively from drought, relative to non-nodulated *M. truncatula*. Proteomic data suggest that phytohormone interactions and enhanced translational regulation play a role in increased leaf maintenance in nodulated plants during drought.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

### 1. Introduction

Future climate scenarios predict an enhanced variability in rainfalls leading to more extreme drought events that develop faster and with greater intensity [1,2]. Limited water availability hampers plant growth [3] and induces leaf senescence [4] leading to substantial losses in agricultural productivity. The 2003 European drought, for example, resulted in a 30% reduction in gross primary production [5]. It is therefore of global concern to stabilize crop yields under adverse environmental conditions and at the same time develop novel ways of sustainable crop management.

Delayed leaf senescence is a valuable trait for crop improvement [6,7]. Plants that, relative to others, show delayed leaf yellowing during aging or in response to environmental cues are called ‘stay-greens’. Several studies showed that the stay-green character was related to higher yield during drought, especially under moderate water limitation (e.g. [8,9]). In forage plants the stay-green trait could in addition be beneficial during the entire growth phase [4]. This was shown for alfalfa (*Medicago sativa*) resulting in enhanced hay nutritional value [10]. Hormonal signaling pathways interacting with sugar signaling are involved in the regulation of leaf senescence [11,12]. For instance, cytokinin is an important inhibitor of leaf senescence [13]. There is indication that this antagonizing effect is related to source–sink relationships [12] which in part are mediated by increased cell wall invertase activity [14,15], an enzyme involved in the phloem unloading of sucrose to sinks. Finally, there are reports on stay-green phenotypes mediated by altered levels of ethylene (ET) [16], jasmonic acid (JA) and salicylic acid [17].

In natural and managed environments plants interact with microorganisms living in the rhizosphere, which may impact on the plant's

\* Corresponding authors.

E-mail addresses: [christiana.staudinger@univie.ac.at](mailto:christiana.staudinger@univie.ac.at) (C. Staudinger), [vlora.mehmeti@univie.ac.at](mailto:vlora.mehmeti@univie.ac.at) (V. Mehmeti-Tershani), [erena.gil@unavarra.es](mailto:erena.gil@unavarra.es) (E. Gil-Quintana), [esther.gonzalez@unavarra.es](mailto:esther.gonzalez@unavarra.es) (E.M. Gonzalez), [florian.hofhansl@univie.ac.at](mailto:florian.hofhansl@univie.ac.at) (F. Hofhansl), [gert.bachmann@univie.ac.at](mailto:gert.bachmann@univie.ac.at) (G. Bachmann), [stefanie.wienkoop@univie.ac.at](mailto:stefanie.wienkoop@univie.ac.at) (S. Wienkoop).

hormonal balance and source–sink relationships. This interaction therefore has the potential to alter the plant's responsiveness to drought [18–21]. Several studies showed that the mutualistic relationship with a subset of free-living soil bacteria, referred to as plant growth promoting rhizobacteria (PGPR), can act as an elicitor of tolerance to drought. For instance, bacteria expressing 1-aminocyclopropane-1-carboxylate (ACC) deaminase changed plant ET levels leading to better performance under drought and better recovery from water deficit [22,23], nitric oxide producing microbes enhanced root growth via auxin signaling [24] and finally, PGPR promoted plant grain yield under drought as a result of increased cell wall elasticity [25]. Mycorrhizal fungi are also reported to alter plant hormonal balances which might impact on the host's drought responsiveness [26–29].

Another economically important interaction with soil microbes is the endosymbiosis formed by legumes and nitrogen-fixing rhizobacteria [30]. After the infection process bacteroids are accommodated in root nodules, provided with photoassimilates in order to fuel nodule maintenance and symbiotic nitrogen fixation. Relative to the fields of PGPR and mycorrhiza research, comprehensive studies dealing with the impact of root nodule symbiosis on plant drought stress tolerance are rare. In one of the first studies on the subject [31], reported that leaves of nodulated (NOD) alfalfa plants were less sensitive to decreasing leaf relative water content, since they maintained higher net photosynthesis and chlorophyll content at moderate stress than nitrogen fertilized, non-nodulated (NN) plants. In a cyclic drought experiment, NOD alfalfa accumulated more biomass as a result of altered leaf ABA/cytokinin balance relative to NN plants [27]. Others also observed an enhanced drought tolerance in nodulated *Phaseolus vulgaris* and *Pisum sativum* on the basis of pod yield or biomass accumulation relative to NN, nitrate-fed plants [32, 33] or an increased proline accumulation in NOD *Lotus corniculatus* when experiencing a dehydration shock [34]. Frechilla and coworkers [33] proposed that an improved photorespiratory regulation during drought conditions partially explains the better performance of NOD plants. Taken together these studies show that nodulation with rhizobacteria can affect the drought response strategies employed by plants, however they raise questions about molecular mechanisms underlying altered performance.

It is conceivable, that differences in N nutritional level may have partially accounted for the enhancement of drought tolerance in NOD legumes. It is well known that the initial N nutritional level of plants depending on N from the soil solution exerts a strong effect on the sensitivity to following water deprivation [35,36]. Notably, low N supply under well-watered conditions increased the stomatal sensitivity to water deficit and to ABA [35]. N deficiency reduced root hydraulic conductivity under well-watered conditions [37]. With increasing leaf N levels drought stressed *Agrostis palustris* showed traits associated with better stress adaptation such as cellular membrane stability, lower osmotic potentials and reduced concentrations of malonyl dialdehyde, the final product of lipid peroxidation [38].

This study therefore aimed at assessing potential differences in plant stress responsiveness to water deficit of NOD and NN *M. truncatula* when subjected to similar stress doses. Plants grown individually in pots were exposed to progressively decreasing substrate water contents. The hypothesis was tested that rhizobial symbiosis induces metabolic and nutritional changes within the host that translate into specific drought stress responsiveness. Another question was, whether these responses were independent of the plant's initial N nutritional level. To address this topic, the responses to severe drought of NOD and NN plants exhibiting various leaf N concentrations were examined. The two most common rhizobial strains (*S. meliloti* and *S. medicae*) were used, previously described to significantly vary in plant total N fixation rate and therefore differentially influence plant growth performance. In order to gain deeper insights about the different molecular strategies, we then studied in more detail drought and recovery of plants nodulated by *S. medicae* compared to NN plants.

## 2. Material and methods

### 2.1. Plant and rhizobia growth conditions

*Medicago truncatula* Gaertn. cv Jemalong A17 as a temperate model legume was chosen to study plant–rhizobia interactions. Seeds were incubated for 7 min in sulfuric acid 98% and surface sterilized for 3 min in 5% sodium hypochloride. After extensive washing, the seeds were placed on 0.8% agar plates and left 3 days for germination in darkness: one day at 4 °C and the following two days at room temperature. Seedlings were then transferred to 1 l pots containing a sterile vermiculite–perlite mixture (2:5, v:v). The plants grew in a climatic chamber equipped with metal halide lamps (Radium HRI-TS 250W/NDL) under controlled environmental conditions (500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 14 h photoperiod,  $22 \pm 2.8$  °C and  $16 \pm 0.5$  °C day and night temperatures;  $59 \pm 10\%$  relative humidity). Plants were watered daily with nutrient solution [39]. During the first week a solution containing 0.5 mM  $\text{NH}_4\text{NO}_3$  (pH 6.2) was used, during the subsequent two weeks plants received 2.5 mM  $\text{NH}_4\text{NO}_3$  (pH 7.4). Three week-old plants were then separated into three groups: non-nodulated (NN) plants receiving 2.5 mM  $\text{NH}_4\text{NO}_3$  and two nodulated (NOD) groups, which were inoculated with either *Sinorhizobium medicae* WSM419 (NOD(e)) or *Sinorhizobium meliloti* 2011 (NOD(i)) receiving 0.5 mM  $\text{NH}_4\text{NO}_3$  during the growth period in order to ensure more similar growth performances. *S. medicae* and *S. meliloti* were chosen as their apparent nitrogenase activity and per plant N fixation rate differ when associated with *M. truncatula* resulting in distinct leaf N concentrations [40,41]. The NN-fertilization was chosen on the basis of preliminary experiments.

*S. medicae* WSM419 and *S. meliloti* 2011 were grown separately at 27 °C for 72 h on a rotary shaker in yeast extract–mannitol medium containing 10 g  $\text{l}^{-1}$  mannitol, 0.5 g  $\text{l}^{-1}$   $\text{K}_2\text{HPO}_4$ , 0.4 g  $\text{l}^{-1}$  yeast extract, 0.2 g  $\text{l}^{-1}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g  $\text{l}^{-1}$  NaCl, pH adjusted to 6.8 [42]. At this time, optical density at 600 nm was around 0.7–0.8, which corresponds to  $\sim 3 \times 10^8$  cells. Each plant designated for nodulation was inoculated with 2 ml of one of the cultures.

### 2.2. Experimental design

After seven weeks of growth, plants were subjected to drought by withholding irrigation. Two different stress experiments were performed. The lethal drought stress experiment comprised two independent experiments consisting of three or five biological replicates, respectively. NN plants and the two rhizobial treatments, NOD(e) and NOD(i), were exposed to lethal drought stress to assess the effect of nodulation and initial leaf N concentration on *M. truncatula* performance under drought, while control plants were watered daily. The time of death was scored, when all leaves were wilted and the plants did not recover upon rehydration. Here, shoot samples were taken from control NN, NOD(i) and NOD(e) plants for metabolite analysis. Second, a drought and rehydration experiment was set up for molecular analyses consisting of four sample sets: leaves of droughted NN and NOD(e) plants and their respective controls. Five sample time points were chosen: five (D5) and nine (D9) days of water withholding and two hours (R10), one day (R11) and three days (R13) of rehydration using nutrient solution, whereof each sample group consisted of five biological replicates.

