Elsevier Editorial System(tm) for
Agricultural Water Management
Manuscript Draft

Manuscript Number: AGWAT10151R1

Title: Do metabolic changes underpin physiological responses to water limitation in alfalfa (Medicago sativa) plants during a regrowth period?

Article Type: VSI: Sustainable Water Use

Keywords: Alfalfa, water stress, metabolite profile, physiology, 15N-labeling

Corresponding Author: Dr. Iker Aranjuelo, Dr

Corresponding Author's Institution: AgriBiology Institute-CSIC

First Author: Iker Aranjuelo, Dr

Order of Authors: Iker Aranjuelo, Dr

Abstract: Drought is one of the most limiting factors on crop productivity under Mediterranean conditions, where the leguminous species alfalfa (Medicago sativa L.) is extensively cultivated. Whereas the effect of drought on plant performance has been widely described at leaf and nodule levels, less attention has been given to plant-nodule interactions and their implication on metabolites exchange during a regrowth period, when water is limiting. For this purpose, physiological characterization and metabolite profiles in different plant organs and nodules were undertaken under water deficit, including regrowth after removal of aerial parts. In order to study in more detail how nitrogen (N) metabolism was affected by water stress, plants were labelled with N-enriched isotopic air (15N2) using especially designed chambers. Water stress affected negatively water status and photosynthetic machinery. Metabolite profile and isotopic composition analyses revealed that, water deficit induced major changes in the accumulation of amino acids (proline, asparagine, histidine, lysine and cysteine), carbohydrates (sucrose, xylose and pinitol) and organic acids (fumarate, succinate and maleic acid) in the nodules in comparison with other organs. The lower 15N-labeling observed in serine, compared with other amino acids, was related with its high turnover rate, which in turn, indicates its potential implication in photorespiration. Isotopic analysis of amino acids also revealed that proline synthesis in the nodule was a local response to water stress and not associated with a feedback inhibition from the leaves.. Water deficit induced extensive reprogramming of whole-plant C and N metabolism, including when the aerial part was removed to trigger regrowth.

CIMMYT MR
International Maize and Wheat Improvement Center

February 2rd, 2018

Dr. Enrique Fernández
Editor-in-chief
Agricultural Water Management

Dear Dr. Fernández,

Thanks a lot for sending us your comments and the comments made by the two referees to our manuscript (AGWAT10151) entitled "Do metabolic changes underpin physiological responses to drought in *Medicago sativa* plants?" written by Molero et al, for consideration in your journal.

Following your recommendation, we have uploaded a new version of our manuscript that we have prepared following the comments made by the referees. You will find below the detailed answer to the comments and suggestions of the decision letter.

All the suggestions made by Reviewer 1 were considered and incorporated in the manuscript and some clarifications are provided in the responses below.
Regarding the comments of Reviewer 2, we have rewritten some parts of the manuscript to make it clearer to the reader. In addition, the title of the manuscript has been slightly modified as recommended.

To our opinion, the present study highligths the effect of water deficit induces extensive reprogramming of whole-plant carbon and nitrogen metabolism in sink and source tissues of alfalfa, respectively when aerial part was removed.

We hope that you will find the new version of the manuscript acceptable for publication in Agricultural Water Management.

Sincerely,

Dr. Gemma Molero | Wheat Physiologist | Global Wheat Program

International Maize and Wheat Improvement Center (CIMMYT)
Carretera México-Veracruz Km. 45, El Batán, Texcoco, México, C.P. 56237
tel: +52 (55) 5804 2004 ext 2254 **skype:** gemmita_m **cel:** +52 1 (55) 4384 8411

REVIEWERS COMMENTS

REVIEWER 1

The major issue with the present form of the manuscript entitled "Do metabolic changes underpin physiological responses to drought in Medicago sativa plants?" is related with the lack of accuracy to define the objective of the study. In the abstract section, the authors mentioned that the intention was "to address the plant-nodule interaction and its implications in metabolites exchange during a regrowth period". However, in the introduction section the objective says: "The objective of this study was to identify possible target specific compounds (soluble sugars, organic acids and amino acids) that may be involved in controlling plant performance under drought conditions, by taking advantage of physiological and isotopic measurements".
This may appear a syntax disparity since in one sentence the emphasis is on the regrowth period and in the other on the drought stress. However, in the experimental system described below, the discrepancy is present again.

The objective have been rewritten to highlight that the main objective is to address the plant-nodule interaction and its implications in metabolites exchange during a regrowth period. This has been modified throughoiut the text and it is also reflected in the new title of the manuscript.

In the materials and methods section the following sentences are shown:
"As described below, when plants were 61 days old and the main root was totally developed we performed 15N2 labeling during 5 days (described below). Immediately after the labeling, the first harvest was undertaken from a subset of four control and four labeled plants (T0)."
This group of plants then is not cut, is without stress and the half part was labeled during 5 days.
Four control and four labelled plants were completelly harvested (aerial part, roots and nodules). In all the remaining plants (control and labelled ones) the aerial part was removed and the drought treatment was imposed in half of the control and half of the labelled plants.
Then, the experimental setup explanation continues as:
"Once the harvest was finished, in all plants, the aboveground part of the remaining plants was cut (to a 5 cm stem height) so to analyze plant regrowth capacity. Parallel with shoot cutting, water stress treatment was imposed."

All plants are cut to 5 cm in height and water stress is imposed at the same time, is difficult to me to understand how can the authors separate the triggered responses of plants to cutting or water stress? Finally, the last sentence states:
"Half plants were kept under optimal irrigation conditions (well watered, WW), whereas in the other half water stress (WS) was imposed through water withholding. A second harvest was performed 8 days after cutting (T8), when the plants were 74 days old."
I consider that a control of plants without cutting and exposed to water stress is lacking, it would allow separating the effects of drought and cutting.
The main objective of the experiment is to evaluate the effect of the drought on regrowing plants, this is why we did not considered to leave uncutted plants to study the effects individually. We do agree that if the objective were to evaluate both effects in a separate way (drought effect or cut effect) this should have been the most appropriate approach. We actually did study the effects of drought (i.e. Aranjuelo et al., 2013, Journal of experimental Botany, 64: 885-897) and cutting (i.e. Aranjuelo et al., 2015, Physiologia Plantarum, 153:91-104) in separate studies. However, in the present study, we were aiming to evaluate the combination of both effects (drought+cutting). Other authors have conducted similar experiments without keeping uncutted alfalfa plants under controlled conditions (Avice et al., 1997, Plant and Soil 188: 189–198) or in the field (Maamouri et al., 2015, Crop and Pasture Science 66: 192-2014).

It is difficult to me to understand the C and N fluxes between de aerial part and the roots and to assign the changes to the drought stress imposition when the aerial part was exposed to cut. I consider that the cut should be responsible a least in some part, of the changes observed.
We can assign the changes in C and N fluxes comparing the control (no drought) with the stressed ones as in both situations aerial part was removed. Therefore, the differences among control and stressed plants are related with drought and not with the cut effect (that exists in both situations).

Besides, the drought stress imposed was mild (since the RWC was reduced in only 8%) as the authors mentioned in some parts, so the title and the objective should be reconsidered.
Gas exchange parameters were also reduced indicating stressfull conditions. For example stomatal conductance was reduced by 36% . Nevertheless, the title has been modified to ‘water limitation’ and we have modified the text to highligth that the drought was mild and changed ‘drought’ by ‘water limitation’ throughout the text.

Therefore, I consider that there are several aspects against the correct interpretation of the discussion and conclusion sections, at least in the present form of the manuscript. The techniques used are accurate but the presentation of the results performed should be carefully revised.
We hope that the present version is now clearer. We have carefully revised the manuscript and added additional information that we consider it makes it more clear and highligths the importance to the study.

Please find in the attached file some specific comments.
All the cooments from the attached file have been addressed in the text, highlights, figures and tables.

REVIEWER 2

Comments/suggestions for authors
This is an interesting manuscript that contributes to our understanding of N2 fixation under water deprivation conditions. The manuscript describes a vital area of study for the use of alfalfa (Medicago sativa) as a useful forage legume in plant stress adaptation studies. The manuscript evaluated the metabolic responses to water stress in alfalfa apical shoots, primary roots and nodules. The authors tried to present some data showing that water deficit induces extensive reprogramming of whole-plant carbon and nitrogen metabolism in sink and source tissues of alfalfa, respectively. They claim that the current study provides a metabolic insight into the impact of water stress on plant growth and maybe adaptation.
Although the general quality of the research is good and the authors interpret their results correctly, however, I have some concerns/remarks/suggestions that might help the authors to improve the current version of the manuscript:

Title
The title is fully reflecting the objectives and the contents of the study. I would recommend the authors to include the common name for your test plant (alfalfa).
We hace included the common name in the title as suggested.
Abstract
The authors tried to present some data showing the metabolite profile and isotopic composition analyses of alfalfa to water stress. Generally, this part is well written by the authors. The Abstract describe the basic information which could be reflected in such concise but informative part of the manuscript. However, the last sentence is far to general and needs some improvement.

We have rewritten the sentence to one more concise and specific of the current study.
Abbreviations (e.g., N, amino acids): Authors should explain at first appearance, and then use the abbreviated forms.
Done.
Maleate (L13): Correct the misspelling and check the whole text, accordingly.
To avoid confusion, we have change it for Maleic acid as it appears in the figures. We have corrected in the whole text accordingly.

Introduction
The authors have presented some relevant literature on the various aspects of the topic studied and the gathered information was presented in a fairly connected fashion. However, the authors must give care/attention to the usage of abbreviations!
We have carefully check all the abbreviations in the text and explain at first appearance, and then use the abbreviated forms
Citation(s) should be given to the first sentence.
Added.

2nd paragraph: Replace "plant-bacteroid" with "plant-rhizobia"
Done.

The use of cites in the article needs revision. For instance, Bacanamwo and Harper (1997) & Neo and Layzell (1997) [P2, L4-5], Lodwig and Poole
(2003) & Lodwig et al. (2003) [P2, L17-18] are some examples of misquotes.
All the references have been carefully reviewed and corrected.

Alternatively, authors should try to give references related to the impact of drought stress.
Some references related with impact of drought stress has been included in the first paragraph of the introduction.

Materials and Methods
The adopted approach is relatively good based on the methodology used.
However, the authors must describe with more clarity the rationale of selecting the alfalfa cultivar "Demnat" for the purpose of this study? I presume that this variety is of economic importance.
A short description about Demnat cultivar has been included.

Was the seeds sterilized prior to germination? If so, mention it in text?

Yes, the information has been included in the text.

How much inoculate was given to the plants and when? It is not totally clear how the inoculation was prepared/performed in the study. Authors must give full information in this regard.
Information has been completed: 'During the first month, plants were inoculated three times a week with 3mL (per plant) of a sucrose solution at 2% containing *Sinorhizobium meliloti* strain 102F78 that was resuspended from agar media.'

Citation(s) must be given to the Hoagland N-free nutrient solution used in this study.
Citation included.

Authors mentioned the words "described below" two times in the same sentence [P2, L13-15]! Please, delete one?
Done.

Consistency requires attention! Authors must check the International Systems (SI) for the units adopted in the manuscript (e.g., ml/mL).
Correct and check the whole text, accordingly.
Corrected.

Reference(s) is needed for the "Pearson correlation method" used for clustering of the GC/TOFMS normalized data.
The sentence has been rewritten as the clustering was based on normal pearson correlation coefficients.

Authors must carefully check the usage of brackets, especially for the references cited in the text.
We have carefully check the usage of brackets and we have not detected any missusage.

Results
Generally, the results are explained in a good quality. The results have been presented in figures and tables, which are basically acceptable.
However, authors must give more attention to the titles/captions/legends of the Tables/Figures. For example: What "†" precisely indicates in Table
1 & 2? This should be explained! Also, I can't see the "C and N isotope composition" data in Table, 1 as mentioned in title/caption.

Added. †marginally significant
Title corrected.
Some titles/captions/legends of the Tables/Figures have been modified.

"GC/TOFMS" or "GC-TOF-MS"? Authors must be consistent in the whole text file!
Corrected.

Instead of "drought", I recommend to use "water stress, water deficit, etc." when describing the results.
Recommendation considered in the text.

Although mentioned there was a significant decrease in stomatal limitation (l) in response to water stress, however, Table 1 shows the reverse.
Please, check and update.
Table 1 shows a higher stomatal limitation for water stressed plants (31.53%) versus control plants (18.12%) resulting from a lower stomatal conductance in WS plants. Therefore, as we have lower gs we expect to have higher values of stomatal limitation.

Once abbreviated, authors must use the abbreviated form in the rest of the text (WW, WS, etc.).
Done.

Citation(s) must be given to "As expected, in shoots,…, reflecting the decrease in photosynthesis and an increase in photorespiration" [P11, 4th paragraph].
Added.

In addition to the profile of different amino acid, I suggest to display a separate figure showing the response of total amino acids.
As the amount of amino acids is presented in Table 2, we consider repetitive to present the figure proposed.

What are the units of amino acid shown in Table 2? nmol/g FW? This should be mentioned!
Included.

Are authors aware of any physiological role for ornithine "Orn" in nodule physiology? If so, please explain briefly.

In higher plants, proline is synthesized from both glutamic acid and ornithine. Under stress conditions, proline was proposed to be synthesized preferentially from glutamic acid (Delauney et al . 1993, J. Biol. Chem. 268: 18673–18678.). However, other studies conclude that the ornithine pathway plays a very significant role in proline synthesis in *M. truncatula* leaves, roots and nodules under salt stress (Verdoy et al., 2006, Plant Cell and Environment, 29: 1913-23). Therefore, in the present study Orn could be invilved in Pro synthesis as reflected in the decrease levels of Orn and increased Pro (Fig. 4).

What does "GC-C-IRMS" stand for? This must be clearly explained in the abbreviation list.
GC-C-IRMS, gas chromatography combustion isotope ratio mass spectrometry
This is already included in the abbreviation list.

The quality of Figure 4 is not so good. For instance, it is hard to recognize the components, such as names, color, etc.. This figure should be designed again with a better resolution.
The figure 4 has been redesigned withg higher resolution for better interpretation

Discussion
Authors hypothesized that N2 fixation decreased under water stress. If this is the case, please explain why nodules biomass significantly increased under water stress conditions?
In our opinion, the increase in nodule biomass might not necessarily translate automatically into more bacteroids, we can have nodules with a larger plant cell fraction and a lower bacteroid fraction. However, it could be also considered that the general decrease in free amino acids (Table 2) can be explained by the decline of N2 fixation rate by nitrogenase in response to mild drought stress (as explained by Zahran, 1999).

Again, the authors must check the usage of abbreviations (e.g., serine/ser)!
Done.

Are the authors aware of any study where proline is synthesized in the nodules under water stress? If yes, please mention it in text?
Yes, we observed an increase in Pro in previous studies (Aranjuelo et al., 2011, Journal of Experimental Botany, 62: 111-23). We have included the citation in the text.

Please show some citations to support the sentence "Ala levels did not change,…, its role in feedback inhibition is unlikely" [P16, 4th paragraph].
There is not a citation we can include. We mention in the introduction that some N compunds could be involved in fewdback inhibition mechanisnms and it is clear that Ala is not one of those.

Apart from the synthesis in the PR, would it be possible to assume that Asn was translocated from the shoots? Please comment on it in the text?

Asparagine is the major transport compound in the xylem from the root to the leaves and in the phloem from the leaves to the developing seeds in a range of plants (Lea et al., 2006, Annals of Applied biology, 150:1-26). In case of legumes, it could be considered that the N flux is more from roots to stems because of N2 fixation. This would mean that there is certainly an Asn flux from belowground to aboveground organs. We recognize that there could be a bidirectional flux, but here we can only look at the net Asn flux. This is why it is more feasible that it was synthetized in the PR and then transported to the shoots.

Conclusions
I think the data presented are solid, and the conclusions are reasonable.
References
References need editing! Some were inconsistently cited according to the standard format of the journal! For example, use correct journal format, e.g., Physiologia Plantarum → Volenec et al. (1996), New Phytologist → Weatherley (1950), watch punctuation! Some references are missing in the list (e.g., Volenec 1993ab).
The bibliography has been carefully reviewed and corrected

REVIEWERS COMMENTS

REVIEWER 1

The major issue with the present form of the manuscript entitled "Do metabolic changes underpin physiological responses to drought in Medicago sativa plants?" is related with the lack of accuracy to define the objective of the study. In the abstract section, the authors mentioned that the intention was "to address the plant-nodule interaction and its implications in metabolites exchange during a regrowth period". However, in the introduction section the objective says: "The objective of this study was to identify possible target specific compounds (soluble sugars, organic acids and amino acids) that may be involved in controlling plant performance under drought conditions, by taking advantage of physiological and isotopic measurements".
This may appear a syntax disparity since in one sentence the emphasis is on the regrowth period and in the other on the drought stress. However, in the experimental system described below, the discrepancy is present again.

The objective have been rewritten to highlight that the main objective is to address the plant-nodule interaction and its implications in metabolites exchange during a regrowth period. This has been modified throughoiut the text and it is also reflected in the new title of the manuscript.

In the materials and methods section the following sentences are shown:
"As described below, when plants were 61 days old and the main root was totally developed we performed 15N2 labeling during 5 days (described below). Immediately after the labeling, the first harvest was undertaken from a subset of four control and four labeled plants (T0)."
This group of plants then is not cut, is without stress and the half part was labeled during 5 days.
Four control and four labelled plants were completelly harvested (aerial part, roots and nodules). In all the remaining plants (control and labelled ones) the aerial part was removed and the drought treatment was imposed in half of the control and half of the labelled plants.
Then, the experimental setup explanation continues as:
"Once the harvest was finished, in all plants, the aboveground part of the remaining plants was cut (to a 5 cm stem height) so to analyze plant regrowth capacity. Parallel with shoot cutting, water stress treatment was imposed."

All plants are cut to 5 cm in height and water stress is imposed at the same time, is difficult to me to understand how can the authors separate the triggered responses of plants to cutting or water stress? Finally, the last sentence states:
"Half plants were kept under optimal irrigation conditions (well watered, WW), whereas in the other half water stress (WS) was imposed through water withholding. A second harvest was performed 8 days after cutting (T8), when the plants were 74 days old."
I consider that a control of plants without cutting and exposed to water stress is lacking, it would allow separating the effects of drought and cutting.
The main objective of the experiment is to evaluate the effect of the drought on regrowing plants, this is why we did not considered to leave uncutted plants to study the effects individually. We do agree that if the objective were to evaluate both effects in a separate way (drought effect or cut effect) this should have been the most appropriate approach. We actually did study the effects of drought (i.e. Aranjuelo et al., 2013, Journal of experimental Botany, 64: 885-897) and cutting (i.e. Aranjuelo et al., 2015, Physiologia Plantarum, 153:91-104) in separate studies. However, in the present study, we were aiming to evaluate the combination of both effects (drought+cutting). Other authors have conducted similar experiments without keeping uncutted alfalfa plants under controlled conditions (Avice et al., 1997, Plant and Soil 188: 189–198) or in the field (Maamouri et al., 2015, Crop and Pasture Science 66: 192-2014).

It is difficult to me to understand the C and N fluxes between de aerial part and the roots and to assign the changes to the drought stress imposition when the aerial part was exposed to cut. I consider that the cut should be responsible a least in some part, of the changes observed.
We can assign the changes in C and N fluxes comparing the control (no drought) with the stressed ones as in both situations aerial part was removed. Therefore, the differences among control and stressed plants are related with drought and not with the cut effect (that exists in both situations).

Besides, the drought stress imposed was mild (since the RWC was reduced in only 8%) as the authors mentioned in some parts, so the title and the objective should be reconsidered.
Gas exchange parameters were also reduced indicating stressfull conditions. For example stomatal conductance was reduced by 36% . Nevertheless, the title has been modified to ‘water limitation’ and we have modified the text to highligth that the drought was mild and changed ‘drought’ by ‘water limitation’ throughout the text.