### 2.3. Drought stress treatment and physiological measurements

Water deficit was imposed by water-withholding for several days. Before the onset of the drying period, pots were watered to pot capacity in order to minimize heterogeneities in substrate water content (SWC). After one day, the pot weight was recorded and served as a reference point for the following experiment. Drought stress was then imposed

by withholding irrigation. To estimate the daily water requirement of control plants, the pots were weighed before the photoperiod on days 0 and 1. The changes in SWC of stressed plants were also estimated gravimetrically, presuming that the contribution of plant growth to pot weight was negligible compared to the total amount of water lost. Predawn leaf xylem water potential was measured when the SWC reached ~50% of the pot capacity (i.e. day seven in the lethal stress experiment and day nine in the drought and rehydration experiment). For each plant, the measurements were performed on mature leaves which were cut and directly sealed in the pressure chamber. Stomatal conductance ( $g_s$ ) was measured 3 h after the onset of the photoperiod (Fig. S1) with a steady state porometer (PMR-4, PP Systems, Hitchin, UK) connected to a data logger (EGM-4, PP Systems). Net photosynthesis was measured using a portable infrared gas analyzer (LI-6200, Li-Cor, Lincoln, NE, USA).

#### 2.4. Anion and cation chromatography

Ultrapure water was added to lyophilized and finely ground leaf material to obtain a 4% solution (v:w). The mixture was subsequently incubated for 30 min at 90 °C. After centrifugation (13,000 rpm, 10 min), the supernatant was collected and again centrifuged (13,000 rpm, 5 min).

For determinations of soluble anion concentrations a 1:20 dilution was analyzed. HPLC was used to separate inorganic anions on an anion exchange column (AS11, 2 × 250 mm) with pre-guard column (AG11, 2 × 50 mm) using a linear KOH gradient (1–10 mM, total run time 30 min), chemical suppression (ASRS 300–2 mm) and conductivity detection (CD-20) on an ion-chromatography system (ICS 3000, Dionex, Vienna, Austria). Data analysis was performed using Chromeleon Version 6.80 SR7 Build 2528.

Soluble cation concentrations were measured using an ion chromatography instrument (881 Compact IC pro, Metrohm, Zofingen, Switzerland) equipped with a conductivity detector. An acidified 1:200 dilution was prepared and 0.2 ml were loaded on a Metrosep C 4 column (150 × 4 mm) and eluted with 34 mM nitric acid/14 mM dipicolinic acid. The results were analyzed using Mag IC Net 2.4 software package.

#### 2.5. Starch content

Leaf starch content was determined enzymatically according to [43] with minor modifications. To remove glucose, leaf material was extracted twice with 80% ethanol (80 °C, 30 min). After centrifugation the pellets were solubilized in 0.5 N NaOH at 95 °C for 30. After acidification with 1N CH<sub>3</sub>COOH, the suspension was incubated for 2 h with amyloglucosidase. The glucose content of the resulting digest was then determined photometrically using glucose oxidase/peroxidase reagent containing o-dianisidine-HCl as chromogenic oxygen acceptor.

#### 2.6. Elemental analysis–isotope ratio mass spectrometry (EA–IR/MS)

Natural abundances of stable isotopes C and N were measured by Elemental Analyzer coupled to Isotopic Ratio Mass Spectrometer (EA1110, CE Instruments; mass spectrometer DELTAplus Finnigan MAT, Thermo Fisher Scientific, Bremen, Germany), according to [44]. For the analysis of bulk leaf, 1.5–2 mg of pulverized material were weighed into tin capsules and introduced into the EA–IR/MS. High purity N<sub>2</sub> reference gas (Air Liquide) was run with each sample. The reference gas was calibrated to the air international standard using IAEA-N-1, IAEA-N-2 and IAEA-NO-3 (IAEA, Vienna, Austria).

#### 2.7. Metabolite and protein extraction

Metabolites were extracted from finely ground and lyophilized leaf material as described previously [45]. The polar phase was then dried in a vacuum concentrator and stored at –20 °C until further use. For

derivatization of metabolites, the dried pellet was dissolved in 20 µl pyridine containing 0.5 mg methoxyamine hydrochloride and incubated on a thermoshaker at 30 °C for 90 min. After addition of 80 µl MSTFA the mixture was agitated at 37 °C for 30 min. A mixture of even n-alkanes (C12–C40) was spiked into the sample prior to measurement allowing for retention time indexing.

Proteins were extracted from the remaining pellet using urea buffer (8 M Urea, 50 mM Hepes, 5 mM PMSF, pH 7.8) and subsequently digested as previously described [46]. The resulting peptide mixture was desalted on C18-SPEC 96-well plates (Varian, Darmstadt, Germany), eluted with methanol, vacuum dried and stored at –20 °C until use.

#### 2.8. Gas chromatography–time-of-flight mass spectrometry (GC–TOF/MS)

Measurements were performed on an Agilent 6890 gas chromatograph (Agilent Technologies, Vienna, Austria) coupled to a LECO Pegasus 4D GC–TOF mass spectrometer (LECO Corporation, Monchengladbach, Germany). When samples were injected, temperature was held for 1 min at 70 °C and then increased with 9 °C per minute until 330 °C were reached. Detector voltage was set to 1600 V and the mass range from 40 to 600 m/z was analyzed with a scan rate of 20 spectra per second. Spectrum deconvolution, base line correction, peak searching, retention time indexing and peak annotation was performed using the LECO–ChromaTOF software in combination with the Golm Metabolome Database spectral library [47]. After visual inspection of the hits with a minimum match factor of 850 and comparison to standard runs, unique fragment ion masses of 47 compounds originating from 32 metabolites were chosen for relative quantification. By matching the chromatograms of all runs against this reference list, peak areas were obtained. Sucrose was excluded from further statistical analysis due to overloading of the detector.

#### 2.9. Liquid chromatograph–linear Trap quadrupole–orbitrap mass spectrometry (LC–LTQ–orbitrap/MS)

Tryptic peptide mixtures were analyzed by LC–MS/MS using a nanoLC–Ultra 1D + (Eksigent, Dublin, CA) system coupled to a LTQ–Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Peptides were dissolved in 0.1% formic acid and 5% acetonitrile and an amount corresponding to 1 µg of protein was loaded onto a Peptide ES–18 column (Supelco 15 cm/0.1 mm, 2.7 µm; Sigma–Aldrich). Analytes were eluted from the column during a 95 min gradient ranging from 98% solvent A (0.1% formic acid) to 55% solvent B (90% acetonitrile, 0.1% formic acid) at a flow rate of 400 nl/min. The mass spectrometer was operated in a data-dependent manner. Full scans (300–1700 m/z) were acquired in the Orbitrap with a maximum injection time of 500 ms and a target automated gain control (AGC) of 1e6 charges. Atmospheric polydimethylcyclsiloxane (m/z 371.101230) was used as a lock mass for internal calibration. Up to seven peaks per cycle were selected for CID fragmentation in the linear ion trap (1e4 target AGC charges, maximum injection time 100 ms). Dynamic exclusion settings were 30 s repeat duration and 60 s exclusion time. Three to five biological replicates were measured.

#### 2.10. Protein identification and computational data analysis

For peptide identification, ProteomeDiscoverer 1.3 (Thermo Fisher Scientific) was used with the Sequest algorithm using the same settings as explained previously [46]. Fragment spectra were searched against in-silico spectra generated from the UniRef100 database of the *M. truncatula* reference proteome ([www.uniprot.org](http://www.uniprot.org), as of 30th October 2014). Methionine oxidation was chosen as variable modification, a maximum of 2 missed cleavages was allowed. Precursor mass tolerance was set to 5 ppm and fragment ion mass tolerance to 0.8 Da. The

Mercator application was used to add functional annotations to the Uniprot protein accessions (<http://mapman.gabipd.org/web/guest/app/mercator>).

Quality control, filtering and statistical analysis were performed in MATLAB R2010b (<http://www.mathworks.com>). The data matrices exported from ProteomeDiscoverer were processed as follows: first, a protein filter was applied. To pass this filter, a protein had to present values in more than half of the observations in at least one treatment. Missing value estimation was then performed in two distinct ways. When a protein was detected in more than half of the observations per treatment, the k-nearest neighborhood method was employed. When a protein was detected only in half or less of the observations per treatment, then a minimum value was imputed. The statistical significance of protein abundance changes among treatments were evaluated by converting the *P*-values from the Kruskal–Wallis test *q*-values in order to correct for multiple testing [48]. The abundance of a protein was judged significantly altered if it met two criteria: (1) adjusted Kruskal–Wallis test *q*-value <0.05 and (2) minimum twofold change.

### 2.11. Statistical analysis

Unless stated otherwise, statistical analysis was conducted using one-way ANOVA. The homogeneity of variances was tested employing Levene's test. All analyses were performed using MATLAB and STATGRAPHICS 16.1.11. (Manugistics, Rockville, MD, U.S.A.)

## 3. Results

### 3.1. Relationships between leaf senescence and rhizobial treatment during lethal drought

The aim of this study was (i) to assess differences in drought susceptibility and responsiveness of NN and NOD *M. truncatula* and (ii) to investigate whether these differences are influenced by initial differences in leaf N content. Therefore, three N regimes were chosen, resulting in distinct leaf N concentrations under well-irrigated conditions. Inoculation with *S. medicae* resulted in the highest leaf N concentration, whilst inoculation with *S. meliloti* resulted in the lowest. With 2.5 mM NH<sub>4</sub>NO<sub>3</sub>-fertilization an intermediate N nutritional level was obtained (%N and C:N ratios in Table 1). This result positively correlated with the relative abundances of amino acids such as asparagine, aspartic acid and glutamine in shoots of well-watered plants (Table S1). The %C and photosynthetic rates were unaffected by the N regime.

Plants showed similar evapotranspirational demands at the start of the experiment (Fig. 1b). During the stress treatment (day seven) older leaves of both NOD plants tended to have slightly lower stomatal conductance than leaves of NN plants of similar age (Fig. S1, *n* = 4, *P* < 0.1, LSD). However, when water was withheld, the rate and extent of substrate desiccation was virtually identical in all three treatments (Fig. 1a) and leaf predawn water potential was also comparable (Fig. 1c).

Both groups of NOD plants, NOD(e) and NOD(i), lost their leaves at similar rates and proportions, while the rate of leaf abscission was

**Table 1**

Effect of different N regimes on photosynthesis (A) and leaf bulk C and N composition under well-watered conditions in seven week old non-nodulated (NN) and nodulated (NOD) *Medicago truncatula*, inoculated with *Sinorhizobium medicae* NOD(e) or *S. meliloti* NOD(i).

	A	N	C	C:N
	μmol m <sup>-2</sup> s <sup>-1</sup>	(%)	(%)	
NN	56.2 (3.04) a	3.19 (0.26) a	43.08 (0.45) a	13.52 (1.56) a
NOD(e)	53.1 (1.02) a	4.19 (0.09) b	39.82 (0.27) a	9.51 (0.16) b
NOD(i)	56.3 (1.82) a	2.31 (0.14) c	39.25 (2.16) a	17.31 (0.45) c

Significant differences between treatments are indicated by distinct letters (*n* = 3–5, ANOVA, Fisher's test, *P* = 0.05). Values in parentheses are standard errors.

**Table 2**

Mean soluble anion and cation concentrations in *Medicago truncatula* leaves under well-watered conditions and effects of N regime (NOD(e) or NN), time (D5, D9, R10, R11, R13) and the interaction term (N × T) as calculated by two-way ANOVA. The percentage shows the global difference in pool size of NN plants relative to NOD(e) plants.

	μmol g <sup>-1</sup> DW		N regime			Time		N × T	
	NOD(e)	NN	(%)	F(1)	<i>P</i>	F(2)	<i>P</i>	F(1,2)	<i>P</i>
Cl <sup>-</sup>	194.9	192.0	-1.4	0.05		18.12	***	0.02	
NO <sub>3</sub> <sup>-</sup>	3.2	0.4	<b>-85.8</b>	22.77	***	0.01		0.77	
SO <sub>4</sub> <sup>2-</sup>	227.3	256.2	12.7	2.22		9.42	**	0.02	
PO <sub>4</sub> <sup>3-</sup>	128.3	104.7	<b>-18.3</b>	17.16	***	16.21	***	7.66	*
Na <sup>+</sup>	24.4	17.8	<b>-27.2</b>	32.32	***	1.28		1.20	
NH <sub>4</sub> <sup>+</sup>	14.8	4.7	<b>-68.1</b>	27.10	***	0.14		3.78	
K <sup>+</sup>	729.1	502.6	<b>-31.1</b>	277.00	***	8.05	*	9.65	**
Ca <sup>2+</sup>	261.0	337.0	<b>29.1</b>	25.51	***	3.33		0.04	
Mg <sup>2+</sup>	345.7	347.1	0.4	0.01		0.54		0.44	

NN: non-nodulated, NOD: nodulated; *n* = 5. Values in the first two columns are means; *n* = 25. Values in bold show significant effects of the N regime. Asterisks indicate levels of significance (\*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001), significant relative changes are shown in boldface.

increased by almost 50% in NN plants during the first 13 days of the experiment (Fig. 2a and Fig. S2). On day 8 the chlorophyll index of NN plants was decreased by 18%, while the index of NOD plants was unaffected or increased (Fig. 2b). The mean SWC at wilting was similar in all treatments ranging from 0.11 to 0.14 g H<sub>2</sub>O g<sup>-1</sup> dried substrate (*n* = 5–7, *P* < 0.05, LSD, see Fig. 1a). Additionally, the three groups of plants did not show a significant divergence in the respective time of death (~15 days, *n* = 5–7, *P* < 0.05, LSD).

### 3.2. Drought and rehydration experiment

To obtain a better understanding of the mechanisms involved, the dynamic system responses to non-lethal drought and rehydration within vital leaves in NN and NOD(e) *M. truncatula* were then studied. *S. medicae* was chosen over *S. meliloti*, due to its higher symbiotic efficiency which resulted in growth performance that was more comparable to NN plants. Plants were subjected to progressively increasing drought stress during nine days followed by a rehydration period of four days. Water withholding resulted in a 58% decrease in SWC on day 9 (D9). At this time point, NN and NOD(e) plants had similar pre-dawn leaf xylem water potentials. No differences in SWC between pots accommodating well-watered control NN and NOD(e) or drought-rehydration treated plants were observed throughout the study (Fig. 3).

### 3.3. Inorganic anion and cation concentrations

The N regime (NN or NOD(e)) had significant effects on the leaf concentrations of soluble anions and cations under well-watered conditions (Table 2). Symbiotically grown plants had significantly higher concentrations of NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, K<sup>+</sup>, Na<sup>+</sup> and PO<sub>4</sub><sup>3-</sup>, whereof the absolute mean difference in K<sup>+</sup> was highest (226 μmol g<sup>-1</sup> DW).

Drought induced decreases in the leaf Cl<sup>-</sup>, PO<sub>4</sub><sup>3-</sup> and K<sup>+</sup> concentrations in both N regimes (Fig. 4). NN plants showed significant differences at the first sampling time point (D5), in NOD(e) plants significant differences were observed from D9 on, except for SO<sub>4</sub><sup>2-</sup>. The decreased concentrations were then completely recovered after three days of rehydration (R13). During the rehydration phase, NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> concentrations substantially increased on R11 and R13 in NN plants relative to controls (the mean (±SE) was 17.9 ± 4.2 and 35 ± 23.05 μmol g<sup>-1</sup> DW, respectively; *n* = 5; *P* < 0.05).

### 3.4. Relative changes in metabolite abundance

Leaf metabolites of the primary C and N metabolism were relatively quantified. Out of 37 metabolites, 17 (the majority of amino acids and

**Table 3**  
Changes in protein abundance of selected protein groups during drought and rehydration in *Medicago truncatula* leaves. The log<sub>2</sub>-fold change of significantly altered proteins is shown (minimum two-fold change,  $P < 0.05$ ; Kruskal–Wallis test after Benjamini–Hochberg adjustment).

Protein group name (% coverage/nr of distinct peptides)	NN			NOD(e)		
	D5/C	D9/C	R11, R13/D	D5/C	D9/C	R11, R13/D
<b>Amino acid metabolism</b>						
G7IAZ2: 2-isopropylmalate synthase (18/7)	–2.59					
G7JTY4: LL-diaminopimelate aminotransferase (29/9)		–2.49	2.36		1.19	
G7J648: Cobalamin-independent methionine synthase (15/9)		3.61				
A0A072UCM6: Glutamate decarboxylase (13/5)					8.68	
<b>Redox</b>						
G7KPG2: Type II peroxiredoxin (42/4)		–1.97	1.78			
G7IE85: Thioredoxin-like protein (30/4)		–2.00				
A0A072VNM9: GDP-D-mannose-3,5-epimerase (41/13)			1.03			
B7FGM0: Type II peroxiredoxin (33/5)					1.78	
I3SA84: Thioredoxin M-type protein (24/4)					1.94	
I3SXR8: Ferredoxin–thioredoxin reductase, variable chain (36/6)					2.35	
G7J9T0: Cytochrome b5-like heme/steroid-binding domain protein (34/3)				3.41		
<b>Hormone metabolism</b>						
<b>Ethylene</b>						
G8A022: 1-aminocyclopropane-1-carboxylate oxidase-like protein (8/2)	2.38					
G7J9K5: 1-aminocyclopropane-1-carboxylate oxidase (19/4)					–9.64	
G7K5B0: Cystathionine beta-synthase (CBS) family protein (25/5)				–1.58	–1.32	
<b>Jasmonate</b>						
Q711Q9: Allene oxide cyclase (30/5)					3.62	
A0A072UMH4: Linoleate 13S-lipoxygenase 2–1, related protein (18/11)					4.86	
A0A072VAE0: Seed linoleate 9S-lipoxygenase (32/22)		–2.63				

organic acids) were significantly enriched in NOD(e) leaves ( $P < 0.05$ ) under control conditions (Fig. 5). Only threitol and myo-inositol had higher concentrations in NN leaves. Relative abundances as well as the number of significant changes of those metabolites were enriched in leaves compared to whole shoots (Table S1).

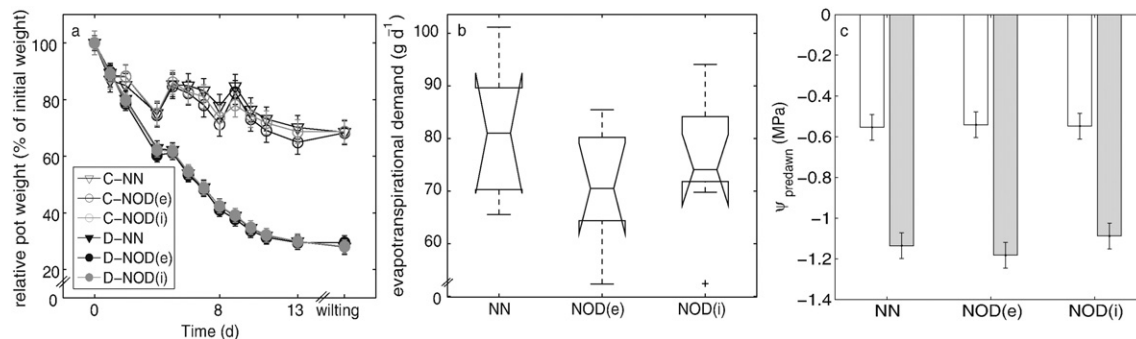
The effects of drought and rehydration on metabolite concentrations are also shown in Fig. 5. There was an accumulation of the sugars fructose, glucose and galactinol along with the progression of drought, independent of the N regime, which still persisted after two hours of rehydration (R10). An accumulation of maltose, myo-inositol, pinitol and threitol was observed in NOD(e) plants. During later stages of recovery, the initial increase of the sugar and polyol levels was followed by a decrease to control levels in the case of NN plants or even below control levels in the case of NOD(e) plants.

With respect to changes in amino acid and organic acid pools NN and NOD(e) plants responded distinctly to changes in water availability: symbiotic plants showed increases in many amino acids during drought (especially in Pro, Asp and Gln). These changes were paralleled by decreases in organic acids. During rehydration, the

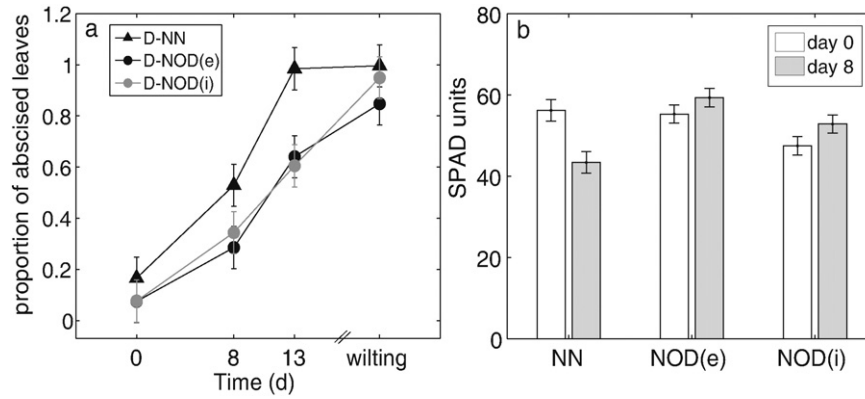
organic acid pool recovered to control levels within one day, followed by amino acids. On the contrary in NN plants, drought induced little changes in the amino acid and organic acid pools. Gradual decreases were observed in Asp and Glu. The pools of Ala and Met were punctually decreased at D5 and D9, respectively. However, most amino acid pool sizes substantially increased during later stages of rehydration (R11 and R13). Taken together, these results suggest that NOD(e) and NN *M. truncatula* differentially regulated their amino acid and organic acid pools during drought and rehydration. Moreover, NOD(e) plants adjusted most metabolite concentrations to control levels, whereas in NN plants 18 metabolites were increased at the last rehydration time point.