Therefore, I consider that there are several aspects against the correct interpretation of the discussion and conclusion sections, at least in the present form of the manuscript. The techniques used are accurate but the presentation of the results performed should be carefully revised.
We hope that the present version is now clearer. We have carefully revised the manuscript and added additional information that we consider it makes it more clear and highligths the importance to the study.

Please find in the attached file some specific comments.
All the cooments from the attached file have been addressed in the text, highlights, figures and tables.

REVIEWER 2

Comments/suggestions for authors
This is an interesting manuscript that contributes to our understanding of N2 fixation under water deprivation conditions. The manuscript describes a vital area of study for the use of alfalfa (Medicago sativa) as a useful forage legume in plant stress adaptation studies. The manuscript evaluated the metabolic responses to water stress in alfalfa apical shoots, primary roots and nodules. The authors tried to present some data showing that water deficit induces extensive reprogramming of whole-plant carbon and nitrogen metabolism in sink and source tissues of alfalfa, respectively. They claim that the current study provides a metabolic insight into the impact of water stress on plant growth and maybe adaptation.
Although the general quality of the research is good and the authors interpret their results correctly, however, I have some concerns/remarks/suggestions that might help the authors to improve the current version of the manuscript:

Title
The title is fully reflecting the objectives and the contents of the study. I would recommend the authors to include the common name for your test plant (alfalfa).
We hace included the common name in the title as suggested.
Abstract
The authors tried to present some data showing the metabolite profile and isotopic composition analyses of alfalfa to water stress. Generally, this part is well written by the authors. The Abstract describe the basic information which could be reflected in such concise but informative part of the manuscript. However, the last sentence is far to general and needs some improvement.

We have rewritten the sentence to one more concise and specific of the current study.
Abbreviations (e.g., N, amino acids): Authors should explain at first appearance, and then use the abbreviated forms.
Done.
Maleate (L13): Correct the misspelling and check the whole text, accordingly.
To avoid confusion, we have change it for Maleic acid as it appears in the figures. We have corrected in the whole text accordingly.

Introduction
The authors have presented some relevant literature on the various aspects of the topic studied and the gathered information was presented in a fairly connected fashion. However, the authors must give care/attention to the usage of abbreviations!
We have carefully check all the abbreviations in the text and explain at first appearance, and then use the abbreviated forms
Citation(s) should be given to the first sentence.
Added.

2nd paragraph: Replace "plant-bacteroid" with "plant-rhizobia"
Done.

The use of cites in the article needs revision. For instance, Bacanamwo and Harper (1997) & Neo and Layzell (1997) [P2, L4-5], Lodwig and Poole
(2003) & Lodwig et al. (2003) [P2, L17-18] are some examples of misquotes.
All the references have been carefully reviewed and corrected.

Alternatively, authors should try to give references related to the impact of drought stress.
Some references related with impact of drought stress has been included in the first paragraph of the introduction.

Materials and Methods
The adopted approach is relatively good based on the methodology used.
However, the authors must describe with more clarity the rationale of selecting the alfalfa cultivar "Demnat" for the purpose of this study? I presume that this variety is of economic importance.
A short description about Demnat cultivar has been included.

Was the seeds sterilized prior to germination? If so, mention it in text?

Yes, the information has been included in the text.

How much inoculate was given to the plants and when? It is not totally clear how the inoculation was prepared/performed in the study. Authors must give full information in this regard.
Information has been completed: 'During the first month, plants were inoculated three times a week with 3mL (per plant) of a sucrose solution at 2% containing *Sinorhizobium meliloti* strain 102F78 that was resuspended from agar media.'

Citation(s) must be given to the Hoagland N-free nutrient solution used in this study.
Citation included.

Authors mentioned the words "described below" two times in the same sentence [P2, L13-15]! Please, delete one?
Done.

Consistency requires attention! Authors must check the International Systems (SI) for the units adopted in the manuscript (e.g., ml/mL).
Correct and check the whole text, accordingly.
Corrected.

Reference(s) is needed for the "Pearson correlation method" used for clustering of the GC/TOFMS normalized data.
The sentence has been rewritten as the clustering was based on normal pearson correlation coefficients.

Authors must carefully check the usage of brackets, especially for the references cited in the text.
We have carefully check the usage of brackets and we have not detected any missusage.

Results
Generally, the results are explained in a good quality. The results have been presented in figures and tables, which are basically acceptable.
However, authors must give more attention to the titles/captions/legends of the Tables/Figures. For example: What "†" precisely indicates in Table
1 & 2? This should be explained! Also, I can't see the "C and N isotope composition" data in Table, 1 as mentioned in title/caption.

Added. †marginally significant
Title corrected.
Some titles/captions/legends of the Tables/Figures have been modified.

"GC/TOFMS" or "GC-TOF-MS"? Authors must be consistent in the whole text file!
Corrected.

Instead of "drought", I recommend to use "water stress, water deficit, etc." when describing the results.
Recommendation considered in the text.

Although mentioned there was a significant decrease in stomatal limitation (l) in response to water stress, however, Table 1 shows the reverse.
Please, check and update.
Table 1 shows a higher stomatal limitation for water stressed plants (31.53%) versus control plants (18.12%) resulting from a lower stomatal conductance in WS plants. Therefore, as we have lower gs we expect to have higher values of stomatal limitation.

Once abbreviated, authors must use the abbreviated form in the rest of the text (WW, WS, etc.).
Done.

Citation(s) must be given to "As expected, in shoots,…, reflecting the decrease in photosynthesis and an increase in photorespiration" [P11, 4th paragraph].
Added.

In addition to the profile of different amino acid, I suggest to display a separate figure showing the response of total amino acids.
As the amount of amino acids is presented in Table 2, we consider repetitive to present the figure proposed.

What are the units of amino acid shown in Table 2? nmol/g FW? This should be mentioned!
Included.

Are authors aware of any physiological role for ornithine "Orn" in nodule physiology? If so, please explain briefly.

In higher plants, proline is synthesized from both glutamic acid and ornithine. Under stress conditions, proline was proposed to be synthesized preferentially from glutamic acid (Delauney et al . 1993, J. Biol. Chem. 268: 18673–18678.). However, other studies conclude that the ornithine pathway plays a very significant role in proline synthesis in *M. truncatula* leaves, roots and nodules under salt stress (Verdoy et al., 2006, Plant Cell and Environment, 29: 1913-23). Therefore, in the present study Orn could be invilved in Pro synthesis as reflected in the decrease levels of Orn and increased Pro (Fig. 4).

What does "GC-C-IRMS" stand for? This must be clearly explained in the abbreviation list.
GC-C-IRMS, gas chromatography combustion isotope ratio mass spectrometry
This is already included in the abbreviation list.

The quality of Figure 4 is not so good. For instance, it is hard to recognize the components, such as names, color, etc.. This figure should be designed again with a better resolution.
The figure 4 has been redesigned withg higher resolution for better interpretation

Discussion
Authors hypothesized that N2 fixation decreased under water stress. If this is the case, please explain why nodules biomass significantly increased under water stress conditions?
In our opinion, the increase in nodule biomass might not necessarily translate automatically into more bacteroids, we can have nodules with a larger plant cell fraction and a lower bacteroid fraction. However, it could be also considered that the general decrease in free amino acids (Table 2) can be explained by the decline of N2 fixation rate by nitrogenase in response to mild drought stress (as explained by Zahran, 1999).

Again, the authors must check the usage of abbreviations (e.g., serine/ser)!
Done.

Are the authors aware of any study where proline is synthesized in the nodules under water stress? If yes, please mention it in text?
Yes, we observed an increase in Pro in previous studies (Aranjuelo et al., 2011, Journal of Experimental Botany, 62: 111-23). We have included the citation in the text.

Please show some citations to support the sentence "Ala levels did not change,…, its role in feedback inhibition is unlikely" [P16, 4th paragraph].
There is not a citation we can include. We mention in the introduction that some N compunds could be involved in fewdback inhibition mechanisnms and it is clear that Ala is not one of those.

Apart from the synthesis in the PR, would it be possible to assume that Asn was translocated from the shoots? Please comment on it in the text?

Asparagine is the major transport compound in the xylem from the root to the leaves and in the phloem from the leaves to the developing seeds in a range of plants (Lea et al., 2006, Annals of Applied biology, 150:1-26). In case of legumes, it could be considered that the N flux is more from roots to stems because of N2 fixation. This would mean that there is certainly an Asn flux from belowground to aboveground organs. We recognize that there could be a bidirectional flux, but here we can only look at the net Asn flux. This is why it is more feasible that it was synthetized in the PR and then transported to the shoots.

Conclusions
I think the data presented are solid, and the conclusions are reasonable.
References
References need editing! Some were inconsistently cited according to the standard format of the journal! For example, use correct journal format, e.g., Physiologia Plantarum → Volenec et al. (1996), New Phytologist → Weatherley (1950), watch punctuation! Some references are missing in the list (e.g., Volenec 1993ab).
The bibliography has been carefully reviewed and corrected

Highlights

- Drought affected negatively leaf CO_2 diffusion and CO_2 fixing machinery.
- At the leaf level drought increased photorespiration.
- Root is less affected by drought than other organs.
- N_2 fixation is regulated at the nodule level.
- Apparent depletion of nodule N_2 fixation of water stressed plants could be linked with the lower amino acid content.
- Water deficit induces extensive reprogramming of whole-plant carbon and nitrogen metabolism in sink and source tissues of alfalfa.

Do metabolic changes underpin physiological responses to ~~drought~~water limitation in alfalfa (*Medicago sativa*) plants during a regrowth period?

Gemma Molero[1,2], Guillaume Tcherkez[3,] Regina Roca[4], Caroline Mauve[5], Llorenç Cabrera-Bosquet[2,6], José Luis Araus[2], Salvador Nogués[2] and Iker Aranjuelo[7]

[1]International Maize and Wheat Improvement Center (CIMMYT), Km. 45, Carretera Mexico-Veracruz, El Batán, Texcoco CP 56237, Mexico;

[2]Secció de Fisologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain;

[3]Research School of Biology, ANU College of Science, Australian National University, 2601 Canberra ACT, Australia

[4]Serveis Científico-Tècnics~~-~~, Universitat de Barcelona, Barcelona, Spain;

[5]Plateforme Métabolisme-Métabolome, Université Paris Sud-XI, Orsay, France;

[6]~~UMR LEPSE~~[6]LEPSE, INRA, Montpellier SupAgro, ~~F-34060,~~Univ Montpellier, Montpellier, France;

[7]Agrobiotechnology Institute (IdAB), CSIC-UPNA-Gobierno de Navarra, Mutilva, Spain.

***Corresponding author**: Gemma Molero Milán, International Maize and Wheat Improvement Center (CIMMYT), Km. 45, Carretera Mexico-Veracruz, El Batán, Texcoco CP 56237, Mexico. Phone number: +52 (55) 5804 2004, email: G.Molero@cgiar.org

Running title: ~~Drought~~Water stress impact on the metabolism of nodulated alfalfa ~~metabolism~~plants.

Submission date: August 30th 2017

Resubmission date: February 2nd 2018

Number of tables: 2

Number of figures: 4 (1 and 3 ~~color~~colour in print)

Total Word count: ~~8647~~9244

Abstract

Drought is one of the most limiting factors on crop productivity under Mediterranean conditions, where the leguminous species alfalfa (*Medicago sativa* L.) is extensively cultivated. Whereas the effect of drought on plant performance has been widely described at leaf and nodule ~~level~~levels, less attention has been ~~addressed~~given to ~~the~~ plant-nodule ~~interaction~~interactions and ~~its implications in~~their implication on metabolites exchange during a regrowth period~~.~~, when water is limiting. For this purpose, physiological characterization and metabolite profiles ~~of~~in different plant organs and nodules were ~~analyzed.~~undertaken under water deficit, including regrowth after removal of aerial parts. In order to study in more detail how nitrogen (N) metabolism was affected by water stress, ~~the~~ plants were ~~labeled~~labelled with N-enriched isotopic air ($^{15}N_2$) using especially designed chambers. Water stress affected negatively water status and photosynthetic machinery. Metabolite profile and isotopic composition analyses revealed that, ~~drought~~water deficit induced major changes in the accumulation of amino acids (~~Pro, Asn, His, Lys~~proline, asparagine, histidine, lysine and ~~Cys~~cysteine), carbohydrates (sucrose, xylose and pinitol) and organic acids (fumarate, succinate and ~~maleate~~maleic acid) in the nodules in comparison with other organs. The lower ^{15}N-labeling observed in ~~Ser~~serine, compared with other amino acids, was related with its high turnover rate, which in turn, indicates its potential implication in photorespiration. Isotopic analysis of amino acids also revealed that ~~Pro~~proline synthesis in the nodule was a local response to ~~drought~~water stress and not associated with a feedback inhibition from the leaves~~. The current study highlighted the fact that isotopic approaches in combination with metabolic profiling are powerful tools to study the turnover rates~~.. Water deficit induced extensive reprogramming of ~~various metabolic intermediates~~whole-plant C and ~~to predict metabolic origin of the compounds as well as to understand metabolic responses to drought in alfalfa~~N metabolism, including when the aerial part was removed to trigger regrowth.

Key words: Alfalfa, ~~drought~~water stress, metabolite profile, physiology, ^{15}N-labeling

Abbreviations:

A, Photosynthetic assimilation; *Arg*, arginine; *AS*, apical shoots; *Asn*, asparagine; *BNF*, biological nitrogen fixation; *C*, carbon; C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; *DW*, dry weigh; *E*, leaf transpiration rate; *F6P*, fructose-6-phosphate; *G6P*, glucose-6-phosphate; *GABA*, gamma-aminobutyric acid; *GC-C-IRMS*, gas chromatography combustion isotope ratio mass spectrometry; *GC-~~TOFMS~~TOF–MS*, gas chromatography with time-of-flight mass spectrometry; *Gln*,

glutamine; g_s, leaf stomatal conductance; *Glu*, glutamate; *Gly*, glycine; J_{max}, maximum electron transport rate contributing to RuBP regeneration; *HPLC*, high performance liquid chromatography; *I*, stomatal limitation; Lys, lysine; Met, methionine; *MEV*, TIGR multi experiment viewer; *MSTFA*, N-methyl-N(trimethylsilyl)trifluoroacetamide; *N*, nitrogen; *Nod*, nodule; *OPA*, *o*-pthaldialdehyde; *Orn*, ornithine; *Pro*, proline; *PPFD*, photosynthetic photon flux density; *PR*, primary roots; *RI*, retention index; *RWC*, relative water content; *Ser*, serine; *T0*, first harvest; *T8*, second harvest; ~~*TCA*~~*TFA*, Trifluoracetic acid; *Thr*, threonine; *TOM*, total organic matter; *Trp*, tryptophan V_{cmax}, carboxylation velocity of Rubisco; *VSP*, vegetative storage proteins; *WW*, well~~–~~-watered, *WS*, water stress; Ψ_s, osmotic potential.

1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the forage crops most extensively cultivated in the Mediterranean region~~. Although alfalfa~~ (Annicchiarico et al., 2011, 2015). Alfalfa is ~~usually grown under irrigation, it is~~a temperate forage frequently exposed to abiotic stresses such as low water availability and high temperature conditions (Walsh, 1995). ~~Moreover~~It is estimated that approximately 70% of yield reduction worldwide is the direct result of environmental stresses (Acquaah, 2012), where drought is considered the main environmental stress in agriculture (Cattivelli et al. 2008). In legumes, water limitation can reduce global N_2 fixation by up to 17 Gt N $year^{-1}$ (Burns and Hardy, 1975). Under drought conditions, alfalfa has a strategy of avoidance by stopping its vegetative growth and accessing water through its deep root system but in general has poor drought resistance and is rapidly affected by water shortage (Sheaffer et al., 1988) resulting in a decrease in yield depending on the severity and duration of drought stress. Alfalfa, similarly other forages, is frequently subjected to above ground organs cutting for animal ~~breeding~~feeding. Such cutting causes important modifications in carbon (C) and nitrogen (N) metabolism (Aranjuelo et al., ~~2014~~2014a) at the different organ levels. During this period, shoot removal requires the mobilization of C and N reserves from roots to shoots (Avice et al., 2003; Aranjuelo et al., ~~2014~~2014a), which means an inversion of source and sink organs due to the disappearance of aerial source organs and the formation of new sinks with developing shoots. Abiotic conditions that limit water availability after shoot removal can have significant effects on the dynamics of regrowth (Erice et al., 2007).

Alfalfa is a ~~leguminous species~~forage legume that stablishes a plant-~~bacteroid~~rhizobia interaction in which plant photosynthesis supplies ~~carbon (~~C~~)~~ to nodules, where it is used by the nitrogenase enzyme in the bacteroid as a source of energy and reducing power to fix nitrogen gas (N_2) (Streeter, 1987). On the other hand, the products of N_2 fixation, either amides or ureids, are exported to the plant via the xylem (Schubert et al., 1995) where they are used for the synthesis of proteins, secondary products and compounds involved in osmotic adjustment under stressful conditions (~~Delauney et al., 1993;~~ Fougère et al., 1991).

Whereas the general effects of drought on leaf gas-exchange in forages (Cornic, 2000; Lawlor, 2002; Aranjuelo et al., 2011) and on the sensitivity of plant-bacteria symbiosis have been extensively studied ~~(Zahran, 1999~~ (Aranjuelo et al., 2014b and references therein), relatively little is known about the effect of water availability in plant-nodule

interactions and its implications in plant functioning and metabolites exchange~~.~~ during a regrowth period. Indeed, some authors reported that the effect of water deficit on plant performance is associated with the deleterious impact of drought on N_2 fixation rather than on photosynthesis itself (~~Castellanos~~Serraj et al., ~~1996~~1999a; Thomas et al., 2004). Previous studies reveal that biological nitrogen fixation (BNF) under drought condition is affected by (1) C supply to nodules (Galvez et al., 2005; Larrainzar et al., 2009); (2) respiration decrease and the resulting lower oxygen (O_2) consumption may locally inhibit nitrogenase activity (Galvez et al., 2005; Aranjuelo et al. 2011) and (3) the accumulation in the nodule of N compounds~~ associated with the decrease in stem N~~ can induce a feedback mechanism (Serraj et al., ~~1999~~1999b). Several molecules like glutamine (Gln) (Neo and Layzell, 1997), ureides (Serraj et al., 2001), and asparagine (Asn) have been suggested to be involved in such a mechanism (Bacanamwo and Harper, 1997)~~ have been suggested to be involved in such a mechanism.~~.

In alfalfa plants, ~~asparagine~~Asn, together with ammonia, is the major organic N compound transported to the plant from the nodule (Groat and Vance, 1981). Some amino acids can be further transported back to the nodule from the shoots as a systemic signal for ~~biological nitrogen fixation (~~BNF~~)~~ regulation under drought conditions (Bacanamwo and Harper, 1997; King and Purcell, 2005; Neo and Layzell, 1997; Serraj et al., 2001). However, studies under drought conditions in~~ the grain legume~~ pea suggest a local signal in addition to the systemic signal involved in BNF activity (Marino et al., 2007). Another point of controversy concerns the different sources of C required for amino acid synthesis. Although organic acids (mainly ~~malate~~maleic acid and succinate) represent an important pool of C skeletons in the bacteroid (Lodwig and Poole, 2003), other studies suggest that some amino acids, like glutamate~~, glutamine, glicine,~~ (Glu), Gln, glycine (Gly), proline (Pro) and tryptophane~~,~~ (Trp), can also be remobilized and thus represent an alternative source of C and energy to nodules (Kohl et al., 1994; Udvardi and Day, 1997; Molero et al. 2011). However, ~~in another study (~~Prell and Poole, (2006~~),~~ suggested that amino acid supply to the bacteroid appears to be related to the synthesis of alanine (Ala) and aspartate~~.~~ (Asp). Disparities amongst results highlight the current uncertainties on the role of amino acids in nodule metabolism and their partitioning through the plant, particularly under drought conditions (Lodwig and Poole, 2003; Lodwig et al., 2003). Some studies suggest that regrowth after shoot removal may be more dependent on the availability of N reserves rather than of C reserves (Avice et al., 1996; Kim et al., 1993; Ourry et al., 1994; Volenec et al., 1996).