### 3.5. Changes in stable C isotopic signature, starch, N and protein content

In response to substrate drying, enrichments of <sup>13</sup>C in the bulk leaf at D9 relative to controls were observed, whereof the enrichment effect tended to be strongest in NOD(e) plants (Fig. 6a). Upon rehydration the  $\delta^{13}\text{C}$  progressively decreased at similar rates under both N regimes.



**Fig. 1.** Drought stress imposition during a lethal drought stress experiment in *M. truncatula*: (a) estimated substrate water content measured gravimetrically, the day of wilting was ~day 15 in all stressed conditions. (b) Plant evapotranspirational demand from day zero to day one. Notches indicate 95% confidence intervals. (c) Predawn leaf xylem water potential, measured on day seven under control (white bars) and drought stressed conditions (gray bars). Values are means (and medians in the boxplot); error bars indicate 95% LSD confidence intervals;  $n = 5$ –7 in (a),  $n = 5$  in (b, c). C: control, D: drought treated, NN: non-nodulated, NOD(e): *S. medicae* nodulated and NOD(i): *S. meliloti* nodulated.



**Fig. 2.** Leaf senescence symptoms in *M. truncatula* induced by water withholding. (a) Leaf abscission rate. The day of wilting was ~day 15 in all conditions. (b) Leaf chlorophyll index at the start of the desiccation period (day zero, white bars) and after eight days of water withholding (gray bars). Values are means; error bars indicate 95% LSD confidence intervals;  $n = 5$ . D: drought treated, NN: non-nodulated, NOD(e): *S. medicae* nodulated.

Compared to NN plants, *M. truncatula* symbiotically grown with *S. medicae* had significantly higher leaf elemental N and protein contents, which were not affected by the irrigation treatment. By contrast, the N and protein contents of NN plants decreased significantly during drought and progressively increased during rehydration (Fig. 6b and c).

Leaf starch concentrations were assessed enzymatically. Interestingly, symbiotic plants had on average 52% lower starch levels than NN plants ( $n = 25$ ,  $P < 0.05$ ). The starch content was not significantly altered by drought or rehydration in both groups of plants, with the exception of drought treated NN plants on R11 (Fig. 6d).

### 3.6. Relative changes in protein abundance

Using a shotgun proteomics approach to study dynamic changes of the leaf soluble proteome, 845 protein groups were identified in the 85 samples assessed (Table S2). The N regime had a significant effect on the abundance of 128 proteins under well-watered conditions (Table S3 and Fig. 7). On the one hand, NOD(e) plants had increased abundances of pyruvate kinase, enzymes involved in ET and JA metabolism and of ribosomal proteins relative to their non-nodulated counterparts. On the other hand, NN plants had higher abundances for most other processes such as of proteins assigned to cellular amino acid and lipid metabolism, starch degradation, sulfur assimilation and protein degradation.

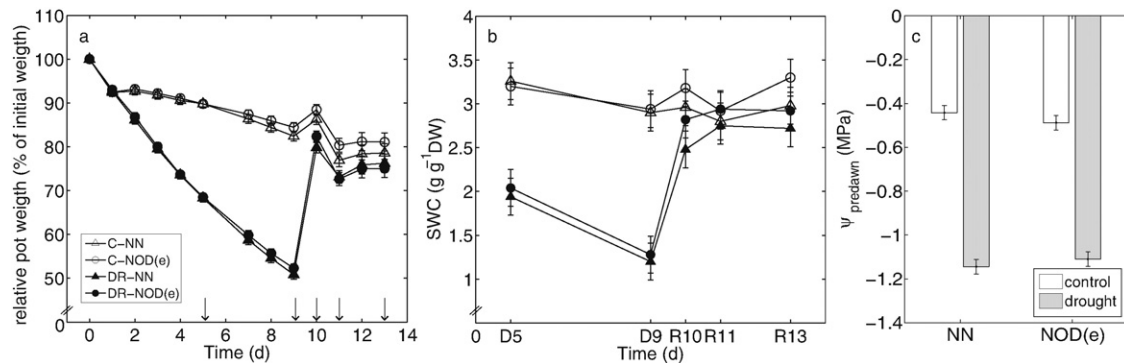
Table S6 shows the number of drought and rehydration responsive proteins in plants of both N regimes. NN and NOD(e) plants had similar drought responsive protein patterns as visualized by PC2 in Fig. 7 but more pronounced for NN plants Fig. 8a: although NOD(e) plant response was significantly delayed compared to NN plants. At D5, the

abundances of ribosomal proteins,  $\alpha$ -amylase and enzymes involved in tetrapyrrole biosynthesis exhibited significant decreases on D5 and D9 in both N-treatments. The NN-specific changes in response to drought included decreased abundances in amino acid synthesizing enzymes and in two more starch degrading enzymes (glucane water dikinase and starch phosphorylase, see Table S5a). The NOD(e)-specific drought responsive proteins were the increase in several redox metabolism enzymes (Tables 3 and S4a). Interestingly, drought-induced changes in enzyme abundance of the senescence related JA and ET metabolism showed opposite directionalities in NOD(e) and NN plants. Symbiotic plants had increased levels of JA biosynthesis enzymes (lipoxygenases, allene oxide synthase and allene oxide cyclase) and decreased levels of enzymes synthesizing ET (ACC oxidase) when compared to well-watered control plants. On the contrary, NN plants decreased the abundance of JA metabolism enzymes and increased the levels of ACC oxidase.

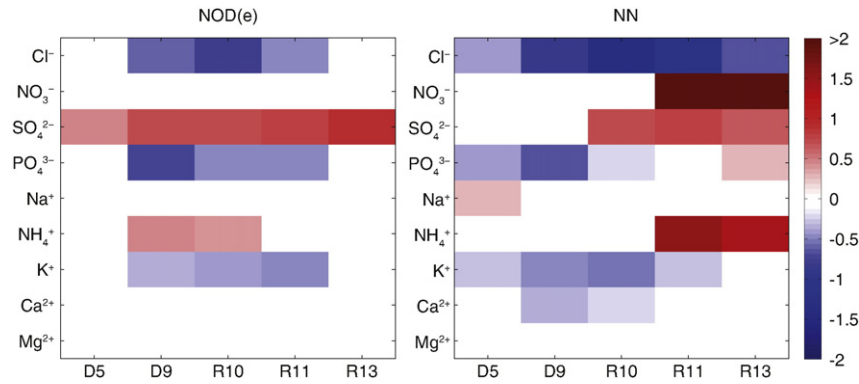
During rehydration distinct responses were observed at R13 between NN and NOD(e) plants (Fig. 7). The number of responsive proteins in NN plants largely exceeded the number of those observed in symbiotic plants (Fig. 8b and Table S6). Mostly ribosomal (up-regulation) and PS (down-regulation) proteins were involved in the distinct pattern of R13 (Fig. 7).

## 4. Discussion

The interaction with various microorganisms is known to alter plant drought stress tolerance as for example plant growth promoting bacteria [49], or vesicular-arbuscular mycorrhiza as reviewed by [19]. However, the impact of root nodule bacteria on legume drought stress



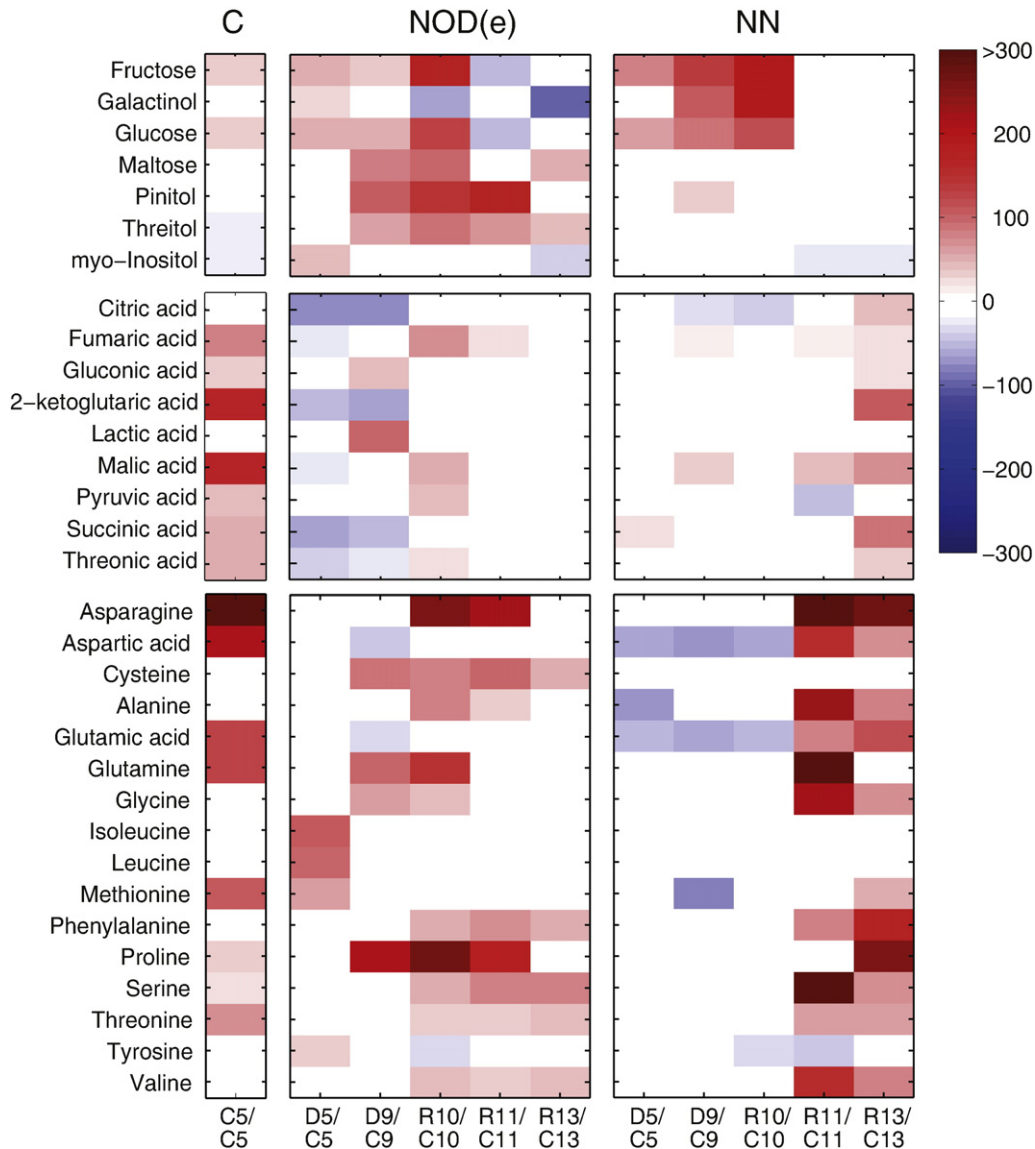
**Fig. 3.** Water status during drought and rehydration. Estimated (a) and absolute (b) SWC and D9 predawn leaf xylem water potential (c). Well-watered (open symbols, C) and treated *Medicago truncatula* (closed symbols, DR) was grown in a vermiculite/perlite mixture. Arrows in (a) designate the sampling time points shown in (b),  $\psi$  was measured on day nine. Values are means; error bars indicate 95% LSD confidence intervals;  $n = 5$ –10 in (a),  $n = 5$  in (b). NN: non-nodulated, NOD(e): *S. medicae* nodulated and NOD(i): *S. meliloti* nodulated.



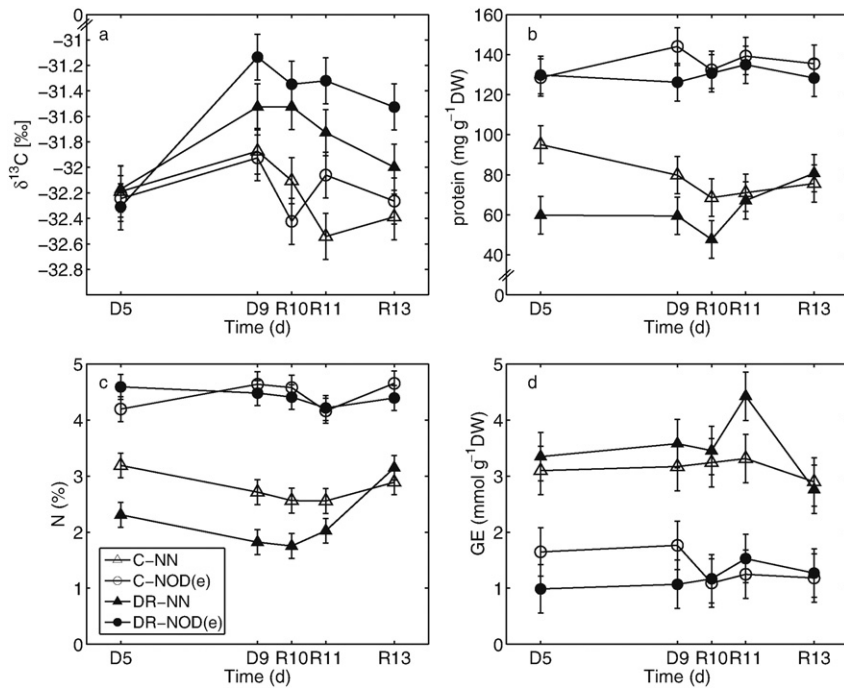
**Fig. 4.** Changes in leaf soluble anion and cation concentrations during drought (D5, D9) and rehydration (R10, R11 and R13) in nodulated (NOD(e)) and non-nodulated (NN) *M. truncatula*. Values are the  $\log_2$ -fold change of treated relative to control plants; statistically significant changes are shown in color and were determined using Fisher's least significant difference after one-way ANOVA ( $n = 5$ ,  $P < 0.05$ ). Initial differences between NOD(e) and NN controls are shown in Table 2.

tolerance is still poorly understood. The elucidation of mechanisms involved is important in order to design strategies for agricultural applications. This study demonstrates that nodulation with *S. meliloti* or

*S. medicae* has the potential to delay drought induced leaf senescence in *M. truncatula* relative to non-nodulated plants solely relying on mineral N fertilizer.



**Fig. 5.** Changes in leaf metabolite content during drought (D5, D9) and rehydration (R10, R11 and R13) in nodulated (NOD(e)) and non-nodulated (NN) *M. truncatula*. The first column shows the relative difference between well-watered NOD(e) and NN plants on day five. Values are the percent change relative to controls; statistically significant changes are shown in color and were determined using Fisher's least significant difference after one-way ANOVA ( $n = 5$ ,  $P < 0.05$ ).



**Fig. 6.** Leaf biochemical composition of *M. truncatula* during a drought (D5, D9) and rehydration (R10, R11, R13) experiment in control (open symbols, C) and treated plants (closed symbols, DR). The graphs show bulk leaf stable isotopic C signature (a), soluble protein content (b), N (c) and starch content (d) in terms of glucose equivalents (GE). Values are means; error bars indicate 95% LSD confidence intervals; n = 5. NN: non-nodulated, NOD(e): *S. medicae* nodulated.

#### 4.1. Leaf senescence is influenced by symbiotic interaction during drought stress

Leaf abscission is one of the strategies that plants may employ in order to reduce transpiring surfaces and postpone the depletion of substrate water reserves. This process is preceded by leaf senescence during which nutrients are remobilized and exported to other plant tissues [50]. Previous studies described a delay of leaf senescence during drought in nodulated alfalfa and common bean relative to their non-symbiotic counterparts [31,32]. In our lethal drought stress experiment,

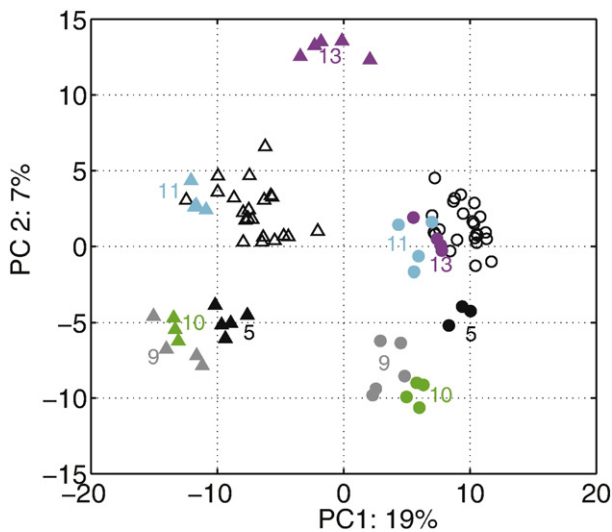
nodulation with *S. medicae* or *S. meliloti* was associated with a delay in stress-induced leaf senescence and abscission (Fig. 2) when compared to NN plants, suggesting that tolerance strategies might be favored by NOD plants, whereas avoidance strategies may be more important in NN plants.

However, it is important to mention that interactions between N nutritional levels and drought stress have been reported. For example, suboptimal N nutrition increases stomatal sensitivity to ABA [51], and reduces the root hydraulic conductivity under well-watered conditions [37]. Several previous studies restricted their comparative analysis to only two N regimes (one NOD and one NN) not allowing for the distinction of the aforementioned factors (e.g. [33,34,52]). Therefore, different N regimes were chosen in order to dissect NN specific from NOD specific drought responses that are not in relation to initial leaf N concentration. Our results show that the symbiotic interaction overlays the effects of initial N nutritional levels described above. In fact, rhizobial symbiosis exerts a strong effect on the plant's strategy utilized when exposed to drought (leaf maintenance or shedding).

To gain more insights into the metabolic changes involved, comparative analysis of NN and symbiotic plants inoculated with *S. medicae* (NOD(e)) during well-watered and during a drought and rehydration regime was then adopted.

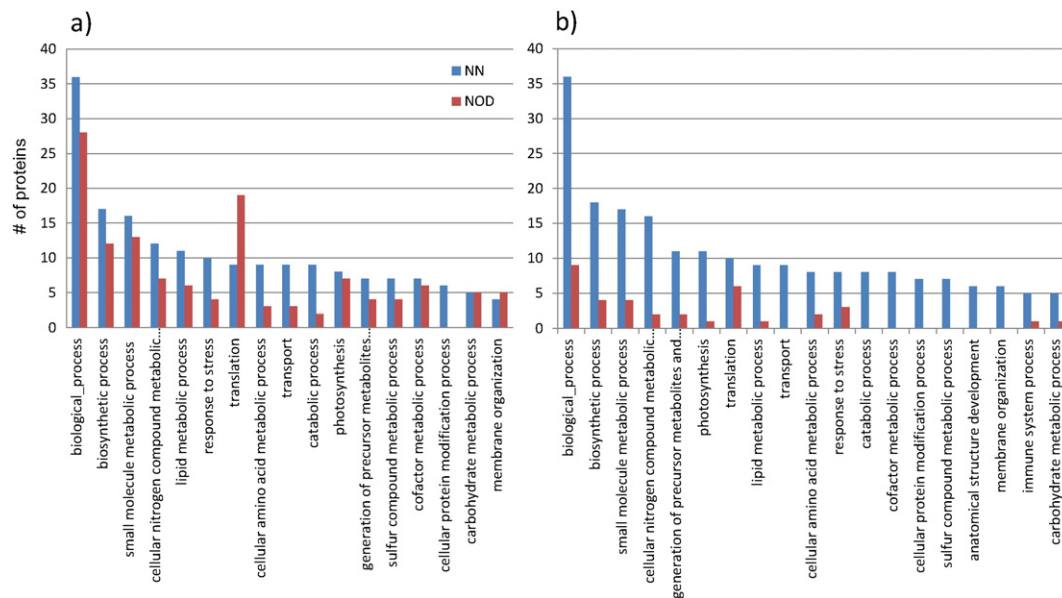
#### 4.2. N regime effects under well-watered conditions

Symbiotic N fixation is an energy costly process fueled by assimilates which are transported from photosynthetic tissues to nodules. Thus, nodulation constitutes an additional sink to the plant. In comparison to NN plants, the enhanced demand for respiratory substrates was concomitant with an increased activity of sucrose phosphate synthetase and decreased starch levels in three out of four soybean cultivars [53]. In this study, NN plants had higher starch levels associated with lower sugar and organic acid levels than NOD(e) plants (Fig. 6D). However, there was no indication that nodulation lead to a substantial sink stimulation of photosynthetic rates (Table 1), as suggested by others [54].