Thus, understanding the exchange of C and N metabolites between plant and nodules is of prime importance, especially under water deficit conditions. ~~Fluxomics~~Fluxomics (*i.e.*the study of the concentration and fluxes of metabolites in an organism) and isotopic tracing can provide insightful information about how different metabolites are exchanged and transferred in a biological system (Tardieu et al 2017; Salon et al 2017). The study of plant metabolites ~~and processes involved in C and N metabolisms~~ can therefore provide new insights on how ~~these metabolites~~specific processes involved in C and N metabolism may confer ~~to plants~~ a better tolerance to water limitation in a context where the aerial part has been removed and, therefore, limiting the C supply to the nodule.

The objective of this study was to identify possible target specific compounds (soluble sugars, organic acids and amino acids) that may be involved in controlling plant performance during a regrowth period under drought conditions, by taking advantage of physiological and isotopic measurements. Here, we focused on the characterization of water availability effects in different organs (leaves, roots and nodules) and carried out metabolic analysis~~ by High Performance Liquid Chromatography (HPLC), Gas Chromatography with Time-of-Flight Mass Spectrometry GC-TOFMS, and Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS). $^{15}N_2$ labeling~~. N-enriched isotopic air ($^{15}N_2$) was used as labelling gas and enabled us to study N fixation in total organic matter (TOM) and individual amino acids and N exchange between different organs.

2. Material and methods

2.1. Experimental design and water status

~~Seeds of alfalfa (*Medicago sativa* L. cv Demnat)~~The alfalfa (*Medicago sativa* L) cultivar Demnat from Morocco, identified as well adaptated to frequent cuts under warm and irrigated conditions (Annicchiarico et al., 2013; Nanni et al., 2014), was selected for the study. Seeds were surface sterilized in 10% commercial bleach for 30 min., and rinsed three times with deionized water. Sterilized seeds were germinated on Petri dishes and planted on 7L white plastic pots filled with sand. Plants were grown at 25/15ºC (day/night) with a photoperiod of 14 hours in growth chambers (Conviron E15, Controlled Environments ltd., Winnipeg, Canada) equipped with fluorescent lamps (SylvaniaDECOR183, Professional-58W, Germany) that provided a photosynthetic photon flux density (PPFD) of *ca*. 400 µmol m^{-2} s^{-1}. During the first month, plants were inoculated three times a week with 3mL (per plant) of a sucrose solution at 2%

containing *Sinorhizobium meliloti* strain 102F78. that was resuspended from agar media. Plants were watered twice a week with Hoagland N-free nutrient solution (Hoagland and Arnon 1950) and once a week with deionized water to avoid salt accumulation in pots. As described below, when plants were 61 days old and the main root was totally developed we performed $^{15}N_2$ labelinglabelling during 5 days (described below).. This plant stage was chosen for labelinglabelling since at this stage, there is an important of C and N compound remobilization from aboveground organs toward taproot that acts as the major storage organ (Avice et al., 1996). Immediately after the labelinglabelling, the first harvest was undertaken from a subset of four control and four labeledlabelled plants (T0). Once the harvest was finished, in all plants, the aboveground part of the remaining plants was cut (to a 5 cm stem height) so to analyzeanalyse plant regrowth capacity. Parallel with shoot cutting, waters stress treatment was imposed. Half plants were kept under optimal irrigation conditions (well--watered, WW), whereas in the other half water stress (WS) was imposed through water withholding. A second harvest was performed 8 days after cutting (T8), when the plants were 74 days old. In each harvest, plants were separated into apical shoot, primary root and nodules. Four plants were collected per treatment and were immediately frozen in liquid N and stored in -80 ºC freezer. A subsample of each organ was separated and dried in an oven during 48 h at 60ºC in order to determine dry weight. Metabolite measurements were conducted in only three replicates per organ and water regime.

Plant water status was evaluated before harvesting by determining apical leaf relative water content (RWC) according to Weatherley, (1950). Osmotic potential was determined in apical shoots, primary roots and nodules using a Wescor 5500 osmometer (Wescor, Logan, Utah, USA) as described by (Ball and Oosterhuis, 2005).

2.2. Leaf gas exchange

Fully-expanded apical leaves were enclosed in a LI-COR 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). Determinations were carried out at 25ºC. Photosynthetic assimilation (A), leaf stomatal conductance (g_s) and leaf transpiration rate (E) were estimated at a saturating PPFD of 1200 µmol $m^{-2}s^{-1}$ using equations developed by von Caemmerer and Farquhar (1981). The ratio intercellular to ambient CO_2 concentration (C_i/C_a) was estimated from net photosynthesis and g_s measurements, according to Farquhar and Sharkey (1982). The gas-exchange response-curve to atmospheric CO_2 concentration was measured from 0 to 1400 µmol mol^{-1} CO_2. Measurements started at 400 µmol mol^{-1} of CO_2, decreased

stepwise through l 250 and 100 until ~~250, 100,~~ 0 µmol mol^{-1} and then restarted at 400 and increased stepwise until 700, 850, 1000 and 1400 µmol mol^{-1}. Estimation of the maximum carboxylation velocity of Rubisco (V_{cmax}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) were made by fitting a maximum likelihood regression below and above inflexion of the A/C_i response using the method of Ethier and Livingston (2004). Stomatal limitation (l), which is the proportionate decrease in light-saturated net CO_2 assimilation attributable to stomata, was calculated according to Farquhar and Sharkey (1982) as $(A_0-A_1)/A_0$, where A_0 is the A at c_i of 360 µmol mol^{-1} and A_1 is A at c_a of 360 µmol mol^{-1}.

2.3 Isotope ~~Labeling~~Labelling

The $^{15}N_2$ ~~labeling~~labelling was conducted at root level in the Conviron E15~~,~~ growth chambers with 10 ‰ enriched $^{15}N_2$. The $^{15}N_2$ gas was prepared in gas sampling bags (SKC, Houston, USA) by mixing $^{15}N_2$ enriched at 99 ATOM % provided by EURISO-TOP (Saint Aubin, France) with ambient air ($\delta^{15}N_2$ at *ca*. 0.35 ‰). The pots containing the plants were placed within a hand-made ~~labeling~~labelling chamber and closed hermetically. The $^{15}N_2$ was then injected in the hand-made chamber using a gas syringe (SGE International Pty Ltd, Australia). The injection of enriched $^{15}N_2$ was conducted twice a day; two and five hours after the beginning of the daily light period, coinciding with the period of largest N_2 fixation activity (Steunou et al., 2008). Then, the ~~labeling~~labelling chambers were removed from the bottom of the pots and the growth chambers were opened and quickly purged with ambient air. Non-~~labeled~~labelled plants were grown in a second growth chamber maintained at ambient N_2 air conditions.

2.4 Metabolite profile analyses

For HPLC analysis of amino acids and GC–~~TOFMS~~TOF–MS profiling, extracts from three of the four replicates were performed as described in Bathellier et al. (2009). The frozen material was ground in liquid N with a pestle and mortar and extracted into 80 % methanol / 20 % water containing ribitol (100 µM) as internal standard. After centrifugation, multiple aliquots were spin-dried under vacuum and stored at -80~~–~~°C. For HPLC analysis of amino acids, aliquots were re-dissolved in water, centrifuged and filtered into autosampler vials prior to automated pre-column derivatization with *o*-pthaldialdehyde (OPA). OPA reagent was made 36 h before first use by dissolving OPA in 200 µl of methanol and adding 1.8 ml 0.5 M sodium borate (pH 9.5) and 40 µl 2-mercaptoethanol. The reagent was filtered into an autosampler vial and used for up to 2 days. Precolumn derivatization was performed in the injection loop by automated

mixing of 10 µl sample and 10 µl OPA reagent, followed by a delay of 2 min prior to injection. The chromatographic separation was performed by gradient elution at 40 ºC using buffer A (20% methanol, 79% sodium acetate, 1% tetrahydrofuran, pH 5.9) and buffer B (80% methanol, 20% sodium acetate, pH 5.9). Buffer flow rate was 0.8 ~~ml~~mL min^{-1} throughout and total run time per injection was 52 min. Peak identity was confirmed by co-elution with authentic standards.

For GC–~~TOFMS~~TOF–MS analysis, methoxyamine was dissolved in pyridine at 20 mg ml^{-1} and 50 ml of this mixture was used to dissolve the dry sample. Following vigorous mixing, samples were incubated for 90 min at 30 ºC with shaking. Then, 80 ml of N-methyl-N(trimethylsilyl)trifluoroacetamide (MSTFA) was added, and the mixture was vortexed, and incubated for 30 min at 37 ºC with shaking. The derivatization mixture was then incubated for 2 h at room temperature. Before loading into the GC autosampler a mix of a series of eight alkanes (chain lengths: C10 to C36) was included to enable identification by retention index (RI) as well as by MS fragmentation pattern.

~~Labeled~~Labelled amino acids with *o*-pthaldialdehyde were separated by reverse-phase HPLC and identified by RI compared to standards. For GC–~~TOFMS~~TOF–MS, metabolite derivatives were identified by comparison of the fragmentation pattern with MS databases using a match ~~cutoff~~cut-off criterion of 750/1,000 and by RI using alkane series as standards. This enabled detection of 78 different metabolites. When standards were available, these were used to test the predicted matches: 59 commercially available compounds were individually ~~analyzed~~analysed by GC–~~TOFMS~~TOF–MS. They were all found to confirm the matches predicted by MS and RI.

For HPLC quantification analysis, amino acid amounts were calculated on linear calibration curves generated for each standard. Values were corrected for the response of the internal standard (ribitol), and quantified on a tissue fresh weight basis. For GC–~~TOFMS~~TOF–MS, integrated peak areas were obtained after deconvolution by the LECO PEGASUS III ChromaTOF® software and quantified using the appropriate software option. These were then normalized to the internal standard peak area for each injection. Principal component and hierarchical clustering analyses were performed using the TIGR Multi Experiment Viewer (MEV) software (Saeed et al., 2003). For each metabolite, the mean was subtracted from each individual value and the result divided by the standard deviation to yield ~~center~~centre-reduced data.

2.5 Determination of $\delta^{15}N$ of amino acids

Stable ^{15}N isotope composition ($\delta^{15}N$) of amino acids was determined as detailed in Molero *et al* (2011). Frozen samples were ground to a fine powder in liquid N and a sub-sample (ranging from 50 to 200 mg fresh weight of tissue) was lyophilized. Extraction of soluble fraction was performed with Trifluoracetic Acid (TFA) 10% (v/v) at ~~4ºC~~4 ºC using a sonicator. The homogenate was centrifuged at 6,000 *g* for 15 minutes at ~~4ºC~~4 ºC. Then the supernatant was collected in Ultrafree-MC 10000 NMWL (*Millipore*, EUA) tubes and purified by centrifugation (13,000 *g* during 45 minutes at ~~4ºC~~4 ºC). Following, 1 ~~mL~~ml of filtered sample was taken and L-norleucine (*Sigma-Aldrich;* Schnelldorf, Germany and St. Quentin Fallavier, France) was added as an internal standard. These fractions were dried under vacuum overnight using a Speed Vac desiccator and stored at -~~20ºC~~20 ºC until further analysis. Then the samples were re-suspended in 1 ~~mL~~ml of HCl 0.1 N (v/v) and passed through a chromatographic column filled with cation exchange resin (Dowex 50W X8 H^+, 200-400 mesh size, Sigma®) allowing extraction of acidic, basic and neutral amino acids.

Amino acids mixture eluted from the column was completely evaporated under heat and dry N obtaining the crystallized amino acids. Derivatization was performed with N-methyl-N-(*tert*.-butyildimethylsilyl)-trifluoroacetamide (Aldrich®) as proposed by (Woo and Chang, 1993; Woo and Lee, 1995). Then the amino acids were derivatized to N(O)-(*tert*.-butyildimethylsilyl) derivatives and the reaction mixture was first injected directly to Gas Chromatography-Mass Spectrometry (GC-MS) in order to separate and to identify the amino acids composition of the samples. The amino acid derivatives were identified by means of their mass spectra (Mass Spectral Library: NIST 05). Then the samples were analyzed by Gas Chromatography Isotope Ratio Mass Spectrometry (GC-C-IRMS) in order to determine $\delta^{15}N$ of individual amino acids.

2.6 Statistical analyses

To evaluate the effect of ~~drought~~water limitation on the measured traits, means were compared by an unpaired *t*-test at the 5% significance level using the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA). GC~~/TOFMS~~–TOF–MS data were normalized with respect to the mean of all organs (Fig. 1A, 1B) or water treatment within organs (Fig. 2). Normalized data were then drawn as a clustered metabolomic array using MeV 4.1 open source software (Saeed et al., 2003) as described above. The clustering was based on the ~~Pearson~~Pearson's correlation ~~method.~~coefficients among the metabolites. In this representation, green ~~color~~colour is proportional to a lower concentration; conversely, the intensity of the red ~~color~~colour is proportional to

higher concentration rates. Significant differences were determined using Student's *t*-test at α = 0.05.

3. Results

~~Drought~~Water limitation effects on plant growth, water status and physiology

At final harvest time (i.e. 8 days after withholding water), water stress was found to have no significant effect on total plant biomass (Table 1). However, a significant increase in nodules biomass and a modest but significant decrease in primary root biomass were observed upon water limitation (Table 1). No significant difference in the percentage of nitrogen was observed in either apical shoots (AS), primary roots (PR) or nodules (Nod) (Table 1). Plants under ~~drought~~water stress treatment showed a significant (P<0.05) decrease of 8.4% in leaf relative water content (RWC) (Table 1). No differences in osmotic potential (Ψ_s) in apical shoots and primary roots were observed. However, a significant decrease in the osmotic potential of nodules was found (Table 1).

Leaf gas exchange measurements (Table 1) performed in apical leaves, 6 days after the beginning of water withholding, revealed a significant decrease in net photosynthetic CO_2 assimilation (A), Rubisco carboxylation maximum capacity (Vc_{max}), RuBP regeneration maximum capacity (J_{max}), stomatal limitation (l) and stomatal conductance (g_s) as a response to water stress.

~~Drought~~Water limitation effects on metabolite profiles from plant and nodule

To ~~analyze~~analyse the effect of water stress on plant and nodule metabolism, non-targeted metabolite profiling was performed by GC-TOF-MS and 78 different metabolites were identified (by reference to their MS data). Comparison of metabolite profiles in AS, PR and Nod grown at WW and WS conditions showed that only 9 of the 78 identified metabolites did not show significant organ or water treatment-dependent effects. Metabolite profiling representation (heatmap) was undertaken between organs (Fig. 1A, 1B) and water treatments (Fig. 2). In order to compare the metabolic composition between organs, each water treatment (WW and WS) was ~~analyzed~~analysed separately (panels 1A and 1B in Fig. 1). Values shown with ~~colors~~colours were ~~uv~~UV-scaled (~~centered~~centred and normalized to standard deviation) for each metabolite. Metabolite contents were normalized with respect to both internal standard (ribitol) and dry mass (see Material and methods) and thus comparisons between organs in Fig. 1 represent differences in relative content per mg DW. Only metabolites showing significant ~~inter-group~~ differences between organs by

ANOVA at the $P < 0.05$ level were retained for the heat map and the hierarchical clustering (using Pearson's correlation coefficient).

Under well-watered conditions, the hierarchical clustering of the 63 significant metabolites formed two clusters (Fig. 1A). Cluster 1 mostly included organic acids, and was made of metabolites in higher concentration in AS as compared to other organs. Cluster 2 was made of different metabolic classes (including sugars and amino acids) at higher concentration in nodules as compared to other organs. Cluster 2 could be sub-divided in several sub-clusters: sugars and Serine (Ser) (2.1), sugars and amino acids (2.2), amino acids, sugar alcohols, organic acids (2.3) and putrescine + maleic acid (2.4).

Under water restriction, 48 metabolites were found to be significantly different between organs (Fig. 1B). Three different clusters were identified according to organ-specific prevalence: compounds with higher concentration in PR (arabinose, ~~malate~~maleic acid and phosphate, cluster 1), AS (myoinositol, ~~serine~~Ser and organic acids, cluster 2) ~~or~~and Nod (various classes, including sugars, cluster 3).

~~The drought effect on metabolites in each individual organ was represented in Fig. 2.~~Figure 2 represents the impact of water deficit on metabolite contect on the different studied organs. In the case of AS, 15 compounds were significantly different between WW and WS. As expected, in shoots, water stress caused a decrease in hexose phosphates and an increase in ~~serine~~Ser and glyceric acids, reflecting the decrease in photosynthesis and an increase in photorespiration~~.~~ (Miller et al., 2010). In PR, 11 metabolites significantly increased upon water deficit (Fig. 2), including sucrose and three metabolites of ~~glutamate~~Glu metabolism (~~glutamine~~Gln, GABA and pyroglutamate). In nodules, 23 metabolites were significantly different between water treatments: 20 increased and included sugars and several amino acids, and only three compounds increased (Pro, sucrose and maleic acid).

Quantitative analysis of differences in amino acids by HPLC

Because the GC–TOF~~-~~MS provides relative contents, amino acids were ~~analyzed~~analysed by targeted HPLC to perform absolute quantitation. This analysis showed that the water regime caused a general increase in amino acid content in PR but a decrease in nodules (Table 2). In agreement with the GC–TOF~~-~~MS analysis, Asp and Ser significantly decreased and increased in AS, respectively, under WS

conditions (Table 2). An increase in amino acid content in PR was observed as a response to ~~drought~~water limitation, so that GABA, Gln, Glu and Trp significantly increased and Asn also tended to increase. In general, all amino acids in nodules decreased with ~~drought~~water stress, but only Arg, Asn, Gln, Glu, Gly, Lys, Met, Orn, Ser and Thr were statistically significant. The most marked decrease (3.2-fold) was observed with Asn.

Isotopic pattern of amino acids revealed by GC~~-~~C~~-~~IRMS

The isotopomic representation of most relevant amino acids involved in the present study is shown in Fig. 4. Each δ^{15}N value of amino acids from ~~labeled~~labelled plants was normalized together with δ^{15}N values of amino acids in control plants. Therefore, green values represent low ^{15}N-enrichment in contrast to red values ~~which~~that represent high ^{15}N -enrichment in amino acids respect control plants (non-~~labeled~~labelled). After ~~labeling~~labelling (T0), PR was the organ containing the most ~~labeled~~labelled amino acids, followed by Nod. At T8, ^{15}N-labeling in amino acids was lower under WW conditions than under ~~water stress~~WS. The cluster shows the isotopic proximity (i.e., with the most similar covariation pattern) between Pro and Glu on the one hand, and between Asp and Asn on the other hand (Fig. 3). In general, the ^{15}N-enrichment was lower in Nod. ~~Eight days after labeling, under~~Under WW conditions, Asp and Asn in Nod and Ser in AS were poorly ~~labeled as~~labelled eight days after labelling compared with other amino acids (green cells). Because of the low levels of Pro, no value of δ^{15}N could be obtained in all organs ~~analyzed~~analysed under well-watered conditions. Under WS conditions, the ^{15}N-labeling in Pro was lower in AS and Nod as compared to PR. Taken as a whole, after ~~8~~eight days under WS conditions, amino acids appeared to be most ^{15}N-enriched in PR, suggesting the remobilization of recently fixed N upon water stress.

4. Discussion

In this study, we used a combination of metabolic profiling and isotopic ~~labeling~~labelling (with gaseous $^{15}N_2$) to investigate the physiological responses of *Medicago sativa* plants subjected to a mild water stress~~.~~ during a regrowth period. With this approach, we examined plant and nodule responses to ~~drought~~water stress at the metabolic level. Physiological, metabolic and isotopic data confirmed a general decrease in C metabolism in nodulated alfalfa plants subjected to water limitation, and a reorchestration of N metabolism.

Leaf gas-exchange and plant-nodule Ψ_s

Gas exchange ~~determinations~~measurements (Table 1) revealed that ~~drought~~water stress decreased photosynthetic activity ~~as a consequence~~because of stomatal (as shown by stomatal limitation data) and non-stomatal processes (Rubisco maximum carboxylation capacity Vc_{max}, and RuBP regeneration capacity, J_{max}), as already found elsewhere (Nogués et al., 2000; Aranjuelo et al., ~~2010~~2011). The decrease in relative water content showed that the worse water status of ~~droughted~~WS plants was involved in the inhibition of photosynthetic machinery (Aranjuelo et al., ~~2010~~2011). The lack of differences in leaf osmotic potential (Ψ_s) despite the change in RWC suggests a low osmoregulatory response to ~~drought~~water stress in leaf cells, and rather a change in matrix and hydrostatic components of total leaf water potential. In other words, plants were here at the stage of ~~drought~~water stress response situated just after stomatal closure initiation, before any important change in leaf water potential (Cornic, 2000; Flexas and Medrano, 2002; Medrano et al., 2002). Accordingly, the leaf response observed here was not accompanied by the accumulation of typical osmolytes like Pro; only myoinositol accumulation being observed (Fig. 2). Similarly, the lack of differences in root Ψ_s ~~is~~was only accompanied by a modest accumulation of sucrose. Conversely, in nodules, the significant decrease in Ψ_s with ~~drought~~water limitation was accompanied by a large increase in Pro and sucrose levels~~.~~, as previously described (Aranjuelo et al., 2011). These results suggest a higher osmotic adjustment in nodules compared with other organs.

Reorchestration of catabolism and N assimilation upon water deficit

In leaves, sucrose levels remained unaltered under ~~drought~~water stress conditions. While, the lower amount of various intermediates of glycolysis, such as glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) could be the consequence of the photosynthetic inhibition, it should be also associated with a decrease in the flux of C to

glycolytic pathway (Fig. 4). The accumulation of pyruvate and the general decrease in Krebs intermediates that was observed under ~~drought~~water-limited conditions ~~may~~might indicate a down-regulation of the Krebs cycle. The accumulation of pyruvate suggests an inhibition of its conversion to acetyl-CoA by pyruvate dehydrogenase. The decrease in palmitic acid, stearic acid and malonic acid (synthesized from acetyl-CoA) may also indicate an inhibition in acetyl-CoA synthesis.

Maleic acid levels are commonly low because of its little involvement in biochemical pathways. However, maleic acid is known to be a competitive inhibitor of several Krebs cycle enzymes (Evans and Garraway, 1984); therefore, its increase under ~~drought~~water limited conditions could be related with the inhibition of Krebs cycle enzymes~~.~~ (as indicated in Fig. 4). The accumulation of Pro in response to ~~drought~~water stress might serve to stabilize protein structure (Schobert and Tschesche, 1978) and is associated with an osmoregulatory function (Hare et al., 1998; Irigoyen et al., 1992). The decrease in Glu can be also explained with the increase in Pro levels, as has been previously reported (Aranjuelo et al., 2011). Similarly to Pro, myo-inositol, which is also associated with an osmoregulatory role (Streeter et al., 2001), was increased in leaves.

Photorespiration may serve as an energy sink, preventing over~~--~~reduction of the photosynthetic electron transport chain and photoinhibition (especially under drought), caused by reduced rates of photosynthetic CO_2 assimilation and thus NADPH utilization (Wingler et al., 1999). ~~Stomatal closure detected in water stressed~~Rubisco, catalyses the reaction of ribulose-1,5 -bisphosphate with either CO_2 or O_2 and thereby initiates CO_2 assimilation and photorespiration, respectively. The balance between the two reactions depends on the relative mole fraction of CO_2 and O_2 at the site of catalysis (chloroplast stroma).Therefore the lower the CO_2 mole fraction, the higher the photorespiration rate is. Stomatal closure detected in WS plants lead to higher level of photorespiration due to lower intercellular CO_2 mole fraction~~.~~ (data not shown)..Moreover, the increase in ~~serine~~Ser and glycerate (intermediates of the photorespiratory cycle) suggest a typical increase in photorespiration rate (Novitskaya et al., 2002). In the other hand, ^{15}N ~~labeling~~labelling conducted in alfalfa plants highlighted the fact that the very low ^{15}N-labeling in leaf Ser (compared with other amino acids) probably comes from the use of non-labelled leaf ~~glutamate~~Glu pool in photorespiration (Martinelli et al., 2007). By contrast, there was a ^{15}N-labeling in Ser in ~~water stressed~~WS plants in other organs, showing that Ser was formed from other metabolic pathways and not translocated inherited from leaves. In other words, Ser

synthesis in roots and nodules was likely achieved by the cytoplasmic pathway from 3-phosphoglycerate, thereby involving transamination from a pool containing ^{15}N.

In general, roots showed a moderate metabolic response to water deficit than the other organs, suggesting a limited impact in pool sizes. Quantification of amino acids by HPLC demonstrated an increase in GABA, Gln, Glu and Trp (Table 2). Metabolomic profiling of roots showed that only 11 compounds were significantly different between WW and WS treatments (Fig. 2). By contrast, isotopomic profiling show that the primary root was the organ with the most ^{15}N enriched free amino acids (Fig. 3). Therefore, it is likely that amino acid pools in PR are associated with a high turn-over; synthesised amino acids being allocated to export (to other organs) or as storage protein synthesis. In fact, PR are believed to have a critical role in N storage and remobilization in alfalfa. For example, defoliation of aerial parts ~~impacts~~affects drastically on N acquisition via a large decrease in nitrogenase activity (Kim et al., 1991, 1993; Ourry et al., 1994) while the production of new shoots during the first days following cutting involves N compounds from PR. In other words, endogenous N reserves in PR, in the form of amino acids and proteins, are used for the regrowth of aerial parts (Ta et al., 1990; Avice et al., 1996), while C reserves (like sugars) are mainly used for sustaining respiratory metabolism of belowground organs (roots and nodules). Specific proteins called vegetative storage proteins (VSPs) have been identified in taproots of alfalfa (Volenec et al., 1996; Ourry et al., 2001; Bewley, 2002). These VSPs can represent up to 40% of the total soluble proteins in the taproot ~~Volenec (1993ab).~~Erice et al., (2007). Furthermore, as observed by previous studies (Hendershot and Volenec, 1993b; Avice et al., 1996a; Corre et al., 1996; Gana et al., 1998) during regrowth, VSPs are degraded, with a rate of remobilization from 60% to 80%, ~~so as~~in order to provide N to re-growing shoots. In our experiment, we hypothesize that PR played a similar role by remobilizing N assimilates and proteins, thereby compensating for the drop in photosynthetic input by shoots caused by water deficit.

In nodules, water stress led to a general decrease in free amino acids (Table 2). This decrease can be explained by the decline of N_2 fixation rate by nitrogenase in response to mild drought stress (Zahran, 1999). Interestingly, Pro was increased in nodules under water deficit (Fig. 4) as previously reported (Aranjuelo et al., 2011). This increase was not related to an import from PR, since Pro is hardly ^{15}N-labelled in nodules (Fig. 3). It is rather synthesized locally by nodule metabolism and as such, it is associated with a decrease in Glu and Asn (Table 2, Fig. 2), suggesting that Glu and Gln

metabolism ~~channeled~~channelled N to Pro synthesis. Glu (precursor of Pro) and Pro are tightly correlated in the isotopomics analysis (r = 0.99 in HCL analysis), suggesting that the ~~turn-over~~turnover of Glu was directly associated with Pro accumulation. In addition to the increased consumption for Pro synthesis, the decrease in Glu pool size in nodules can also be explained by the decrease in GOGAT activity, which is the key enzyme of Glu biosynthesis in alfalfa nodules (Temple et al., 1998). In fact, this enzyme activity appears to be particularly sensitive to drought stress (Ramos et al., 1999).

Sugar and N exchange and ~~signaling~~signalling at the whole plant level

The accumulation of sucrose levels in nodules has been shown to be associated with the decrease in nodule sucrose synthase activity in soybean (Gordon et al., 1997) and pea (Galvez et al., 2005; Gonzalez et al., 1998). Recent studies suggest that the accumulation of sucrose in alfalfa nodules is caused by a still active import of sucrose from the shoot, together with a limitation of sucrose consumption within nodules due to the impairment of respiratory activity (Naya et al., 2007). Also, sucrose has been shown to play an important ~~osmoregularoty~~osmoregulatory role in *S. meliloti* (Gouffi et al., 1998). Dicarboxylic acids inherited from the host plant by bacteroids provide the main reduced carbon source (C skeletons) supporting N_2 fixation (Lodwig and Poole, 2003). Under ~~water stress~~WS conditions, where the dicarboxylate input from PR and shoots is limited (general decrease in the TCA pathway, see above), Pro synthesis in the nodule consumes a significant part of carbon skeletons available locally, and Pro can in turn become an alternative source of reduced C to bacteroid under more favorable conditions (Curtis et al., 2004). This simply explains why there was a general decrease in other aminoacids and organic acids in nodules in the WS treatment. In addition, Pro was unlikely to have been transported from leaves or roots since in that case~~,~~ it would have inherited a substantial ^{15}N signal~~, but~~. However, the fact that Pro was rather synthesized within the nodule, supports the assumption that symbiotic N_2 fixation under drought is mainly driven by local metabolism and thus, maybe, not controlled by a systemic N signal (Marino et al., 2007).

Elevated levels of nitrogenous compounds, including ureides and amino acids, have been proposed to play a role in the decline of symbiotic N_2 fixation in legumes in response to water deficit. For instance, the accumulation of free amino acids has been shown to be involved in the feedback inhibition of symbiotic N_2 fixation in alfalfa (Schubert et al., 1995) and soybean (Serraj et al., ~~1999~~1999b) subject to drought (King and Purcell, 2005). That is, nodule ureides and Asp, together with several amino acids in leaves, represent candidate molecules for feedback inhibition of symbiotic N_2 fixation

in alfalfa and soybean. However, in the present work, Asp declined in nodules, suggesting that other compounds could be play the role of feedback inhibitor, such as Pro (Curtis et al., 2004). In fact, Pro in nodules was likely synthesized locally (see above) and leaves did not accumulate Pro. The sole amino acid exhibiting a rather similar isotopic enrichment between leaves, roots and nodules was Ala, suggesting that Ala could be exchanged between plant organs. However, Ala levels did not change significantly under water deficit in nodules and therefore, its role in feedback inhibition is unlikely.

Asn is the main N-transporter in *M. sativa* (Groat and Vance, 1981; Vance et al., 1994). The decrease in Asn levels in nodules is likely originated from the decrease in N_2 fixation caused by water deficit. This might lead to a decrease in Asn export to the plant. However, contrary to expectations, Asn levels in roots and leaves did not decrease and furthermore, Asn was mostly enriched in PR but not in nodules. It thus appears more likely that PR synthesized Asn from N fixed before water stress treatment (T0) and that there was limited Asn exchange upon water deficit.

5. Conclusions

Using a combination of metabolomics and ^{15}N-~~labeling~~labelling, we could follow the metabolism of amino acids during water stress. Our data are in agreement with the assumption that N fixation in nodules is controlled locally. Although water deficit affected negatively photosynthetic activity, sucrose of circulating sugars did not change significantly in shoots, and thus photoassimilate limitation is unlikely to be the cause of decrease N_2 fixing activity. We rather suggest here that some amino acids, in particular Pro, could represent a candidate compounds exerting feedback inhibition on nodule activity when water deficit leads to a decline in nodule water potential. We nevertheless recognize that our study was limited to few amino acids in isotopic analyses and so the exchange of nitrogen found here was probably not fully representative. Further studies will be conducted with more sensitive techniques such as high resolution LC-MS that allow analysis of isotopic patterns.

Acknowledgements

The authors would like to acknowledge the comments and suggestions of Dr. Florence Baptist ~~which~~that were valuable in improving the quality of our manuscript.

Funding

This study was supported in part by the European research project PERMED (INCO-CT-2004-509140), the Spanish Ministry of Science and Technology (CGL2009-13079-C02) and i-COOP-*suelos y legumbres* (2016SU0016) funded by the National Research Council. GM was the recipient of a doctoral fellowship (*Programa Nacional de Formación de Personal Universitario*) sponsored by the Spanish Ministry of Education and Science.

Figure legends

Figure 1A and 1B. Variations in the metabolite profiles from apical shoots (AS), primary roots (PR) and nodules (Nod.) of *Medicago sativa* subjected to ~~drought.~~water deficit. Hierarchically clustered heat maps of the mean ~~centered~~centred-reduced values of metabolites that were found to be significantly different between organs at (A) well~~-~~-watered conditions (WW) and (B) water stress (WS). Three replicates are presented per organ. Intensity of *red* and *green* indicates increase and decrease relative to the mean, according to the ~~color~~colour scale at the top.

Figure 2. Hierarchically clustered heat maps of the mean ~~centered~~centred-reduced values of metabolites that were found to be significantly different between treatments (WW~~, well-watered~~ and WS~~, water stress~~) in apical shoots (AS), primary roots (PR) and nodules (Nod). Each column represents one replicate per organ and water regime. Intensity of *red* and *green* indicates increase and decrease relative to the mean, respectively, according to the ~~color~~colour scale at the top of the heat map.

Figure 3. Isotopomic representation of ^{15}N-enrichment in most relevant amino acids. Values were normalized with δ^{15}N-values of control plants. ~~0~~Zero means ^{15}N-enrichment of amino acids at first harvested (T0). Then, WW and WS is referred to plants 8 days after ~~labeling~~labelling (T8). Intensity of *red* is associated with higher ^{15}N-enrichment respect non~~-labeled~~-labelled plants. *Green* ~~color~~colour indicates few ~~labeling~~labelling respect control. Data are the mean of at least two replicates.

Figure 4. Mapping of metabolite concentrations obtained by GC-TOF~~-~~-MS onto plant biosynthetic pathways. Full bars represent ~~well-watered~~WW conditions whereas open bars represent ~~water stress~~WS. The first pair of bars correspond to apical shoots (AS WW and AS WS), the second pair of bars to primary roots (PR WW and PR WS), and the third pair correspond to nodules (Nod WW, Nod WS). Asterisks (*) represent significance between treatments by GC-TOF. [*] represent significance observed by HPLC in spite of GC-TOF did not detect differences. ~~The metabolites~~Metabolites, which were significant in Figure 3 but do not find linkage in the pathways shown in this figure, are phosphoric acid and monomethylphosphate: these compounds are not shown. Maleic acid could inhibit Krebs cycle enzymes as indicated in the figure.

References

Acquaah, G. 2012. Principles of Plant Genetics and Breeding. (Acquaah, G., Ed.) (Second Ed.). Wiley-Blackwell, Oxford, UK.

Annicchiarico P., Pecetti L., Abdelguerfi A., Bouizgaren A., Carroni A.M., Hayek T., … Mezni M. 2011. Adaptation of landrace and variety germplasm and selection strategies for lucerne in the Mediterranean basin. Field Crops Research 120, 283–291.

Annicchiarico, P., Pecetti, L., Tava, A. 2013. Physiological and morphological traits associated with adaptation of lucerne (*Medicago sativa*) to severely drought-stressed and to irrigated environments. Annals of Applied Biology 162: 27-40.

Annicchiarico P., Barrett B., Brummer E.C., Julier B., Marshall A.H. 2015. Achievements and Challenges in Improving Temperate Perennial Forage Legumes. Critical Reviews in Plant Sciences 34, 327–380.

Aranjuelo, I., Molero, G., Erice, G., Avice, J.C., Nogués, S. 2011. Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.) Journal of Experimental Botany 62, 111-123.

Aranjuelo, I., Molero, G., Erice, G., Aldasoro, J., Arrese-Igor, C., Nogués, S. ~~2014~~2014a. Effect of shoot on remobilization of carbon and nitrogen during regrowth of nitrogen-fixing alfalfa. Physiologia Plantarum 153: 91-104.

~~Araus, J.L. 2004. The problems of sustainable water use in the Mediterranean and research requirements for agriculture. Annals of Applied Biology 144, 259-272.~~

~~Araus, J.L., Slafer, G.A., Reynolds, M.P., Royo, C. 2002. Plant breeding and drought in C3 cereals: What should we breed for? Annals of Botany 89, 925-940.~~

~~Araus, J.L., Slafer, G.A., Royo, C., Serret, M.D. 2008. Breeding for yield potential and stress adaptation in cereals. Critical Reviews in Plant Sciences 27, 377-412.~~

Aranjuelo I, Arrese-Igor C, Molero G. 2014b. Nodule performance within a changing environmental context. Journal of Plant Physiology 171, 1076–1090.

Avice, J.C., Ourry, A., Lemaire, G., Boucaud, J. 1996. Nitrogen and carbon flows estimated by N-15 and C-13 pulse-chase labeling during regrowth of alfalfa. Plant Physiology 112, 281-290.

Avice, J.C., Le Dily, F., Goulas, E., Noquet, C., Meuriot, F., Volenec, J.J. 2003. Vegetative storage proteins in overwintering storage organs of forage legumes: roles and regulation. Canadian Journal of Botany; 81:1198–212.

Bacanamwo, M., Harper, J.E. 1997. The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. Physiologia Plantarum 100, 371-377.

Ball, R.A., Oosterhuis, D.M. 2005. Measurement of root and leaf osmotic potential using the vapor-pressure osmometer. Environmental and Experimental Botany 53, 77-84.

Bathellier, C., Tcherkez, G., Mauve, C., Bligny, R., Gout, E., Ghashghaie J. 2009. On the resilience of nitrogen assimilation by intact roots under starvation, as revealed by

isotopic and metabolomic techniques. Rapid Communications in Mass Spectrometry 23, 2847-2856.

Bewley, J.D. 2002. Root storage proteins, with particular reference to taproots. Canadian Journal of Botany 80, 321-329.

Burns, R.C., Hardy, R.W.F. 1975. Nitrogen fixation in bacteria and higher plants. Molecular Biology, Biochemistry and Biophysics 21, 39–60.

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A.M., Francia, E., Marè. C., Tondelli, A., Stanca, A. M. 2008. ~~Castellanos, M.I., Chauvet, A., Deschamps, A., Barreau, C. 1996. PCR methods for identification and specific detection of probiotic lactic acid bacteria. Current Microbiology 33, 100-103.~~

Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. Field Crops Research 105, 1–14.

Cornic, G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture - not by affecting ATP synthesis. Trends in Plant Science 5, 187-188.

Corre, N., Bouchart, V., Ourry, A., Boucaud, J. 1996. Mobilization of nitrogen reserves during regrowth of defoliatied *Trifolium repens* L., and identification of potential vegetative storage proteins. Journal of Experimental Botany 301, 1111-1118.

Curtis, J., Shearer, G., Kohl, D.H. 2004. Bacteroid proline catabolism affects N-2 fixation rate of drought-stressed soybeans. Plant Physiology 136, 3313-3318.

~~Delauney, A.J., Hu, C.A.A., Kishor, P.B.K., Verma, D.P.S. 1993. Cloning of Ornithine Delta-Aminotransferase Cdna from Vigna-Aconitifolia by Transcomplementation in Escherichia-Coli and Regulation of Proline Biosynthesis. Journal of Biological Chemistry 268, 18673-18678.~~

Erice, G., Irigoyen, J.J., Sánchez-Díaz, M., Avice, J.C., Ourry A. 2007. Effect of drought, elevated CO_2 and temperature on accumulation of N and vegetative storage proteins (VSP) in taproot of nodulated alfalfa before and after cutting. Plant Science 179, 903-912.

Ethier, G.J., Livingston, N.J. 2004. On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. Plant Cell and Environment 27, 137-153.

Evans, R.C., Garraway, M.O. 1984. Effect of Nitrogen-Source and Vitamins on Ethanol and Pyruvate Production by Bipolaris-Maydis Race-T. Mycologia 76, 515-522.

Farquhar, G.D., Sharkey, T.D. 1982. Stomatal Conductance and Photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 33, 317-345.

Flexas, J., Medrano, H. 2002. Drought-inhibition of photosynthesis in C-3 plants, Stomatal and non-stomatal limitations revisited. Annals of Botany 89, 183-189.