**Fig. 7.** PCA scores plot of 620 protein abundances as affected by drought and rehydration in NN (triangles) and NOD(e) (circles) *Medicago truncatula*. Open symbols represent well-watered control samples of all five timepoints. Black and gray symbols indicate drought stressed (D5 and D9), colored symbols indicate rehydrated plants (R10, R11 and R13). PC1 and PC2 account for 19% and 7% of the total variance, respectively.





**Fig. 8.** GO enrichment annotation of proteins with significant abundance changes during drought (a) and recovery (b). GO numbers of accessions from significantly changed protein where retrieved from the AgBase (v 2.0) [76] using GO Retriever. The Slim id output file of the Biological Process was used for the enrichment visualization.

Here, the N regime had little effect on photosynthesis but it exerted a strong effect on plant daytime C allocation between starch and soluble sugars.

Additional sink organs, such as nodules, might also increase the sucrose loading into the phloem. Interestingly, NOD(e) plants showed substantially higher  $K^+$  concentrations compared to NN plants. By contributing to pH stabilization across membranes and to formation of osmotic potential,  $K^+$  is essential for sucrose phloem loading and mass flow-driven solute transport in the vascular tissues [55]. Previous studies demonstrated the importance of K in the functioning of root nodule symbiosis. Indeed, NOD legumes receiving adequate K nutrition supplied more sugars to nodules, thereby increasing the rates of N fixation, when compared to K-deficient plants [56,57]. This allows for the conclusion that NOD(e) *M. truncatula* accumulates more  $K^+$  in order to optimize assimilate transport to belowground organs.

Root infection by rhizobia induces signaling pathways reminiscent of those induced during plant defense, which are both driven by phytohormonal changes. ET and JA are negative regulators of nodulation by inhibiting the plant's response to rhizobial Nod factors [58,59]. Recently, [28] showed that ET and JA pathways in poplar roots associated with ectomycorrhiza were induced to limit fungal growth during later stages of colonization. In addition, infection with rhizobia results in significant increases in plant ET levels [60,61]. There is evidence that ET-dependent pathways regulate nodule numbers in temperate legumes such as the genera *Medicago*, *Pisum* and *Vicia*, whereas the ET-mediated suppression of nodulation is less pronounced in tropical legumes [62]. In line with this reports, our proteomic analysis of *M. truncatula* leaves provides some indication that ET and JA pathways were systemically induced by nodulation. ET and JA are also known to induce leaf senescence [63], however, without implication to the well-watered NOD(e) plants (day 0 in Fig. 2a). Phytohormone interactions during water deficit are currently intensely examined [64,65] However, their exact role needs to be further investigated.

#### 4.3. Rhizobial effects on leaf osmolyte production and hormone metabolism during drought

The observed delay in leaf senescence of NOD plants suggests that senescence inhibiting hormones such as cytokinin might play a more

important role than JA. There are some reports on rhizobia-derived cytokinin in the xylem of legumes [66]. Furthermore, there were decreased abundances of proteins involved in ET synthesis supporting the observation of a delayed leaf senescence phenotype mediated by rhizobial symbiosis (symbiosis-induced stay-green phenotype; SISG).

Water deficits have been shown to increase the concentrations of C rich compounds such as soluble carbohydrates, several amino acids and organic acids, many of which are osmolytes and considered to counteract the deleterious effects of cellular water deficits (reviewed by [3]). In both, NN and NOD(e) plants the drought stress treatment led to the accumulation of glucose and fructose, as also observed by others [33,67,68]. Pinitol is a major carbohydrate in legumes which also acts as an osmolyte [69,70]. The concentration of pinitol however, only increased in NOD(e) plants but not in NOD(i) plants of our previous study [46]. In another study, pinitol increased only at very low water potentials ( $\sim -3$  MPa, day seven) in NN *M. truncatula* [68].

A massive investment of reserves into the synthesis of osmolytes, such as proline, was previously observed in NOD alfalfa exposed to drought [71]. Here, the N regime exerted a strong effect on amino acid levels during drought. The concentrations of Asp and Glu were significantly decreased in NN plants. Glu is a precursor of proline biosynthesis and Asp is formed by transamination involving 2-oxoglutarate and Glu. The depletion of Asp and Glu together with the decrease in leaf N and soluble protein content (Fig. 6b and c) suggests that protein derived N was remobilized and exported to other actively growing tissues. Indeed, a previous study showed that proline concentrations decreased in shoots of NN *M. truncatula* within six days of water withholding, while substantial amounts accumulated in roots [46]. Our previous findings match with the present data such that drought induced increases in amino acid concentrations (including proline) were restricted to NOD plants. This finding, together with the observation of decreasing protein levels in NN plants suggests an improved osmoprotective capacity in NOD plants. Moreover, the elevated  $K^+$  levels observed in NOD(e) relative to NN plants under well-watered conditions might have contributed to turgor maintenance during drought. Thus, metabolite data confirm that the differential drought response is influenced by plant–rhizobia interaction.

#### 4.4. Rhizobial effect on a distinct pattern of the leaf protein synthesis apparatus under well-watered conditions. Cause or consequence of the SISG effect?

Noteworthy, our previous study [46] revealed a priming effect of NOD(i) plants. Under well-watered conditions the drought responsive shoot proteins showed induced levels compared to those of the NN plants. We hypothesized that this priming mechanism was involved in the induction of reduced leaf senescence. Here, the levels of NOD(e) leaf proteins, responsive to drought, also showed induced levels compared to those of NN plants, supporting our previous hypothesis. In particular, several ribosomal leaf proteins, involved in protein synthesis and general drought stress response, exhibited induced levels in NOD(e) plants under well-watered conditions compared to NN plants. This demonstrates an important regulation of the protein synthesis apparatus as a consequence of severe drought. Therefore, our study indicates that nodulation, independent of the N fixation efficiency of the microsymbiont, induces abundance increases in drought stress relevant proteins.

#### 4.5. Evidence for a more effective drought stress recovery in nodulated plants

As expected, plants resumed leaf biomass accumulation upon rehydration as evidenced by rapidly decreasing sugar concentrations and the depletion of  $^{13}\text{C}$  in the bulk leaf tissue (Figs. 5 and 6a). Others also reported that sugars are among the first rehydration-responsive metabolites [72,73].

Apart from sugars, the N regime had a strong effect on the plant's molecular responses to rehydration. N fixation as well as  $\text{NO}_3^-$  assimilation are drought sensitive processes, which are rapidly inhibited by water deficits [73,74]. The considerable increase in Asp suggests that N fixation in NOD(e) plant was already induced after 2 h of rehydration, since Asp is one of the major N transport forms in *Medicago* [75]. The onset of  $\text{NO}_3^-$  assimilation was delayed in NN plants, as indicated by increases in  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations 1 d after rehydration, which were concomitant with an increase in amino acids relative to controls.

In contrast to NOD(e) plants, in rehydrated NN plants a more pronounced decrease of photosynthetic proteins at R13 was observed along with increase of the synthesis apparatus. These data suggest that NN plants invested in newly synthesized leaves. Thus, from a metabolic and proteomic perspective, our results demonstrate that at the one hand NOD(e) plants exhibited a faster response to rehydration enabling the plants to adjust metabolite and protein abundances to control levels; on the other hand NN plants responded primarily with de-novo synthesis of proteins which may have been limited by ribosomal availability and more energy demanding re-sprouting efforts. These data again emphasize a possible role of enhanced protein synthesis capacity induced by rhizobia that might be relevant for delayed leaf senescence in NOD plants.

#### 4.6. Summary and concluding remarks

Symbiotic interaction with *Sinorhizobium* spp. induced physiological, nutritional and metabolic changes that exerted a strong effect on the drought response strategy used by *M. truncatula*. Nodulated plants maintained their foliage through initially increased concentrations of drought responsive proteins and potassium and through shifts in the carbon partitioning between starch and sugars under well-watered conditions. They furthermore show enhanced investment in osmolyte synthesis and accumulation during drought. Non-nodulated plants, in contrast, tended to avoid water deficits through accelerated leaf senescence and abscission. In response to rehydration the metabolism of symbiotic plants recovered faster to control levels and potentially at lower metabolic cost, than non-nodulated plants.

Moreover, our results show that the delay in drought-induced leaf senescence was independent of the initial leaf N and amino acid levels under well-watered conditions. Plant growth and N availability as well as N fixation efficiency are positively correlated [40,41]. Thus, this study provides evidence that the N fixation efficiency is not relevant for the symbiotically induced effect but rather an initial N-regime-independent rhizobial interaction mechanism. This mechanism has an important positive effect on yield in mild and transient drought scenarios. Thus, the *Sinorhizobium*–*Medicago* interaction is a good model to further investigate the relevance of N fixation for the delayed drought-induced leaf senescence mechanism, which we also call Symbiosis Induced Stay-Green (SISG) phenotype.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2016.01.006>.

#### Conflict of interest

This work was funded by the Austrian Science Foundation FWF [P23441-B20] and the faculty of Life Sciences, University of Vienna.

No conflict of interest.

#### Acknowledgments

We thank Thomas Nägele for fruitful discussions. Reinhard Turetschek for help during sampling. We acknowledge the work of Margarete Watzka at the EA/IR-MS instrument. We are grateful to Jason Terpolilli for providing the *S. medicae* WSM419 strain and to the gardeners of the University of Vienna for watering the plants. Christiana Staudinger was supported by the Austrian Science Foundation FWF [P23441-B20] and a PhD completion grant of the Faculty of Life Sciences, University of Vienna.