Fougère, F., Lerudulier, D., Streeter, J.G. 1991. Effects of Salt Stress on Amino-Acid, Organic-Acid, and Carbohydrate-Composition of Roots, Bacteroids, and Cytosol of Alfalfa (*Medicago* ~~*Sativa*~~ *sativa* L). Plant Physiology 96, 1228-1236.

Galvez, L., Gonzalez, E.M., Arrese-Igor, C. 2005. Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. Journal of Experimental Botany 56, 2551-2561.

Gana, J.A., Kalengamaliro, N.E., Cunningham, S.M., Volenec, J.J. 1998. Expression of β-amylase from alfalfa taproots. Plant Physiology 118, 1495-1506.

Gonzalez, E.M., Aparicio-Tejo, P.M., Gordon, A.J., Minchin, F.R., Royuela, M., Arrese-Igor, C. 1998. Water-deficit effects on carbon and nitrogen metabolism of pea nodules. Journal of Experimental Botany 49, 1705-1714.

Gordon, A.J., Minchin, F.R., Skot, L., James, C.L. 1997. Stress-induced declines in soybean N-2 fixation are related to nodule sucrose synthase activity. Plant Physiology 114, 937-946.

Gouffi, K., Pichereau, V., Rolland, J.P., Thomas, D., Bernard, T., Blanco, C. 1998. Sucrose is a ~~nonaccumulated~~non-accumulated osmoprotectant in *Sinorhizobium meliloti*. Journal of Bacteriology 180, 5044-5051.

Groat, R.G., Vance, C.P. 1981. Root Nodule Enzymes of Ammonia Assimilation in Alfalfa (*Medicago-Sativa* L) - Developmental Patterns and Response to Applied Nitrogen. Plant Physiology 67, 1198-1203.

Hare, P.D., Cress, W.A., Van Staden J. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell and Environment 21, 535-553.

Hendershot, K.L., Volenec, J.J. 1993. Taproot nitrogen accumulation and use in overwintering alfalfa (*Medicago sativa* L.). Journal of Plant Physiology 141, 68-74.

Hoagland, D.R., Arnon, D.I. 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347, 1–32.

Irigoyen, J.J., Emerich, D.W., Sanchez-Diaz, M. 1992. Alfalfa Leaf Senescence Induced by Drought Stress - Photosynthesis, Hydrogen-Peroxide Metabolism, Lipid-Peroxidation and Ethylene Evolution. Physiologia Plantarum 84, 67-72.

Kim, T.H., Ourry, A., Boucaud, J., Lemaire, G. 1991. Changes in the source-sink relationship for nitrogen during regrowth of lucerne (*Medicago sativa* L.) following removal of shoots. Australian Journal of Plant Physiology 18, 593-602.

Kim, T.H., Ourry, A., Boucaud, J., Lemaire, G. 1993. Partitioning of nitrogen derived from N_2 fixation and reserves in nodulated *Medicago sativa* L. during regrowth. Journal of experimental Botany 44, 555-562.

King, C.A., Purcell, L.C. 2005. Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiology 137, 1389-1396.

Kohl, D.H., Straub, P.F., Shearer, G. 1994. Does Proline Play A Special Role in Bacteroid Metabolism~~.~~? Plant Cell and Environment 17, 1257-1262.

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., Ladrera, R., Arrese-Igor, C., Weckwerth, W., Gonzalez, E.M. 2009. Carbon Metabolism and Bacteroid Functioning Are Involved in the Regulation of Nitrogen Fixation in *Medicago truncatula* Under Drought and Recovery. Molecular Plant-Microbe Interactions 22, 1565-1576.

Lawlor, D.W. 2002. Limitation to photosynthesis in water-stressed leaves, Stomata vs. metabolism and the role of ATP. Annals of Botany 89, 871-885.

Lluch, C., Carrero, J.J., Tejera, N., Ocaña, A. 2002. Interacción planta-microorganismo del suelo, simbiosis Fijadoras de Nitrógeno. In Ecofisiología Vegetal, eds. Reigosa JM, Pedrol N, Sánchez A Editorial, Paraninfo, pps. 331-360, Madrid

Lobell, D.B., Burke, M.B., Tebaldi, C., Mastrandrea, M.D., Falcon, W.P., Naylor, R.L. 2008. Prioritizing climate change adaptation needs for food security in 2030. Science 319, 607-610.

Lodwig, E., Poole, P. 2003. Metabolism of Rhizobium bacteroids. Critical Reviews in Plant Sciences 22, 37-78.

Lodwig, E.M., Hosie, A.H.F., Bordes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J.A., Poole, P.S. 2003. Amino-acid cycling drives nitrogen fixation in the legume - Rhizobium symbiosis. Nature 422, 722-726.

Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., Gonzalez, E.M. 2007. Nitrogen fixation control under drought stress. Localized or systemic? Plant Physiology 143, 1968-1974.

Martinelli, T., Whittaker, A., Masclaux-Daubresse C., Farrant, J.M., Brilli, F., Loreto, F., Vazzana, C. 2007. Evidence for the presence of photorespiration in desiccation-sensitive leaves of the C-4 'resurrection' plant *Sporobolus stapfianus* during dehydration stress. Journal of Experimental Botany 58, 3929-3939.

McNeill, A.M., Hood, R.C., Wood, M. 1994. Direct measurement of nitrogen fixation by *Trifolium-repens* L and *Alnus-glutinosa* L using $^{15}N_2$. Journal of Experimental Botany 45, 749-755

Medrano, H., Escalona, J.M., Bota, J., Gulias, J., Flexas, J. 2002. Regulation of photosynthesis of C-3 plants in response to progressive drought, Stomatal conductance as a reference parameter. Annals of Botany 89, 895-905.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell and Environment 33, 453–467.

Molero, G., Aranjuelo, I., Teixidor, P., Araus, J.L. Nogués, S. 2011. Measurement of ^{13}C and ^{15}N isotope labeling by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry to study amino acid fluxes in a symbiotic plant - microbe association. Rapid Communications in Mass Spectrometry. In press 25, 599-607.

Nanni, V., Paolini, M., Innocenti, A., Mulè, P., Vargiu, M. 2014. Abstract Evaluation of dormancy and winter hardiness of alfalfa *(Medicago sativa* L.) experimental lines, obtained by crossing parental lines characterized within the framework of the PERMED project. *In Quantitative traits breeding for multifunctional grasslands and turf.* (eds D. Sokolović, C. Huyghe & J. Radović), Springer, Dordrecht, the Netherlands.

Naya, L., Ladrera, R., Ramos, J., Gonzalez, E.M., Arrese-Igor, C., Minchin, F.R., Becana, M. 2007. The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiology 144, 1104-1114.

Neo, H.H., Layzell, D.B. 1997. Phloem glutamine and the regulation of O-2 diffusion in legume nodules. Plant Physiology 113, 259-267.

Nogués, S., Baker N.R. 2000. Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation. Journal of Experimental Botany 51, 1309- 1317.

Novitskaya, L., Trevanion, S.J., Driscoll, S., Foyer, C.H., Noctor, G. 2002. How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. Plant Cell and Environment 25, 821-835.

Ourry, A., Kim, T.H., Boucaud, J. 1994. Nitrogen reserve mobilization during regrowth of *Medicago sativa* L., relationships between their availability and regrowth yield. Plant Physiology 105, 831-837.

Ourry, A., MacDuff, J., Volenec, J.J., Gaudillère, J.P. 2001. Nitrogen traffic during plant growth and development. In, Lea PJ, Morot-Gaudry JF. (Eds), Plant Nitrogen. Springer Verlag. pp 255-273.

~~Passioura, J.B. 2002. Environmental biology and crop improvement. Functional Plant Biology 29, 537-546.~~

Prell, J., Poole, P. 2006. Metabolic changes of rhizobia in legume nodules. Trends in Microbiology 14, 161-168.

Ramos, M.L.G., Gordon, A.J., Minchin, F.R., Sprent, J.I., Parsons, R. 1999. Effect of water stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris* L.). Annals of Botany 83, 57-63.

Saeed, A., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J. 2003. TM4, A free, open-source system for microarray data management and analysis. BioTechniques 34, 374-378.

Salon, C., Avice, J.C., Colombie, S., Dieuaide-Noubhani, M., Gallardo, K., Jeudy, C., Ourry, A., Prudent, M~~,~~, Voisinm A., Rolin, D. 2017. Fluxomics links cellular functional analyses to whole-plant phenotyping. Journal of Experimental Botany 68, 2083-2098.

Schobert, B., Tschesche, H. 1978. Unusual Solution Properties of Proline and Its Interaction with Proteins. Biochimica et Biophysica Acta 541, 270-277.

Schubert, S., Serraj, R., Pliesbalzer, E., Mengel, K. 1995. Effect of Drought Stress on Growth, Sugar Concentrations and Amino-Acid Accumulation in N-2-Fixing Alfalfa (*Medicago Sativa*). Journal of Plant Physiology 146, 541-546.

Serraj, R., Sinclair, T.R., Purcell, L.C. 1999a. Symbiotic N2 fixation response to drought. Journal of Experimental Botany 50, 143–155.

Serraj, R., Vadez, V., Denison, R.F., Sinclair, T.R. ~~1999~~1999b. Involvement of ureides in nitrogen fixation inhibition in soybean. Plant Physiology 119, 289-296.

Serraj, R., Vadez, V., Sinclair, T.R. 2001. Feedback regulation of symbiotic N-2 fixation under drought stress. Agronomie 21, 621-626.

~~Sprent, J.I. 2001. Nodulation in legumes. Royal Botanic Gardens, Kew, U.K.~~

Sheaffer, C. C., Tanner, C. B. & Kirkham, M. B. 1988. Alfalfa water relations and irrigation. In: Hanson, A.A., Barnes, D.K. & Hill, R.R., Jr. eds. Alfalfa and alfalfa improvement. Madison, Wisconsin: American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Vol. 29, 373-409.

Steunou, A.S., Jensen, S.I., Brecht, E., Becraft, E.D., Bateson, M.M., Kilian, O., Bhaya, D., Ward, D.M., Peters, J.W., Grossman, A.R., Kuhl, M. 2008. Regulation of nif gene expression and the energetics of N-2 fixation over the diel cycle in a hot spring microbial mat. Isme Journal 2, 364-378.

~~Stitt, M., Muller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., Scheible, W.R., Krapp, A. 2002. Steps towards an integrated view of nitrogen metabolism. Journal of Experimental Botany 53, 959-970.~~

Streeter, J.G. 1987. Effect of Nitrate on the Organic-Acid and Amino-Acid-Composition of Legume Nodules. Plant Physiology 85, 774-779.

Streeter, J.G., Lohnes, D.G., Fioritto, R.J. 2001. Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. Plant Cell and Environment 24, 429-438.

Ta, T.C., MacDowall, F.D.H., Frais, M.A. 1990. Utilization of carbon and nitrogen reserves of alfalfa roots in supporting N_2-fixation and shoot regrowth. Plant Soil. 127, 231-236.

Tardieu, F., Cabrera-Bosquet, L., Pridmore, T., Bennett, M. 2017 Plant Phenomics, From Sensors to Knowledge. Current Biology 27, R770-R783.

Temple, S.J., Vance, C.P., Gantt, J.S. 1998. Glutamate synthase and nitrogen assimilation. Trends in Plant Science 3, 51-56.

The World Bank. 2007. World development report 2008. Agriculture for development. Washington DC. http,//siteresources.worldbank.org/ INTWDR2008/Resources/ WDR_00_book.pdf. 364 pp

Thomas, Robertson, M.J, Fukai, S., Peoples, M.B. 2004. The effect of timing and severity of water deficit on growth, development, yield accumulation and nitrogen fixation of mungbean. Field Crops Research 86, 67-80.

Udvardi, M.K., Day, D.A. 1997. Metabolite transport across symbiotic membranes of legume nodules. Annual Review of Plant Physiology and Plant Molecular Biology 48, 493-523.

Vance, C.P., Gregerson, R.G., Robinson, D.L., Miller, S.S., Gantt, J.S. 1994. Primary Assimilation of Nitrogen in Alfalfa Nodules - Molecular-Features of the Enzymes Involved. Plant Science 101, 51-64.

Volenec, J.J., Ourry, A., Joern, B.C. 1996. A role for nitrogen reserves in forage regrowth and stress tolerance. ~~Physiol.~~Physiologia Plantarum 97, 185-193.

Von Caemmerer, S., Farquhar, G.D. 1981. Some ~~Relationships Between~~relationships between the ~~Biochemistry~~biochemistry of ~~Photosynthesis~~photosynthesis and the ~~Gas Exchange~~gas exchange of *Phaseolus* ~~Vulgaris~~ *vulgaris* Cultivar Hawkesbury-Wonder Leaves. Planta (Heidelberg) 153, 376-387.

Walsh, K.B. 1995. Physiology of the Legume Nodule and Its Response to Stress. Soil Biology & Biochemistry 27, 637-655.

Weatherley, P. 1950. Studies in the water relations of the cotton plant. 1. The field measurement of water deficits in leaves. New ~~Phytol~~Phytologist 49, 81-97.

Wingler, A., Quick, W.P., Bungard, R.A., Bailey, K.J., Lea, P.J., Leegood, R.C. 1999. The role of photorespiration during drought stress, an analysis utilizing barley mutants with reduced activities of photorespiratory enzymes. Plant Cell and Environment 22, 361-373.

Woo, K.L., Chang, D.K. 1993. Determination of 22 Protein Amino-Acids As N(O)-Tert-Butyldimethylsilyl Derivatives by Gas-Chromatography. Journal of Chromatography 638, 97-107.

Woo, K.L., Lee, D.S. 1995. Capillary Gas-Chromatographic Determination of Proteins and Biological Amino-Acids As N(O)-Tert-Butyldimethylsilyl Derivatives. Journal of Chromatography B-Biomedical Applications 665, 15-25.

Zahran, H.H. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews 63, 968-989.

Tables

Click here to download Tables: Tables (AGWAT10151).docx

Table 1. Plant growth, water status and N content of *Medicago* sativa plants at harvesting T8 under well-watered (WW) and water stressed conditions (WS). Gas exchange determinations (photosynthetic rates, A; Rubisco carboxylation maximum capacity, Vc_{max}; RuBP regeneration rate, J_{max}; stomatal limitation, l and stomatal conductance, g_s) were conducted at T8. The water availability effect is in terms of relative water content (RWC) and leaf osmotic potential (Ψ_s). Measurements were conducted at the end of the experiment, when plants were 3 months old. Data are the mean of at least 3 replicates. Within each water treatment values with asterisks are significantly different according to the *t*-test, $P<0.05$. †marginally significant.

	WW	WS
Plant growth		
Total biomass (g DW)	1.23	1.34
Apical shoot (g DW)	0.20	0.28
Primary root (g DW)	0.73	0.42†
Nodule (g DW)	0.03	0.08*
Apical shoot N (%)	6.2	5.1
Primary root N (%)	2.5	2.2
Nodule N (%)	6.9	6.0
Water status		
Leaf RWC (%)	87.3	79.9*
Apical shoot Ψs (MPa)	-1.12	-1.17
Primary root Ψs (MPa)	-0.99	-1.00
Nodule Ψs (MPa)	-1.33	-1.49*
Gas exchange		
A (µmol m^{-2} s^{-1})	19.85	14.90*
Vc_{max}(µmol m^{-2} s^{-1})	111.12	51.77*
J_{max} (µmol m^{-2} s^{-1})	195.59	87.34*
l (%)	18.12	31.53*
g_s (mmol H_2O m^{-2} s^{-1})	0.36	0.23*

Table 2. Quantification (nmol/gDW) of amino acids in apical shoots, primary roots and nodule of *M. sativa* under well-watered (WW) and water stressed conditions (WS) determined by HPLC. Data are the mean of at least 3 replicates. Within each water treatment values with asterisks are significantly different according to the *t*-test, $P<0.05$. †marginally significant.

	Apical shoots		Primary roots		Nodules	
	WW	WS	WW	WS	WW	WS
Ala	9.5	9.5	3.6	11.5	91.0	54.8
Arg	0.5	0.6	3.4	2.7	85.3	29.8*
Asn	53.9	48.8	45.9	139.1	1489.6	470.9*
Asp	9.0	3.6*	5.6	12.4	37.1	19.6
β-Ala	0.8	1.0	0.3	0.9	4.0	2.9
GABA	1.9	2.4	3.0	9.9*	27.2	24.5
Gln	5.3	3.8	1.2	2.8*	26.3	10.2*
Glu	34.7	21.1*	6.0	12.4†	131.8	71.3*
Gly	1.1	1.6	1.2	2.3	15.2	8.9*
hSer	1.7	1.9	0.8	1.3	4.9	3.0
Ile	0.5	1.8	1.1	2.4	17.8	10.1
Leu	1.0	1.9	1.0	2.0	14.6	8.8
Lys	0.5	1.2	0.7	1.1	20.9	10.8†
Met	0.4	0.3	0.1	0.4	3.2	1.6*
Orn	0.3	0.2*	0.2	0.4	2.4	0.8*
Phe	1.3	1.2	0.5	0.9	17.7	8.0
Ser	15.7	24.1*	3.8	11.5	33.7	20.8*
Thr	2.6	4.9*	2.2	5.1	20.9	13.2*
Trp	0.2	0.7	2.8	5.4*	39.5	22.3
Val	1.6	3.3	1.7	4.7	25.1	18.0

Figure

Figure 1.

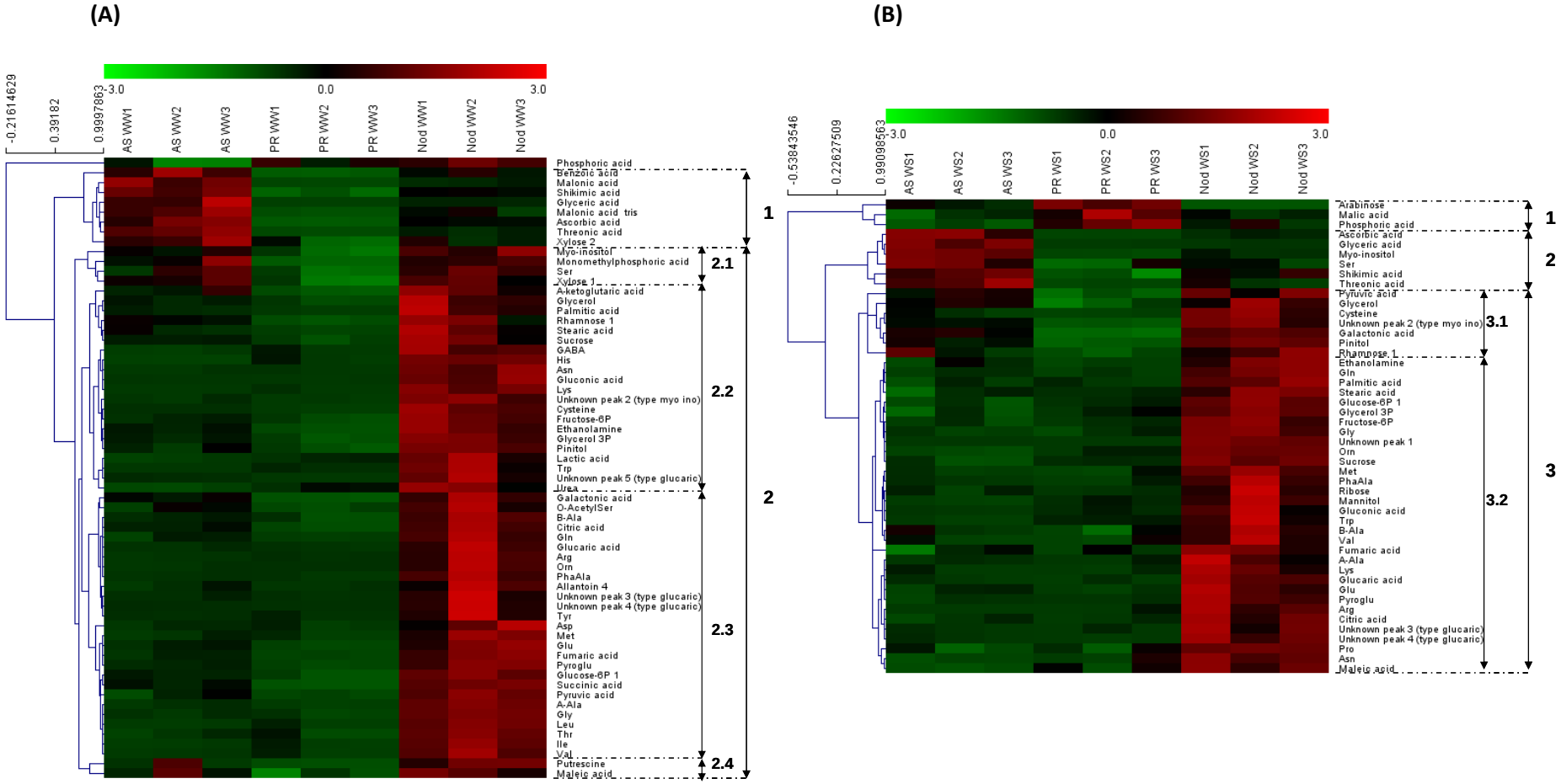


Figure 2.

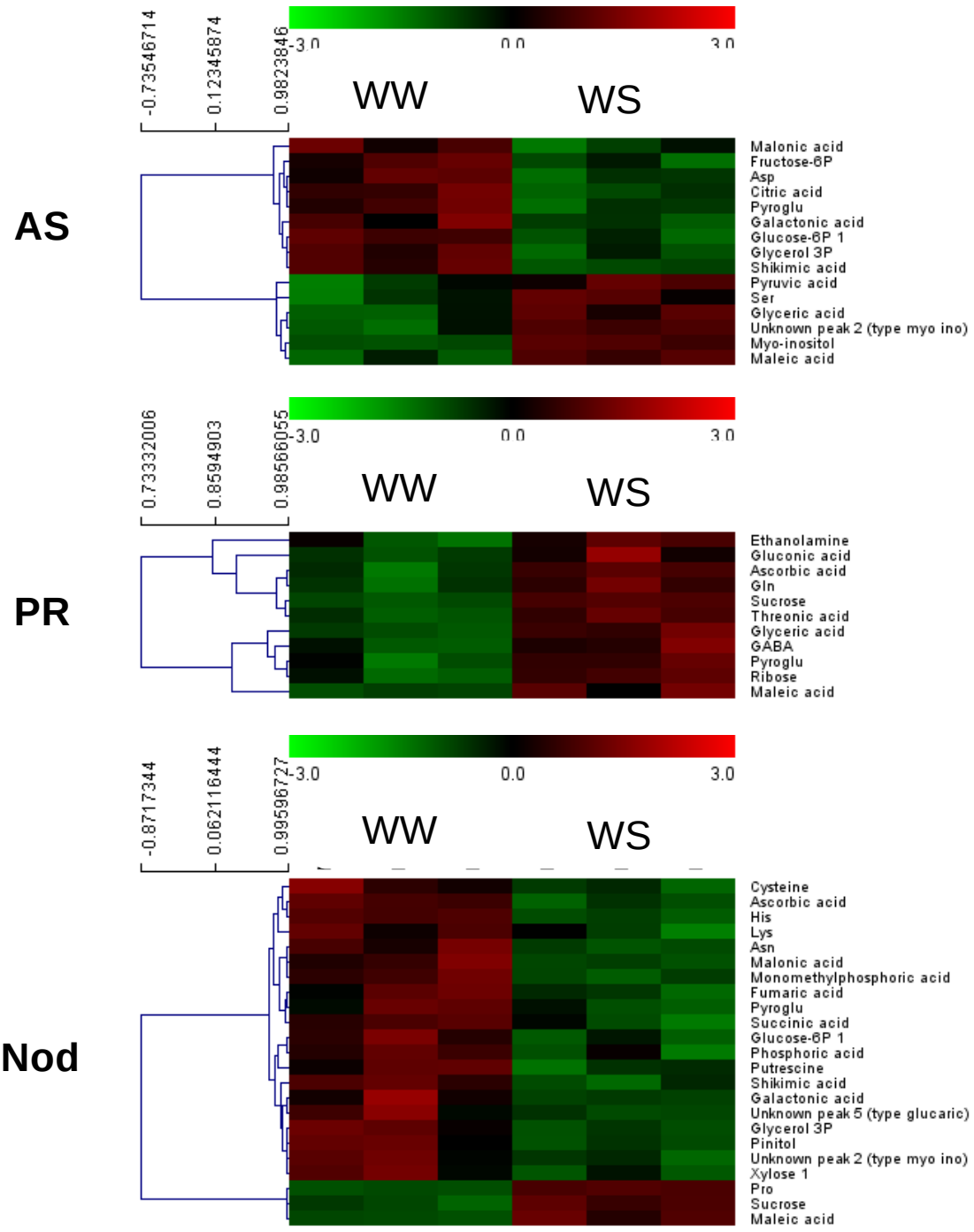

AS
-0.73546714
0.12345874
0.9823846
-3.0
0.0
3.0
WW
WS
Malonic acid
Fructose-6P
Asp
Citric acid
Pyroglu
Galactonic acid
Glucose-6P 1
Glycerol 3P
Shikimic acid
Pyruvic acid
Ser
Glyceric acid
Unknown peak 2 (type myo ino)
Myo-inositol
Maleic acid
PR
0.73332006
0.8594903
0.98566055
-3.0
0.0
3.0
WW
WS
Ethanolamine
Gluconic acid
Ascorbic acid
Gln
Sucrose
Threonic acid
Glyceric acid
GABA
Pyroglu
Ribose
Maleic acid
Nod
-0.8717344
0.062116444
0.99596727
-3.0
0.0
3.0
WW
WS
Cysteine
Ascorbic acid
His
Lys
Asn
Malonic acid
Monomethylphosphoric acid
Fumaric acid
Pyroglu
Succinic acid
Glucose-6P 1
Phosphoric acid
Putrescine
Shikimic acid
Galactonic acid
Unknown peak 5 (type glucaric)
Glycerol 3P
Pinitol
Unknown peak 2 (type myo ino)
Xylose 1
Pro
Sucrose
Maleic acid

Figure 3.

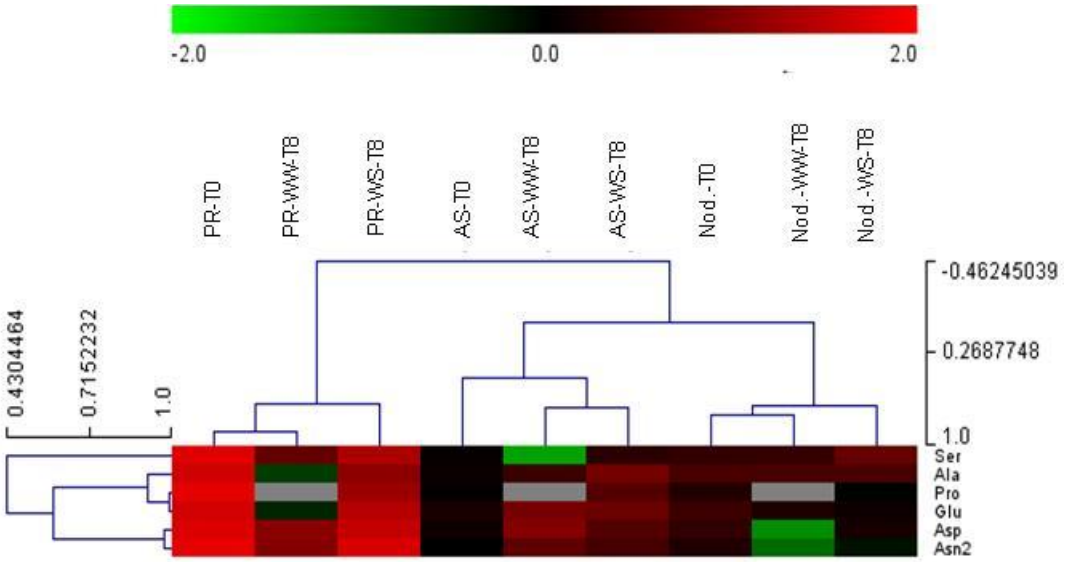
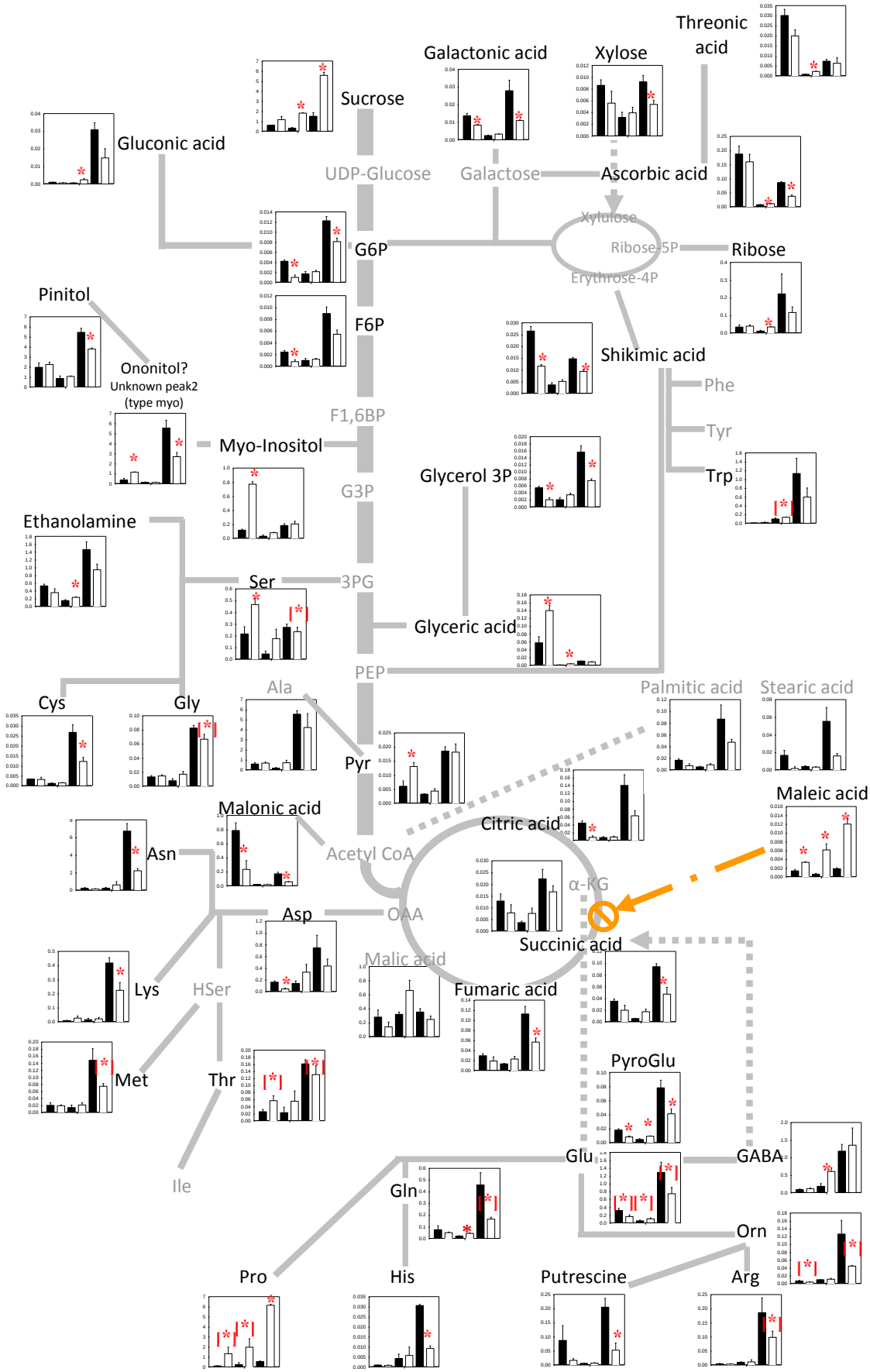

-2.0
0.0
2.0
PR-T0
PR-WW-T8
PR-WS-T8
AS-T0
AS-WW-T8
AS-WS-T8
Nod.-T0
Nod.-WW-T8
Nod.-WS-T8
0.4304464
0.7152232
1.0
-0.46245039
0.2687748
1.0
Ser
Ala
Pro
Glu
Asp
Asn2

Figure 4.


Threonic acid
Galactonic acid
Xylose
Sucrose
Gluconic acid
UDP-Glucose
Galactose
Ascorbic acid
Xylulose
Ribose-5P
Ribose
G6P
Erythrose-4P
Pinitol
F6P
Shikimic acid
Ononitol?
Unknown peak2
(type myo)
Phe
F1,6BP
Tyr
Myo-Inositol
Trp
Glycerol 3P
G3P
Ethanolamine
Ser
3PG
Glyceric acid
PEP
Cys
Gly
Ala
Palmitic acid
Stearic acid
Pyr
Maleic acid
Malonic acid
Citric acid
Asn
Acetyl CoA
α-KG
Asp
OAA
Succinic acid
Malic acid
Lys
HSer
Fumaric acid
PyroGlu
Met
Thr
Glu
GABA
Ile
Gln
Orn
Pro
His
Putrescine
Arg

Do metabolic changes underpin physiological responses to water limitation in alfalfa (*Medicago sativa*) plants during a regrowth period?

Gemma Molero[1,2], Guillaume Tcherkez[3,] Regina Roca[4], Caroline Mauve[5], Llorenç Cabrera-Bosquet[2,6], José Luis Araus[2], Salvador Nogués[2] and Iker Aranjuelo[7]

[1]International Maize and Wheat Improvement Center (CIMMYT), Km. 45, Carretera Mexico-Veracruz, El Batán, Texcoco CP 56237, Mexico;

[2]Secció de Fisologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain;

[3]Research School of Biology, ANU College of Science, Australian National University, 2601 Canberra ACT, Australia

[4]Serveis Científico-Tècnics, Universitat de Barcelona, Barcelona, Spain;

[5]Plateforme Métabolisme-Métabolome, Université Paris Sud-XI, Orsay, France;

[6]LEPSE, INRA, Montpellier SupAgro, Univ Montpellier, Montpellier, France;

[7]Agrobiotechnology Institute (IdAB), CSIC-UPNA-Gobierno de Navarra, Mutilva, Spain.

*__Corresponding author__: Gemma Molero Milán, International Maize and Wheat Improvement Center (CIMMYT), Km. 45, Carretera Mexico-Veracruz, El Batán, Texcoco CP 56237, Mexico. Phone number: +52 (55) 5804 2004, email: G.Molero@cgiar.org

Running title: Water stress impact on the metabolism of nodulated alfalfa plants.

Submission date: August 30th 2017

Resubmission date: February 2nd 2018

Number of tables: 2

Number of figures: 4 (1 and 3 colour in print)

Total Word count: 9247

Abstract

Drought is one of the most limiting factors on crop productivity under Mediterranean conditions, where the leguminous species alfalfa (*Medicago sativa* L.) is extensively cultivated. Whereas the effect of drought on plant performance has been widely described at leaf and nodule levels, less attention has been given to plant-nodule interactions and their implication on metabolites exchange during a regrowth period, when water is limiting. For this purpose, physiological characterization and metabolite profiles in different plant organs and nodules were undertaken under water deficit, including regrowth after removal of aerial parts. In order to study in more detail how nitrogen (N) metabolism was affected by water stress, plants were labelled with N-enriched isotopic air ($^{15}N_2$) using especially designed chambers. Water stress affected negatively water status and photosynthetic machinery. Metabolite profile and isotopic composition analyses revealed that, water deficit induced major changes in the accumulation of amino acids (proline, asparagine, histidine, lysine and cysteine), carbohydrates (sucrose, xylose and pinitol) and organic acids (fumarate, succinate and maleic acid) in the nodules in comparison with other organs. The lower ^{15}N-labeling observed in serine, compared with other amino acids, was related with its high turnover rate, which in turn, indicates its potential implication in photorespiration. Isotopic analysis of amino acids also revealed that proline synthesis in the nodule was a local response to water stress and not associated with a feedback inhibition from the leaves.. Water deficit induced extensive reprogramming of whole-plant C and N metabolism, including when the aerial part was removed to trigger regrowth.

Key words: Alfalfa, water stress, metabolite profile, physiology, ^{15}N-labeling

Abbreviations:

A, Photosynthetic assimilation; *Arg*, arginine; *AS*, apical shoots; *Asn*, asparagine; *BNF*, biological nitrogen fixation; *C*, carbon; C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; *DW*, dry weigh; *E*, leaf transpiration rate; *F6P*, fructose-6-phosphate; *G6P*, glucose-6-phosphate; *GABA*, gamma-aminobutyric acid; *GC-C-IRMS*, gas chromatography combustion isotope ratio mass spectrometry; *GC-TOF–MS*, gas chromatography with time-of-flight mass spectrometry; *Gln*, glutamine; g_s, leaf stomatal conductance; *Glu*, glutamate; *Gly*, glycine; J_{max}, maximum electron transport rate contributing to RuBP regeneration; *HPLC*, high performance liquid chromatography; *I*, stomatal limitation; Lys, lysine; Met, methionine; *MEV*, TIGR multi experiment viewer; *MSTFA*, N-methyl- N(trimethylsilyl)trifluoroacetamide; *N*, nitrogen; *Nod*, nodule; *OPA*, *o*-pthaldialdehyde; *Orn*, ornithine; *Pro*, proline; *PPFD*, photosynthetic photon flux density; *PR*, primary roots; *RI*, retention index; *RWC*, relative water content; *Ser*, serine; *T0*, first harvest; *T8*, second harvest;

TFA, Trifluoracetic acid; *Thr*, threonine; *TOM*, total organic matter; *Trp*, tryptophan V_{cmax}, carboxylation velocity of Rubisco; *VSP*, vegetative storage proteins; *WW*, well-watered, *WS*, water stress; Ψ_s, osmotic potential.

1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the forage crops most extensively cultivated in the Mediterranean region (Annicchiarico et al., 2011, 2015). Alfalfa is a temperate forage frequently exposed to abiotic stresses such as low water availability and high temperature conditions (Walsh, 1995). It is estimated that approximately 70% of yield reduction worldwide is the direct result of environmental stresses (Acquaah, 2012), where drought is considered the main environmental stress in agriculture (Cattivelli et al. 2008). In legumes, water limitation can reduce global N_2 fixation by up to 17 Gt N $year^{-1}$ (Burns and Hardy, 1975). Under drought conditions, alfalfa has a strategy of avoidance by stopping its vegetative growth and accessing water through its deep root system but in general has poor drought resistance and is rapidly affected by water shortage (Sheaffer et al., 1988) resulting in a decrease in yield depending on the severity and duration of drought stress. Alfalfa, similarly other forages, is frequently subjected to above ground organs cutting for animal feeding. Such cutting causes important modifications in carbon (C) and nitrogen (N) metabolism (Aranjuelo et al., 2014a) at the different organ levels. During this period, shoot removal requires the mobilization of C and N reserves from roots to shoots (Avice et al., 2003; Aranjuelo et al., 2014a), which means an inversion of source and sink organs due to the disappearance of aerial source organs and the formation of new sinks with developing shoots. Abiotic conditions that limit water availability after shoot removal can have significant effects on the dynamics of regrowth (Erice et al., 2007).

Alfalfa is a forage legume that stablishes a plant-rhizobia interaction in which plant photosynthesis supplies C to nodules, where it is used by the nitrogenase enzyme in the bacteroid as a source of energy and reducing power to fix nitrogen gas (N_2) (Streeter, 1987). On the other hand, the products of N_2 fixation, either amides or ureids, are exported to the plant via the xylem (Schubert et al., 1995) where they are used for the synthesis of proteins, secondary products and compounds involved in osmotic adjustment under stressful conditions (Fougère et al., 1991).