#### References

- [1] IPCC, Fourth assessment report: synthesis report, [http://ipcc.ch/publications\\_and\\_data/ar4/syr/en/contents.html2007](http://ipcc.ch/publications_and_data/ar4/syr/en/contents.html2007) (Publ. Online).
- [2] K.E. Trenberth, A. Dai, G. van der Schrier, P.D. Jones, J. Barichivich, K.R. Briffa, et al., Global warming and changes in drought, *Nat. Clim. Chang.* 4 (2013) 17–22, <http://dx.doi.org/10.1038/nclimate2067>.
- [3] B. Muller, F. Pantin, M. Génard, O. Turc, S. Freixes, M. Piques, et al., Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs, *J. Exp. Bot.* 62 (2011) 1715–1729, <http://dx.doi.org/10.1093/jxb/erq438>.
- [4] P.L. Gregersen, A. Culetic, L. Boschian, K. Krupinska, Plant senescence and crop productivity, *Plant Mol. Biol.* 82 (2013) 603–622, <http://dx.doi.org/10.1007/s11103-013-0013-8>.
- [5] P. Ciais, M. Reichstein, N. Viovy, A. Granier, J. Oögé, V. Allard, et al., Europe-wide reduction in primary productivity caused by the heat and drought in 2003, *Nature* 437 (2005) 529–533, <http://dx.doi.org/10.1038/nature03972>.
- [6] H. Thomas, H. Ougham, The stay-green trait, *J. Exp. Bot.* 65 (2014) 3889–3900, <http://dx.doi.org/10.1093/jxb/eru037>.
- [7] Y. Sakuraba, S. Schelbert, S.-Y. Park, S.-H. Han, B.-D. Lee, C.B. Andrés, et al., STAY-GREEN and chlorophyll catabolic enzymes interact at light-harvesting complex II for chlorophyll detoxification during leaf senescence in *Arabidopsis*, *Plant Cell* 24 (2012) 507–518, <http://dx.doi.org/10.1105/tpc.111.089474>.
- [8] A.K. Borrell, G.L. Hammer, R.G. Henzell, Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield, *Crop Sci.* 40 (2000) 1037–1048.
- [9] J.L. Rolando, D.A. Ramírez, W. Yactayo, P. Monneveux, R. Quiroz, Leaf greenness as a drought tolerance related trait in potato (*Solanum tuberosum* L), *Environ. Exp. Bot.* 110 (2015) 27–35, <http://dx.doi.org/10.1016/j.envexpbot.2014.09.006>.
- [10] C. Zhou, L. Han, C. Pislariu, J. Nakashima, C. Fu, Q. Jiang, et al., From model to crop: functional analysis of a STAY-GREEN gene in the model legume *Medicago truncatula* and effective use of the gene for alfalfa improvement, *Plant Physiol.* 157 (2011) 1483–1496, <http://dx.doi.org/10.1104/pp.111.185140>.
- [11] T. Roitsch, M.-C. González, Function and regulation of plant invertases: sweet sensations, *Trends Plant Sci.* 9 (2004) 606–613, <http://dx.doi.org/10.1016/j.tplants.2004.10.009>.
- [12] H. Thomas, Senescence, ageing and death of the whole plant, *New Phytol.* 197 (2013) 696–711, <http://dx.doi.org/10.1111/nph.12047>.
- [13] P.J. Zwack, A.M. Rashotte, Cytokinin inhibition of leaf senescence, *Plant Signal. Behav.* 8 (2013), e24737 <http://dx.doi.org/10.4161/psb.24737>.
- [14] Y. Jin, D.-A. Ni, Y.-L. Ruan, Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level, *Plant Cell* 21 (2009) 2072–2089, <http://dx.doi.org/10.1105/tpc.108.063719>.