Whereas the general effects of drought on leaf gas-exchange in forages (Cornic, 2000; Lawlor, 2002; Aranjuelo et al., 2011) and on the sensitivity of plant-bacteria symbiosis have been extensively studied (Aranjuelo et al., 2014b and references therein), relatively little is known about the effect of water availability in plant-nodule interactions and its implications in plant functioning and metabolites exchange during a regrowth period. Indeed, some authors reported that the effect of water deficit on plant

performance is associated with the deleterious impact of drought on N_2 fixation rather than on photosynthesis itself (Serraj et al., 1999a; Thomas et al., 2004). Previous studies reveal that biological nitrogen fixation (BNF) under drought condition is affected by (1) C supply to nodules (Galvez et al., 2005; Larrainzar et al., 2009); (2) respiration decrease and the resulting lower oxygen (O_2) consumption may locally inhibit nitrogenase activity (Galvez et al., 2005; Aranjuelo et al. 2011) and (3) the accumulation in the nodule of N compounds can induce a feedback mechanism (Serraj et al., 1999b). Several molecules like glutamine (Gln) (Neo and Layzell, 1997), ureides (Serraj et al., 2001), and asparagine (Asn) have been suggested to be involved in such a mechanism (Bacanamwo and Harper, 1997).

In alfalfa plants, Asn, together with ammonia, is the major organic N compound transported to the plant from the nodule (Groat and Vance, 1981). Some amino acids can be further transported back to the nodule from the shoots as a systemic signal for BNF regulation under drought conditions (Bacanamwo and Harper, 1997; King and Purcell, 2005; Neo and Layzell, 1997; Serraj et al., 2001). However, studies under drought conditions in pea suggest a local signal in addition to the systemic signal involved in BNF activity (Marino et al., 2007). Another point of controversy concerns the different sources of C required for amino acid synthesis. Although organic acids (mainly maleic acid and succinate) represent an important pool of C skeletons in the bacteroid (Lodwig and Poole, 2003), other studies suggest that some amino acids, like glutamate (Glu), Gln, glycine (Gly), proline (Pro) and tryptophane (Trp), can also be remobilized and thus represent an alternative source of C and energy to nodules (Kohl et al., 1994; Udvardi and Day, 1997; Molero et al. 2011). However, Prell and Poole, (2006 suggested that amino acid supply to the bacteroid appears to be related to the synthesis of alanine (Ala) and aspartate (Asp). Disparities amongst results highlight the current uncertainties on the role of amino acids in nodule metabolism and their partitioning through the plant, particularly under drought conditions (Lodwig and Poole, 2003; Lodwig et al., 2003). Some studies suggest that regrowth after shoot removal may be more dependent on the availability of N reserves rather than of C reserves (Avice et al., 1996; Kim et al., 1993; Ourry et al., 1994; Volenec et al., 1996).

Thus, understanding the exchange of C and N metabolites between plant and nodules is of prime importance, especially under water deficit conditions. Fluxomics (*i.e.*the study of the concentration and fluxes of metabolites in an organism) and isotopic tracing can provide insightful information about how different metabolites are exchanged and transferred in a biological system (Tardieu et al 2017; Salon et al

2017). The study of plant metabolites can therefore provide new insights on how specific processes involved in C and N metabolism may confer a better tolerance to water limitation in a context where the aerial part has been removed and, therefore, limiting the C supply to the nodule.

The objective of this study was to identify possible target specific compounds (soluble sugars, organic acids and amino acids) that may be involved in controlling plant performance during a regrowth period under drought conditions, by taking advantage of physiological and isotopic measurements. Here, we focused on the characterization of water availability effects in different organs (leaves, roots and nodules) and carried out metabolic analysis. N-enriched isotopic air ($^{15}N_2$) was used as labelling gas and enabled us to study N fixation in total organic matter (TOM) and individual amino acids and N exchange between different organs.

2. Material and methods

2.1. Experimental design and water status

The alfalfa (*Medicago sativa* L) cultivar Demnat from Morocco, identified as well adaptated to frequent cuts under warm and irrigated conditions (Annicchiarico et al., 2013; Nanni et al., 2014), was selected for the study. Seeds were surface sterilized in 10% commercial bleach for 30 min., and rinsed three times with deionized water. Sterilized seeds were germinated on Petri dishes and planted on 7L white plastic pots filled with sand. Plants were grown at 25/15ºC (day/night) with a photoperiod of 14 hours in growth chambers (Conviron E15, Controlled Environments ltd., Winnipeg, Canada) equipped with fluorescent lamps (SylvaniaDECOR183, Professional-58W, Germany) that provided a photosynthetic photon flux density (PPFD) of *ca*. 400 µmol m^{-2} s^{-1}. During the first month, plants were inoculated three times a week with 3mL (per plant) of a sucrose solution at 2% containing *Sinorhizobium meliloti* strain 102F78 that was resuspended from agar media. Plants were watered twice a week with Hoagland N-free nutrient solution (Hoagland and Arnon 1950) and once a week with deionized water to avoid salt accumulation in pots. As described below, when plants were 61 days old and the main root was totally developed we performed $^{15}N_2$ labelling during 5 days. This plant stage was chosen for labelling since at this stage, there is an important C and N remobilization from aboveground organs toward taproot that acts as the major storage organ (Avice et al., 1996). Immediately after the labelling, the first harvest was undertaken from a subset of four control and four labelled plants (T0). Once the harvest was finished, the aboveground part of the remaining plants was cut

(to a 5 cm stem height) so to analyse plant regrowth capacity. Parallel with shoot cutting, waters stress treatment was imposed. Half plants were kept under optimal irrigation conditions (well-watered, WW), whereas in the other half water stress (WS) was imposed through water withholding. A second harvest was performed 8 days after cutting (T8), when plants were 74 days old. In each harvest, plants were separated into apical shoot, primary root and nodules. Four plants were collected per treatment and were immediately frozen in liquid N and stored in -80 ºC freezer. A subsample of each organ was separated and dried in an oven during 48 h at 60ºC in order to determine dry weight. Metabolite measurements were conducted in only three replicates per organ and water regime.

Plant water status was evaluated before harvesting by determining apical leaf relative water content (RWC) according to Weatherley, (1950). Osmotic potential was determined in apical shoots, primary roots and nodules using a Wescor 5500 osmometer (Wescor, Logan, Utah, USA) as described by (Ball and Oosterhuis, 2005).

2.2. Leaf gas exchange

Fully-expanded apical leaves were enclosed in a LI-COR 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). Determinations were carried out at 25ºC. Photosynthetic assimilation (A), leaf stomatal conductance (g_s) and leaf transpiration rate (E) were estimated at a saturating PPFD of 1200 µmol $m^{-2}s^{-1}$ using equations developed by von Caemmerer and Farquhar (1981). The ratio intercellular to ambient CO_2 concentration (C_i/C_a) was estimated from net photosynthesis and g_s measurements, according to Farquhar and Sharkey (1982). The gas-exchange response-curve to atmospheric CO_2 concentration was measured from 0 to 1400 µmol mol^{-1} CO_2. Measurements started at 400 µmol mol^{-1} of CO_2, decreased stepwise through l 250 and 100 until 0 µmol mol^{-1} and then restarted at 400 and increased stepwise until 700, 850, 1000 and 1400 µmol mol^{-1}. Estimation of the maximum carboxylation velocity of Rubisco (V_{cmax}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) were made by fitting a maximum likelihood regression below and above inflexion of the A/C_i response using the method of Ethier and Livingston (2004). Stomatal limitation (l), which is the proportionate decrease in light-saturated net CO_2 assimilation attributable to stomata, was calculated according to Farquhar and Sharkey (1982) as $(A_0-A_1)/A_0$, where A_0 is the A at c_i of 360 µmol mol^{-1} and A_1 is A at c_a of 360 µmol mol^{-1}.

2.3 Isotope Labelling

The $^{15}N_2$ labelling was conducted at root level in the Conviron E15 growth chambers with 10 ‰ enriched $^{15}N_2$. The $^{15}N_2$ gas was prepared in gas sampling bags (SKC, Houston, USA) by mixing $^{15}N_2$ enriched at 99 ATOM % provided by EURISO-TOP (Saint Aubin, France) with ambient air ($\delta^{15}N_2$ at *ca*. 0.35 ‰). The pots containing the plants were placed within a hand-made labelling chamber and closed hermetically. The $^{15}N_2$ was then injected in the hand-made chamber using a gas syringe (SGE International Pty Ltd, Australia). The injection of enriched $^{15}N_2$ was conducted twice a day; two and five hours after the beginning of the daily light period, coinciding with the period of largest N_2 fixation activity (Steunou et al., 2008). Then, the labelling chambers were removed from the bottom of the pots and the growth chambers were opened and quickly purged with ambient air. Non-labelled plants were grown in a second growth chamber maintained at ambient N_2 air conditions.

2.4 Metabolite profile analyses

For HPLC analysis of amino acids and GC–TOF–MS profiling, extracts from three of the four replicates were performed as described in Bathellier et al. (2009). The frozen material was ground in liquid N with a pestle and mortar and extracted into 80 % methanol / 20 % water containing ribitol (100 µM) as internal standard. After centrifugation, multiple aliquots were spin-dried under vacuum and stored at -80°C. For HPLC analysis of amino acids, aliquots were re-dissolved in water, centrifuged and filtered into autosampler vials prior to automated pre-column derivatization with *o*-pthaldialdehyde (OPA). OPA reagent was made 36 h before first use by dissolving OPA in 200 µl of methanol and adding 1.8 ml 0.5 M sodium borate (pH 9.5) and 40 µl 2-mercaptoethanol. The reagent was filtered into an autosampler vial and used for up to 2 days. Precolumn derivatization was performed in the injection loop by automated mixing of 10 µl sample and 10 µl OPA reagent, followed by a delay of 2 min prior to injection. The chromatographic separation was performed by gradient elution at 40 ºC using buffer A (20% methanol, 79% sodium acetate, 1% tetrahydrofuran, pH 5.9) and buffer B (80% methanol, 20% sodium acetate, pH 5.9). Buffer flow rate was 0.8 mL min^{-1} throughout and total run time per injection was 52 min. Peak identity was confirmed by co-elution with authentic standards.

For GC–TOF–MS analysis, methoxyamine was dissolved in pyridine at 20 mg ml^{-1} and 50 ml of this mixture was used to dissolve the dry sample. Following vigorous mixing, samples were incubated for 90 min at 30 ºC with shaking. Then, 80 ml of N-methyl-N(trimethylsilyl)trifluoroacetamide (MSTFA) was added, and the mixture was vortexed, and incubated for 30 min at 37 ºC with shaking. The derivatization mixture was then

incubated for 2 h at room temperature. Before loading into the GC autosampler a mix of a series of eight alkanes (chain lengths: C10 to C36) was included to enable identification by retention index (RI) as well as by MS fragmentation pattern.

Labelled amino acids with *o*-pthaldialdehyde were separated by reverse-phase HPLC and identified by RI compared to standards. For GC–TOF–MS, metabolite derivatives were identified by comparison of the fragmentation pattern with MS databases using a match cut-off criterion of 750/1,000 and by RI using alkane series as standards. This enabled detection of 78 different metabolites. When standards were available, these were used to test the predicted matches: 59 commercially available compounds were individually analysed by GC–TOF–MS. They were all found to confirm the matches predicted by MS and RI.

For HPLC quantification analysis, amino acid amounts were calculated on linear calibration curves generated for each standard. Values were corrected for the response of the internal standard (ribitol), and quantified on a tissue fresh weight basis. For GC–TOF–MS, integrated peak areas were obtained after deconvolution by the LECO PEGASUS III ChromaTOF® software and quantified using the appropriate software option. These were then normalized to the internal standard peak area for each injection. Principal component and hierarchical clustering analyses were performed using the TIGR Multi Experiment Viewer (MEV) software (Saeed et al., 2003). For each metabolite, the mean was subtracted from each individual value and the result divided by the standard deviation to yield centre-reduced data.

2.5 Determination of $\delta^{15}N$ of amino acids

Stable ^{15}N isotope composition ($\delta^{15}N$) of amino acids was determined as detailed in Molero *et al* (2011). Frozen samples were ground to a fine powder in liquid N and a sub-sample (ranging from 50 to 200 mg fresh weight of tissue) was lyophilized. Extraction of soluble fraction was performed with Trifluoracetic Acid (TFA) 10% (v/v) at 4 ºC using a sonicator. The homogenate was centrifuged at 6,000 *g* for 15 minutes at 4 ºC. Then the supernatant was collected in Ultrafree-MC 10000 NMWL (*Millipore*, EUA) tubes and purified by centrifugation (13,000 *g* during 45 minutes at 4 ºC). Following, 1 ml of filtered sample was taken and L-norleucine (*Sigma-Aldrich;* Schnelldorf, Germany and St. Quentin Fallavier, France) was added as an internal standard. These fractions were dried under vacuum overnight using a Speed Vac desiccator and stored at -20 ºC until further analysis. Then the samples were re-suspended in 1 ml of HCl 0.1 N (v/v) and passed through a chromatographic column filled with cation exchange resin

(Dowex 50W X8 H^+, 200-400 mesh size, Sigma®) allowing extraction of acidic, basic and neutral amino acids.

Amino acids mixture eluted from the column was completely evaporated under heat and dry N obtaining the crystallized amino acids. Derivatization was performed with N-methyl-N-(*tert*.-butyildimethylsilyl)-trifluoroacetamide (Aldrich®) as proposed by (Woo and Chang, 1993; Woo and Lee, 1995). Then the amino acids were derivatized to N(O)-(*tert*.-butyildimethylsilyl) derivatives and the reaction mixture was first injected directly to Gas Chromatography-Mass Spectrometry (GC-MS) in order to separate and to identify the amino acids composition of the samples. The amino acid derivatives were identified by means of their mass spectra (Mass Spectral Library: NIST 05). Then the samples were analyzed by Gas Chromatography Isotope Ratio Mass Spectrometry (GC-C-IRMS) in order to determine $\delta^{15}N$ of individual amino acids.

2.6 Statistical analyses

To evaluate the effect of water limitation on the measured traits, means were compared by an unpaired *t*-test at the 5% significance level using the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA). GC–TOF–MS data were normalized with respect to the mean of all organs (Fig. 1A, 1B) or water treatment within organs (Fig. 2). Normalized data were then drawn as a clustered metabolomic array using MeV 4.1 open source software (Saeed et al., 2003) as described above. The clustering was based on the Pearson's correlation coefficients among the metabolites. In this representation, green colour is proportional to a lower concentration; conversely, the intensity of the red colour is proportional to higher concentration rates. Significant differences were determined using Student's *t*-test at $\alpha = 0.05$.

3. Results

Water limitation effects on plant growth, water status and physiology

At final harvest time (i.e. 8 days after withholding water), water stress was found to have no significant effect on total plant biomass (Table 1). However, a significant increase in nodules biomass and a modest but significant decrease in primary root biomass were observed upon water limitation (Table 1). No significant difference in the percentage of nitrogen was observed in either apical shoots (AS), primary roots (PR) or nodules (Nod) (Table 1). Plants under water stress treatment showed a significant ($P<0.05$) decrease of 8.4% in leaf relative water content (RWC) (Table 1). No differences in osmotic potential (Ψ_s) in apical shoots and primary roots were observed.

However, a significant decrease in the osmotic potential of nodules was found (Table 1).

Leaf gas exchange measurements (Table 1) performed in apical leaves, 6 days after the beginning of water withholding, revealed a significant decrease in net photosynthetic CO_2 assimilation (A), Rubisco carboxylation maximum capacity (Vc_{max}), RuBP regeneration maximum capacity (J_{max}), stomatal limitation (l) and stomatal conductance (g_s) as a response to water stress.

Water limitation effects on metabolite profiles from plant and nodule

To analyse the effect of water stress on plant and nodule metabolism, non-targeted metabolite profiling was performed by GC-TOF-MS and 78 different metabolites were identified (by reference to their MS data). Comparison of metabolite profiles in AS, PR and Nod grown at WW and WS conditions showed that only 9 of the 78 identified metabolites did not show significant organ or water treatment-dependent effects. Metabolite profiling representation (heatmap) was undertaken between organs (Fig. 1A, 1B) and water treatments (Fig. 2). In order to compare the metabolic composition between organs, each water treatment (WW and WS) was analysed separately (panels 1A and 1B in Fig. 1). Values shown with colours were UV-scaled (centred and normalized to standard deviation) for each metabolite. Metabolite contents were normalized with respect to both internal standard (ribitol) and dry mass (see Material and methods) and thus comparisons between organs in Fig. 1 represent differences in relative content per mg DW. Only metabolites showing significant differences between organs by ANOVA at the $P < 0.05$ level were retained for the heat map and the hierarchical clustering (using Pearson's correlation coefficient).

Under well-watered conditions, the hierarchical clustering of the 63 significant metabolites formed two clusters (Fig. 1A). Cluster 1 mostly included organic acids, and was made of metabolites in higher concentration in AS as compared to other organs. Cluster 2 was made of different metabolic classes (including sugars and amino acids) at higher concentration in nodules as compared to other organs. Cluster 2 could be sub-divided in several sub-clusters: sugars and Serine (Ser) (2.1), sugars and amino acids (2.2), amino acids, sugar alcohols, organic acids (2.3) and putrescine + maleic acid (2.4).

Under water restriction, 48 metabolites were found to be significantly different between organs (Fig. 1B). Three different clusters were identified according to organ-specific

prevalence: compounds with higher concentration in PR (arabinose, maleic acid and phosphate, cluster 1), AS (myoinositol, Ser and organic acids, cluster 2) and Nod (various classes, including sugars, cluster 3).

Figure 2 represents the impact of water deficit on metabolite contect on the different studied organs. In the case of AS, 15 compounds were significantly different between WW and WS. As expected, in shoots, water stress caused a decrease in hexose phosphates and an increase in Ser and glyceric acids, reflecting the decrease in photosynthesis and an increase in photorespiration (Miller et al., 2010). In PR, 11 metabolites significantly increased upon water deficit (Fig. 2), including sucrose and three metabolites of Glu metabolism (Gln, GABA and pyroglutamate). In nodules, 23 metabolites were significantly different between water treatments: 20 increased and included sugars and several amino acids, and only three compounds increased (Pro, sucrose and maleic acid).

Quantitative analysis of differences in amino acids by HPLC

Because the GC–TOF–MS provides relative contents, amino acids were analysed by targeted HPLC to perform absolute quantitation. This analysis showed that the water regime caused a general increase in amino acid content in PR but a decrease in nodules (Table 2). In agreement with the GC–TOF–MS analysis, Asp and Ser significantly decreased and increased in AS, respectively, under WS conditions (Table 2). An increase in amino acid content in PR was observed as a response to water limitation, so that GABA, Gln, Glu and Trp significantly increased and Asn also tended to increase. In general, all amino acids in nodules decreased with water stress, but only Arg, Asn, Gln, Glu, Gly, Lys, Met, Orn, Ser and Thr were statistically significant. The most marked decrease (3.2-fold) was observed with Asn.

Isotopic pattern of amino acids revealed by GC–C–IRMS

The isotopomic representation of most relevant amino acids involved in the present study is shown in Fig. 4. Each $\delta^{15}N$ value of amino acids from labelled plants was normalized together with $\delta^{15}N$ values of amino acids in control plants. Therefore, green values represent low ^{15}N-enrichment in contrast to red values that represent high ^{15}N -enrichment in amino acids respect control plants (non-labelled). After labelling (T0), PR was the organ containing the most labelled amino acids, followed by Nod. At T8, ^{15}N-labeling in amino acids was lower under WW conditions than under WS. The cluster shows the isotopic proximity (i.e., with the most similar covariation pattern) between

Pro and Glu on the one hand, and between Asp and Asn on the other hand (Fig. 3). In general, the ^{15}N-enrichment was lower in Nod. Under WW conditions, Asp and Asn in Nod and Ser in AS were poorly labelled eight days after labelling compared with other amino acids (green cells). Because of the low levels of Pro, no value of δ^{15}N could be obtained in all organs analysed under well-watered conditions. Under WS conditions, the ^{15}N-labeling in Pro was lower in AS and Nod as compared to PR. Taken as a whole, after eight days under WS conditions, amino acids appeared to be most ^{15}N-enriched in PR, suggesting the remobilization of recently fixed N upon water stress.