- [15] M.E.B. Lara, M.-C. Gonzalez Garcia, T. Fatima, R. Ehness, T.K. Lee, R. Proels, et al., Extracellular invertase is an essential component of cytokinin-mediated delay of senescence, *Plant Cell* 16 (2004) 1276–1287, <http://dx.doi.org/10.1105/tpc.1018929>.
- [16] V. Grbic, A.B. Bleeker, Ethylene regulates the timing of leaf senescence in *Arabidopsis*, *Plant J.* 8 (1995) 595–602, <http://dx.doi.org/10.1046/j.1365-313X.1995.8040595.x>.
- [17] Y. Miao, U. Zentgraf, The antagonist function of *Arabidopsis* WRKY53 and ESR/ESP in leaf senescence is modulated by the jasmonic and salicylic acid equilibrium, *Plant Cell* 19 (2007) 819–830, <http://dx.doi.org/10.1105/tpc.106.042705>.
- [18] J. Yang, J.W. Kloepper, C.M. Ryu, Rhizosphere bacteria help plants tolerate abiotic stress, *Trends Plant Sci.* 14 (2009) 1–4, <http://dx.doi.org/10.1016/j.tplants.2008.10.004>.
- [19] R. Augé, Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis, *Mycorrhiza* 11 (2001) 3–42, <http://link.springer.com/article/10.1007/s005720100097> (accessed September 16, 2014).
- [20] N.J. Atkinson, P.E. Urwin, The interaction of plant biotic and abiotic stresses: from genes to the field, *J. Exp. Bot.* 63 (2012) 3523–3543, <http://dx.doi.org/10.1093/jxb/ers100>.
- [21] C. Dimkpa, T. Weinand, F. Asch, Plant–rhizobacteria interactions alleviate abiotic stress conditions, *Plant Cell Environ.* 32 (2009) 1682–1694, <http://dx.doi.org/10.1111/j.1365-3040.2009.02028.x>.
- [22] S. Mayak, T. Tirosh, B.R. Glick, Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers, *Plant Sci.* 166 (2004) 525–530.
- [23] A.A. Belimov, I.C. Dodd, N. Hontzeas, J.C. Theobald, V.I. Safronova, W.J. Davies, Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling, *New Phytol.* 181 (2009) 413–423, <http://dx.doi.org/10.1111/j.1469-8137.2008.02657.x>.
- [24] C. Molina-Favero, C.M. Creus, M. Simontacchi, S. Puntarulo, L. Lamattina, Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato, *Mol. Plant-Microbe Interact.* 21 (2008) 1001–1009, <http://dx.doi.org/10.1094/MPMI-21-7-1001>.
- [25] C.M. Creus, R.J. Sueldo, C.A. Barassi, Water relations and yield in *Azospirillum*-inoculated wheat exposed to drought in the field, *Can. J. Bot.* 82 (2004) 273–281, <http://dx.doi.org/10.1139/b03-119>.
- [26] R. Aroca, P. Vernieri, J.M. Ruiz-Lozano, Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery, *J. Exp. Bot.* 59 (2008) 2029–2041, <http://dx.doi.org/10.1093/jxb/ern057>.
- [27] N. Goicoechea, M.C. Antolín, M. Sanchez-Diaz, Gas exchange is related to the hormone balance in mycorrhizal or nitrogen-fixing alfalfa subjected to drought, *Physiol. Plant.* 100 (1997) 989–997, <http://dx.doi.org/10.1111/j.1399-3054.1997.tb00027.x>.
- [28] J.M. Plett, A. Khachane, M. Ouassou, B. Sundberg, A. Kohler, F. Martin, Ethylene and jasmonic acid act as negative modulators during mutualistic symbiosis between *Laccaria bicolor* and *Populus* roots, *New Phytol.* 202 (2014) 270–286, <http://dx.doi.org/10.1111/nph.12655>.
- [29] B. Sánchez-Romera, J.M. Ruiz-Lozano, Á.M. Zamarreño, J.M. García-Mina, R. Aroca, Arbuscular mycorrhizal symbiosis and methyl jasmonate avoid the inhibition of root hydraulic conductivity caused by drought, *Mycorrhiza* (2015) <http://dx.doi.org/10.1007/s00572-015-0650-7>.
- [30] P.H. Graham, C.P. Vance, Legumes: importance and constraints to greater use, *Plant Physiol.* 131 (2003) 872–877, <http://dx.doi.org/10.1104/pp.017004>.
- [31] M.C. Antolín, J. Yoller, M. Sanchez-Diaz, Effects of temporary drought on nitrate-fed and nitrogen-fixing alfalfa plants, *Plant Sci.* 107 (1995) 159–165.
- [32] A. Lodeiro, P. González, A. Hernández, L. Balagué, G. Favelukes, Comparison of drought tolerance in nitrogen-fixing and inorganic nitrogen-grown common beans, *Plant Sci.* 154 (2000) 31–41 (<http://www.ncbi.nlm.nih.gov/pubmed/10725556>).
- [33] S. Frechilla, E.M. González, M. Royuela, F.R. Minchin, P.M. Aparicio-Tejo, C. A. Rrese-Igor, Source of nitrogen nutrition (nitrogen fixation or nitrate assimilation) is a major factor involved in pea response to moderate water stress, *J. Plant Physiol.* 157 (2000) 609–617, [http://dx.doi.org/10.1016/S0176-1617\(00\)80003-6](http://dx.doi.org/10.1016/S0176-1617(00)80003-6).
- [34] O. Borsani, P. Díaz, J. Monza, Proline is involved in water stress responses of *Lotus corniculatus* nitrogen fixing and nitrate fed plants, *J. Plant Physiol.* 155 (1999) 269–273, [http://dx.doi.org/10.1016/S0176-1617\(99\)80018-2](http://dx.doi.org/10.1016/S0176-1617(99)80018-2).
- [35] J.W. Radin, R. Ackerson, Water relations of cotton plants under nitrogen deficiency: III. Stomatal conductance, photosynthesis, and abscisic acid accumulation during drought, *Plant Physiol.* 67 (1981) 115–119 (<http://onlinelibrary.wiley.com/doi/10.1111/j.1399-3054.1981.tb00893.x/full> (accessed December 23, 2014)).
- [36] J.W. Radin, J.S. Boyer, Control of leaf expansion by nitrogen nutrition in sunflower plants, *Plant Physiol.* 69 (1982) 771–775, <http://dx.doi.org/10.1104/pp.69.4771>.
- [37] D.T. Clarkson, Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress, *J. Exp. Bot.* 51 (2000) 61–70, <http://dx.doi.org/10.1093/jxb/51.342.61>.
- [38] H. Saneoka, R.E.A. Mogaheib, G.S. Premachandra, K. Fujita, Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relations in *Agrostis palustris* Huds. *Environ. Exp. Bot.* 52 (2004) 131–138, <http://dx.doi.org/10.1016/j.envexpbot.2004.01.011>.
- [39] H. Evans, Symbiotic nitrogen fixation in legume nodules, in: T.C. Moore (Ed.), *Res. Exp. Plant Physiol. a Lab. Man.* Springer Verlag 1974, pp. 417–426.
- [40] J.J. Terpolilli, G.W. O'Hara, R.P. Tiwari, M.J. Dilworth, J.G. Howieson, The model legume *Medicago truncatula* A17 is poorly matched for N<sub>2</sub> fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021, *New Phytol.* 179 (2008) 62–66, <http://dx.doi.org/10.1111/j.1469-8137.2008.02464.x>.
- [41] E. Larrainzar, E. Gil-Quintana, A. Seminario, C. Arrese-Igor, E.M. González, Nodule carbohydrate catabolism is enhanced in the *Medicago truncatula* A17-*Sinorhizobium medicae* WSM419 symbiosis, *Front. Microbiol.* 5 (2014) 447, <http://dx.doi.org/10.3389/fmicb.2014.00447>.
- [42] J.M. Vincent, A manual for the practical study of the root-nodule bacteria. IBP Handbook, 15, Blackwell Scientific Publications, Oxford and Edinburgh, 1970.
- [43] T. Nägele, A.G. Heyer, Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana*. *New Phytol.* 198 (2013) 777–787, <http://dx.doi.org/10.1111/nph.12201>.
- [44] M. Watzka, K. Buchgraber, W. Wanek, Natural 15N abundance of plants and soils under different management practices in a montane grassland, *Soil Biol. Biochem.* 38 (2006) 1564–1576.
- [45] H. Doerfler, D. Lyon, T. Nägele, X. Sun, L. Fragner, F. Hadacek, et al., Granger causality in integrated GC–MS and LC–MS metabolomics data reveals the interface of primary and secondary metabolism, *Metabolomics* 9 (2013) 564–574.
- [46] C. Staudinger, V. Mehmeti, R. Turetschek, D. Lyon, V. Egelhofer, S. Wienkoop, Possible role of nutritional priming for early salt and drought stress responses in *Medicago truncatula*, *Front. Plant Sci.* 3 (2012) 285, <http://dx.doi.org/10.3389/fpls.2012.00285>.
- [47] J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmüller, et al., GMD@CSB.DB: the Golm Metabolome Database, *Bioinformatics* 21 (2005) 1635–1638, <http://dx.doi.org/10.1093/bioinformatics/bti236>.
- [48] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, *J. R. Stat. Soc. Ser. B* 57 (1995) 289–300.
- [49] J. Bresson, F. Varoquaux, T. Bontpart, B. Touraine, D. Vile, The PGPR strain *Phyllobacterium brassicacearum* STM196 induces a reproductive delay and physiological changes that result in improved drought tolerance in *Arabidopsis*, *New Phytol.* 200 (2013) 558–569, <http://dx.doi.org/10.1111/nph.12383>.
- [50] A. Wingler, M. Mares, N. Pourtau, Spatial patterns and metabolic regulation of photosynthetic parameters during leaf senescence, *New Phytol.* 161 (2004) 781–789, <http://dx.doi.org/10.1111/j.1469-8137.2003.00996.x>.
- [51] J.W. Radin, L.L. Parker, G. Guinn, Water relations of cotton plants under nitrogen deficiency: V. Environmental control of abscisic acid accumulation and stomatal sensitivity to abscisic acid, *Plant Physiol.* 2 (1982) 1066–1070.
- [52] E. Kirova, N. Tzvetkova, I. Vaseva, G. Ignatov, Photosynthetic responses of nitrate-fed and nitrogen-fixing soybeans to progressive water stress, *J. Plant Nutr.* 31 (2008) 445–458, <http://dx.doi.org/10.1080/01904160801894988>.
- [53] S.C. Huber, D.W. Israel, N. Carolina, Biochemical basis for partitioning of photosynthetically fixed carbon between starch and sucrose in soybean (*Glycine max* Merr.), *Plant Physiol.* 69 (1982) 691–696.
- [54] G. Kaschuk, T.W. Kuyper, P.A. Leffelaar, M. Hungria, K.E. Giller, Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biol. Biochem.* 41 (2009) 1233–1244, <http://dx.doi.org/10.1016/j.soilbio.2009.03.005>.
- [55] H. Marschner, E.A. Kirkby, I. Cakmak, Effect of mineral nutritional status on shoot–root partitioning of photoassimilates and cycling of mineral nutrients, *J. Exp. Bot.* 47 (1996) 1255–1263, [http://dx.doi.org/10.1093/jxb/47.Special\\_Issue.1255](http://dx.doi.org/10.1093/jxb/47.Special_Issue.1255).
- [56] K. Mengel, M.R. Haghparast, K. Koch, The effect of potassium on the fixation of molecular nitrogen by root nodules of, *Vicia faba*, *Plant Physiol.* 54 (1974) 535–538 (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=367448&tool=pmcentrez&rendertype=abstract>).
- [57] M. Collins, S.H. Duke, Influence of potassium-fertilization rate and form on photosynthesis and N<sub>2</sub> fixation of alfalfa, *Crop Sci.* 21 (1981) 481, <http://dx.doi.org/10.2135/cropsci1981.001183X002100040001x>.
- [58] J. Sun, V. Cardoza, D.M. Mitchell, L. Bright, G.E.D. Oldroyd, J.M. Harris, Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation, *Plant J.* 46 (2006) 961–970, <http://dx.doi.org/10.1111/j.1365-313X.2006.02751.x>.
- [59] G.E.D. Oldroyd, J.A. Downie, Coordinating nodule morphogenesis with rhizobial infection in legumes, *Annu. Rev. Plant Biol.* 59 (2008) 519–546, <http://dx.doi.org/10.1146/annurev.arplant.59.032607.092839>.
- [60] K.H. Lee, T.A. LaRue, Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv Sparkle, *Plant Physiol.* 100 (1992) 1759–1763.
- [61] F. Ligeró, C. Lluch, J. Olivares, Evolution of ethylene from eoots of *Medicago sativa* plants inoculated with *Rhizobium meliloti*, *J. Plant Physiol.* 125 (1986) 361–365.
- [62] J.S. Schmidt, J.E. Harper, T.K. Hoffman, A.F. Bent, Regulation of soybean nodulation independent of ethylene signaling, *Plant Physiol.* 119 (1999) 951–960.
- [63] Y. Guo, S. Gan, Leaf senescence: signals, execution, and regulation, *Curr. Top. Dev. Biol.* 71 (2005) 83–112.
- [64] M. Riemann, R. Dhakarey, M. Hazman, B. Miro, A. Kohli, P. Nick, Exploring jasmonates in the hormonal network of drought and salinity, *Plant Physiol.* 6 (2015) 1–16, <http://dx.doi.org/10.3389/fpls.2015.01077>.
- [65] V.A. Muñoz-Espinoza, M.F. López-Climent, J.A. Casaretto, A. Gómez-Cadenas, Water stress responses of tomato mutants impaired in hormone biosynthesis reveal abscisic acid, jasmonic acid and salicylic acid interactions, *Front. Plant Sci.* 6 (2015) 1–14, <http://dx.doi.org/10.3389/fpls.2015.00997>.
- [66] N.M. Upadhyaya, C.W. Parker, D.S. Letham, K.F. Scott, P.J. Dart, Evidence for cytokinin involvement in *Rhizobium* (IC3342)-induced leaf curl syndrome of pigeon pea (*Cajanus cajan* Millsp.), *Plant Physiol.* 95 (1991) 1019–1025, <http://dx.doi.org/10.1104/pp.95.4.1019>.
- [67] D.H. Sanchez, F. Schwabe, A. Erban, M.K. Udvardi, J. Kopka, Comparative metabolomics of drought acclimation in model and forage legumes, *Plant Cell Environ.* 35 (2012) 136–149, <http://dx.doi.org/10.1111/j.1365-3040.2011.02423.x>.
- [68] J.-Y. Zhang, M.H. Cruz DE Carvalho, I. Torres-Jerez, Y. Kang, S.N. Allen, D.V. Huhman, et al., Global reprogramming of transcription and metabolism in *Medicago truncatula* during progressive drought and after rewatering, *Plant Cell Environ.* 37 (2014) <http://dx.doi.org/10.1111/pce.12328>.

- [69] J.G. Streeter, D.G. Lohnes, R.J. Fioritto, Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance, *Plant Cell Environ.* 24 (2001) 429–438, <http://dx.doi.org/10.1046/j.1365-3040.2001.00690.x>.
- [70] A.R. Reddy, K.V. Chaitanya, M. Vivekanandan, Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants, *J. Plant Physiol.* 161 (2004) 1189–1202, <http://dx.doi.org/10.1016/j.jplph.2004.01.013>.
- [71] I. Aranjuelo, G. Molero, G. Erice, J.C. Avicé, S. Nogués, Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.), *J. Exp. Bot.* 62 (2011) 111–123, <http://dx.doi.org/10.1093/jxb/erq249>.
- [72] Y. Kang, Y. Han, I. Torres-Jerez, M. Wang, Y. Tang, M. Monteros, et al., System responses to long-term drought and re-watering of two contrasting alfalfa varieties, *Plant J.* 68 (2011) 871–889, <http://dx.doi.org/10.1111/j.1365-313X.2011.04738.x>.
- [73] E. Larrainzar, S. Wienkoop, C. Scherling, S. Kempa, R. Ladrera, C. Arrese-Igor, et al., Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery, *Mol. Plant-Microbe Interact.* 22 (2009) 1565–1576, <http://dx.doi.org/10.1094/MPMI-22-12-1565>.
- [74] C. Fresneau, J. Ghashghaie, G. Cornic, Drought effect on nitrate reductase and sucrose-phosphate synthase activities in wheat (*Triticum durum* L.): role of leaf internal CO<sub>2</sub>, *J. Exp. Bot.* 58 (2007) 2983–2992.
- [75] P.J. Lea, L. Sodek, M.A.J. Parry, P.R. Shewry, N.G. Halford, Asparagine in plants, *Ann. Appl. Biol.* 150 (2007) 1–26, <http://dx.doi.org/10.1111/j.1744-7348.2006.00104.x>.
- [76] F.M. McCarthy, N. Wang, G.B. Magee, B. Nanduri, M.L. Lawrence, E.B. Camon, et al., AgBase : a functional genomics resource for agriculture, *BMC Genomics* 7 (2006) 229, <http://dx.doi.org/10.1186/1471-2164-7-229>.