4. Discussion

In this study, we used a combination of metabolic profiling and isotopic labelling (with gaseous $^{15}N_2$) to investigate the physiological responses of *Medicago sativa* plants subjected to a mild water stress during a regrowth period. With this approach, we examined plant and nodule responses to water stress at the metabolic level. Physiological, metabolic and isotopic data confirmed a general decrease in C metabolism in nodulated alfalfa plants subjected to water limitation, and a reorchestration of N metabolism.

Leaf gas-exchange and plant-nodule Ψ_s

Gas exchange measurements (Table 1) revealed that water stress decreased photosynthetic activity because of stomatal (as shown by stomatal limitation data) and non-stomatal processes (Rubisco maximum carboxylation capacity Vc_{max}, and RuBP regeneration capacity, J_{max}), as already found elsewhere (Nogués et al., 2000; Aranjuelo et al., 2011). The decrease in relative water content showed that the worse water status of WS plants was involved in the inhibition of photosynthetic machinery (Aranjuelo et al., 2011). The lack of differences in leaf osmotic potential (Ψ_s) despite the change in RWC suggests a low osmoregulatory response to water stress in leaf cells, and rather a change in matrix and hydrostatic components of total leaf water potential. In other words, plants were here at the stage of water stress response situated just after stomatal closure initiation, before any important change in leaf water potential (Cornic, 2000; Flexas and Medrano, 2002; Medrano et al., 2002). Accordingly, the leaf response observed here was not accompanied by the accumulation of typical osmolytes like Pro; only myoinositol accumulation being observed (Fig. 2). Similarly, the lack of differences in root Ψ_s was only accompanied by a modest accumulation of sucrose. Conversely, in nodules, the significant decrease in Ψ_s with water limitation was accompanied by a large increase in Pro and sucrose levels, as previously

described (Aranjuelo et al., 2011). These results suggest a higher osmotic adjustment in nodules compared with other organs.

Reorchestration of catabolism and N assimilation upon water deficit

In leaves, sucrose levels remained unaltered under water stress conditions. While, the lower amount of various intermediates of glycolysis, such as glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) could be the consequence of the photosynthetic inhibition, it should be also associated with a decrease in the flux of C to glycolytic pathway (Fig. 4). The accumulation of pyruvate and the general decrease in Krebs intermediates that was observed under water-limited conditions might indicate a down-regulation of the Krebs cycle. The accumulation of pyruvate suggests an inhibition of its conversion to acetyl-CoA by pyruvate dehydrogenase. The decrease in palmitic acid, stearic acid and malonic acid (synthesized from acetyl-CoA) may also indicate an inhibition in acetyl-CoA synthesis.

Maleic acid levels are commonly low because of its little involvement in biochemical pathways. However, maleic acid is known to be a competitive inhibitor of several Krebs cycle enzymes (Evans and Garraway, 1984); therefore, its increase under water limited conditions could be related with the inhibition of Krebs cycle enzymes (as indicated in Fig. 4). The accumulation of Pro in response to water stress might serve to stabilize protein structure (Schobert and Tschesche, 1978) and is associated with an osmoregulatory function (Hare et al., 1998; Irigoyen et al., 1992). The decrease in Glu can be also explained with the increase in Pro levels, as has been previously reported (Aranjuelo et al., 2011). Similarly to Pro, myo-inositol, which is also associated with an osmoregulatory role (Streeter et al., 2001), was increased in leaves.

Photorespiration may serve as an energy sink, preventing over-reduction of the photosynthetic electron transport chain and photoinhibition (especially under drought), caused by reduced rates of photosynthetic CO_2 assimilation and thus NADPH utilization (Wingler et al., 1999). Rubisco, catalyses the reaction of ribulose-1,5 - bisphosphate with either CO_2 or O_2 and thereby initiates CO_2 assimilation and photorespiration, respectively. The balance between the two reactions depends on the relative mole fraction of CO_2 and O_2 at the site of catalysis (chloroplast stroma).Therefore the lower the CO_2 mole fraction, the higher the photorespiration rate is. Stomatal closure detected in WS plants lead to higher level of photorespiration due to lower intercellular CO_2 mole fraction (data not shown)..Moreover, the increase in Ser and glycerate (intermediates of the photorespiratory cycle) suggest a typical increase in

photorespiration rate (Novitskaya et al., 2002). In the other hand, ^{15}N labelling conducted in alfalfa plants highlighted the fact that the very low ^{15}N-labeling in leaf Ser (compared with other amino acids) probably comes from the use of non-labelled leaf Glu pool in photorespiration (Martinelli et al., 2007). By contrast, there was a ^{15}N-labeling in Ser in WS plants in other organs, showing that Ser was formed from other metabolic pathways and not translocated inherited from leaves. In other words, Ser synthesis in roots and nodules was likely achieved by the cytoplasmic pathway from 3-phosphoglycerate, thereby involving transamination from a pool containing ^{15}N.

In general, roots showed a moderate metabolic response to water deficit than the other organs, suggesting a limited impact in pool sizes. Quantification of amino acids by HPLC demonstrated an increase in GABA, Gln, Glu and Trp (Table 2). Metabolomic profiling of roots showed that only 11 compounds were significantly different between WW and WS treatments (Fig. 2). By contrast, isotopomic profiling show that the primary root was the organ with the most ^{15}N enriched free amino acids (Fig. 3). Therefore, it is likely that amino acid pools in PR are associated with a high turn-over; synthesised amino acids being allocated to export (to other organs) or as storage protein synthesis. In fact, PR are believed to have a critical role in N storage and remobilization in alfalfa. For example, defoliation of aerial parts affects drastically on N acquisition via a large decrease in nitrogenase activity (Kim et al., 1991, 1993; Ourry et al., 1994) while the production of new shoots during the first days following cutting involves N compounds from PR. In other words, endogenous N reserves in PR, in the form of amino acids and proteins, are used for the regrowth of aerial parts (Ta et al., 1990; Avice et al., 1996), while C reserves (like sugars) are mainly used for sustaining respiratory metabolism of belowground organs (roots and nodules). Specific proteins called vegetative storage proteins (VSPs) have been identified in taproots of alfalfa (Volenec et al., 1996; Ourry et al., 2001; Bewley, 2002). These VSPs can represent up to 40% of the total soluble proteins in the taproot Erice et al., (2007). Furthermore, as observed by previous studies (Hendershot and Volenec, 1993b; Avice et al., 1996a; Corre et al., 1996; Gana et al., 1998) during regrowth, VSPs are degraded, with a rate of remobilization from 60% to 80%, in order to provide N to re-growing shoots. In our experiment, we hypothesize that PR played a similar role by remobilizing N assimilates and proteins, thereby compensating for the drop in photosynthetic input by shoots caused by water deficit.

In nodules, water stress led to a general decrease in free amino acids (Table 2). This decrease can be explained by the decline of N_2 fixation rate by nitrogenase in response

to mild drought stress (Zahran, 1999). Interestingly, Pro was increased in nodules under water deficit (Fig. 4) as previously reported (Aranjuelo et al., 2011). This increase was not related to an import from PR, since Pro is hardly ^{15}N-labelled in nodules (Fig. 3). It is rather synthesized locally by nodule metabolism and as such, it is associated with a decrease in Glu and Asn (Table 2, Fig. 2), suggesting that Glu and Gln metabolism channelled N to Pro synthesis. Glu (precursor of Pro) and Pro are tightly correlated in the isotopomics analysis (r = 0.99 in HCL analysis), suggesting that the turnover of Glu was directly associated with Pro accumulation. In addition to the increased consumption for Pro synthesis, the decrease in Glu pool size in nodules can also be explained by the decrease in GOGAT activity, which is the key enzyme of Glu biosynthesis in alfalfa nodules (Temple et al., 1998). In fact, this enzyme activity appears to be particularly sensitive to drought stress (Ramos et al., 1999).

Sugar and N exchange and signalling at the whole plant level

The accumulation of sucrose levels in nodules has been shown to be associated with the decrease in nodule sucrose synthase activity in soybean (Gordon et al., 1997) and pea (Galvez et al., 2005; Gonzalez et al., 1998). Recent studies suggest that the accumulation of sucrose in alfalfa nodules is caused by a still active import of sucrose from the shoot, together with a limitation of sucrose consumption within nodules due to the impairment of respiratory activity (Naya et al., 2007). Also, sucrose has been shown to play an important osmoregulatory role in *S. meliloti* (Gouffi et al., 1998). Dicarboxylic acids inherited from the host plant by bacteroids provide the main reduced carbon source (C skeletons) supporting N_2 fixation (Lodwig and Poole, 2003). Under WS conditions, where the dicarboxylate input from PR and shoots is limited (general decrease in the TCA pathway, see above), Pro synthesis in the nodule consumes a significant part of carbon skeletons available locally, and Pro can in turn become an alternative source of reduced C to bacteroid under more favorable conditions (Curtis et al., 2004). This simply explains why there was a general decrease in other aminoacids and organic acids in nodules in the WS treatment. In addition, Pro was unlikely to have been transported from leaves or roots since in that case it would have inherited a substantial ^{15}N signal. However, the fact that Pro was rather synthesized within the nodule, supports the assumption that symbiotic N_2 fixation under drought is mainly driven by local metabolism and thus, maybe, not controlled by a systemic N signal (Marino et al., 2007).

Elevated levels of nitrogenous compounds, including ureides and amino acids, have been proposed to play a role in the decline of symbiotic N_2 fixation in legumes in

response to water deficit. For instance, the accumulation of free amino acids has been shown to be involved in the feedback inhibition of symbiotic N_2 fixation in alfalfa (Schubert et al., 1995) and soybean (Serraj et al., 1999b) subject to drought (King and Purcell, 2005). That is, nodule ureides and Asp, together with several amino acids in leaves, represent candidate molecules for feedback inhibition of symbiotic N_2 fixation in alfalfa and soybean. However, in the present work, Asp declined in nodules, suggesting that other compounds could be play the role of feedback inhibitor, such as Pro (Curtis et al., 2004). In fact, Pro in nodules was likely synthesized locally (see above) and leaves did not accumulate Pro. The sole amino acid exhibiting a rather similar isotopic enrichment between leaves, roots and nodules was Ala, suggesting that Ala could be exchanged between plant organs. However, Ala levels did not change significantly under water deficit in nodules and therefore, its role in feedback inhibition is unlikely.

Asn is the main N-transporter in *M. sativa* (Groat and Vance, 1981; Vance et al., 1994). The decrease in Asn levels in nodules is likely originated from the decrease in N_2 fixation caused by water deficit. This might lead to a decrease in Asn export to the plant. However, contrary to expectations, Asn levels in roots and leaves did not decrease and furthermore, Asn was mostly enriched in PR but not in nodules. It thus appears more likely that PR synthesized Asn from N fixed before water stress treatment (T0) and that there was limited Asn exchange upon water deficit.

5. Conclusions

Using a combination of metabolomics and ^{15}N-labelling, we could follow the metabolism of amino acids during water stress. Our data are in agreement with the assumption that N fixation in nodules is controlled locally. Although water deficit affected negatively photosynthetic activity, sucrose of circulating sugars did not change significantly in shoots, and thus photoassimilate limitation is unlikely to be the cause of decrease N_2 fixing activity. We rather suggest here that some amino acids, in particular Pro, could represent a candidate compounds exerting feedback inhibition on nodule activity when water deficit leads to a decline in nodule water potential. We nevertheless recognize that our study was limited to few amino acids in isotopic analyses and so the exchange of nitrogen found here was probably not fully representative. Further studies will be conducted with more sensitive techniques such as high resolution LC-MS that allow analysis of isotopic patterns.

Acknowledgements

The authors would like to acknowledge the comments and suggestions of Dr. Florence Baptist that were valuable in improving the quality of our manuscript.

Funding

This study was supported in part by the European research project PERMED (INCO-CT-2004-509140), the Spanish Ministry of Science and Technology (CGL2009-13079-C02) and i-COOP-*suelos y legumbres* (2016SU0016) funded by the National Research Council. GM was the recipient of a doctoral fellowship (*Programa Nacional de Formación de Personal Universitario*) sponsored by the Spanish Ministry of Education and Science.

Figure legends

Figure 1A and 1B. Variations in the metabolite profiles from apical shoots (AS), primary roots (PR) and nodules (Nod.) of *Medicago sativa* subjected to water deficit. Hierarchically clustered heat maps of the mean centred-reduced values of metabolites that were found to be significantly different between organs at (A) well-watered conditions (WW) and (B) water stress (WS). Three replicates are presented per organ. Intensity of *red* and *green* indicates increase and decrease relative to the mean, according to the colour scale at the top.

Figure 2. Hierarchically clustered heat maps of the mean centred-reduced values of metabolites that were found to be significantly different between treatments (WW and WS) in apical shoots (AS), primary roots (PR) and nodules (Nod). Each column represents one replicate per organ and water regime. Intensity of *red* and *green* indicates increase and decrease relative to the mean, respectively, according to the colour scale at the top of the heat map.

Figure 3. Isotopomic representation of ^{15}N-enrichment in most relevant amino acids. Values were normalized with δ^{15}N-values of control plants. Zero means ^{15}N-enrichment of amino acids at first harvested (T0). Then, WW and WS is referred to plants 8 days after labelling (T8). Intensity of *red* is associated with higher ^{15}N-enrichment respect non-labelled plants. *Green* colour indicates few labelling respect control. Data are the mean of at least two replicates.

Figure 4. Mapping of metabolite concentrations obtained by GC-TOF–MS onto plant biosynthetic pathways. Full bars represent WW conditions whereas open bars represent WS. The first pair of bars correspond to apical shoots (AS WW and AS WS), the second pair of bars to primary roots (PR WW and PR WS), and the third pair correspond to nodules (Nod WW, Nod WS). Asterisks (*) represent significance between treatments by GC-TOF. [*] represent significance observed by HPLC in spite of GC-TOF did not detect differences. Metabolites, which were significant in Figure 3 but do not find linkage in the pathways shown in this figure, are phosphoric acid and monomethylphosphate: these compounds are not shown. Maleic acid could inhibit Krebs cycle enzymes as indicated in the figure.

References

Acquaah, G. 2012. Principles of Plant Genetics and Breeding. (Acquaah, G., Ed.) (Second Ed.). Wiley-Blackwell, Oxford, UK.

Annicchiarico P., Pecetti L., Abdelguerfi A., Bouizgaren A., Carroni A.M., Hayek T., … Mezni M. 2011. Adaptation of landrace and variety germplasm and selection strategies for lucerne in the Mediterranean basin. Field Crops Research 120, 283–291.

Annicchiarico, P., Pecetti, L., Tava, A. 2013. Physiological and morphological traits associated with adaptation of lucerne (*Medicago sativa*) to severely drought-stressed and to irrigated environments. Annals of Applied Biology 162: 27-40.

Annicchiarico P., Barrett B., Brummer E.C., Julier B., Marshall A.H. 2015. Achievements and Challenges in Improving Temperate Perennial Forage Legumes. Critical Reviews in Plant Sciences 34, 327–380.

Aranjuelo, I., Molero, G., Erice, G., Avice, J.C., Nogués, S. 2011. Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.) Journal of Experimental Botany 62, 111-123.

Aranjuelo, I., Molero, G., Erice, G., Aldasoro, J., Arrese-Igor, C., Nogués, S. 2014a. Effect of shoot on remobilization of carbon and nitrogen during regrowth of nitrogen-fixing alfalfa. Physiologia Plantarum 153: 91-104.

Aranjuelo I, Arrese-Igor C, Molero G. 2014b. Nodule performance within a changing environmental context. Journal of Plant Physiology 171, 1076–1090.

Avice, J.C., Ourry, A., Lemaire, G., Boucaud, J. 1996. Nitrogen and carbon flows estimated by N-15 and C-13 pulse-chase labeling during regrowth of alfalfa. Plant Physiology 112, 281-290.

Avice, J.C., Le Dily, F., Goulas, E., Noquet, C., Meuriot, F., Volenec, J.J. 2003. Vegetative storage proteins in overwintering storage organs of forage legumes: roles and regulation. Canadian Journal of Botany; 81:1198–212.

Bacanamwo, M., Harper, J.E. 1997. The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. Physiologia Plantarum 100, 371-377.

Ball, R.A., Oosterhuis, D.M. 2005. Measurement of root and leaf osmotic potential using the vapor-pressure osmometer. Environmental and Experimental Botany 53, 77-84.

Bathellier, C., Tcherkez, G., Mauve, C., Bligny, R., Gout, E., Ghashghaie J. 2009. On the resilience of nitrogen assimilation by intact roots under starvation, as revealed by isotopic and metabolomic techniques. Rapid Communications in Mass Spectrometry 23, 2847-2856.

Bewley, J.D. 2002. Root storage proteins, with particular reference to taproots. Canadian Journal of Botany 80, 321-329.

Burns, R.C., Hardy, R.W.F. 1975. Nitrogen fixation in bacteria and higher plants. Molecular Biology, Biochemistry and Biophysics 21, 39–60.

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A.M., Francia, E., Marè. C., Tondelli, A., Stanca, A. M. 2008. Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. Field Crops Research 105, 1–14.

Cornic, G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture - not by affecting ATP synthesis. Trends in Plant Science 5, 187-188.

Corre, N., Bouchart, V., Ourry, A., Boucaud, J. 1996. Mobilization of nitrogen reserves during regrowth of defoliatied *Trifolium repens* L., and identification of potential vegetative storage proteins. Journal of Experimental Botany 301, 1111-1118.

Curtis, J., Shearer, G., Kohl, D.H. 2004. Bacteroid proline catabolism affects N-2 fixation rate of drought-stressed soybeans. Plant Physiology 136, 3313-3318.

Erice, G., Irigoyen, J.J., Sánchez-Díaz, M., Avice, J.C., Ourry A. 2007. Effect of drought, elevated CO_2 and temperature on accumulation of N and vegetative storage proteins (VSP) in taproot of nodulated alfalfa before and after cutting. Plant Science 179, 903-912.

Ethier, G.J., Livingston, N.J. 2004. On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. Plant Cell and Environment 27, 137-153.

Evans, R.C., Garraway, M.O. 1984. Effect of Nitrogen-Source and Vitamins on Ethanol and Pyruvate Production by Bipolaris-Maydis Race-T. Mycologia 76, 515-522.

Farquhar, G.D., Sharkey, T.D. 1982. Stomatal Conductance and Photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 33, 317-345.

Flexas, J., Medrano, H. 2002. Drought-inhibition of photosynthesis in C-3 plants, Stomatal and non-stomatal limitations revisited. Annals of Botany 89, 183-189.

Fougère, F., Lerudulier, D., Streeter, J.G. 1991. Effects of Salt Stress on Amino-Acid, Organic-Acid, and Carbohydrate-Composition of Roots, Bacteroids, and Cytosol of Alfalfa (*Medicago sativa* L). Plant Physiology 96, 1228-1236.

Galvez, L., Gonzalez, E.M., Arrese-Igor, C. 2005. Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. Journal of Experimental Botany 56, 2551-2561.

Gana, J.A., Kalengamaliro, N.E., Cunningham, S.M., Volenec, J.J. 1998. Expression of β-amylase from alfalfa taproots. Plant Physiology 118, 1495-1506.

Gonzalez, E.M., Aparicio-Tejo, P.M., Gordon, A.J., Minchin, F.R., Royuela, M., Arrese-Igor, C. 1998. Water-deficit effects on carbon and nitrogen metabolism of pea nodules. Journal of Experimental Botany 49, 1705-1714.

Gordon, A.J., Minchin, F.R., Skot, L., James, C.L. 1997. Stress-induced declines in soybean N-2 fixation are related to nodule sucrose synthase activity. Plant Physiology 114, 937-946.

Gouffi, K., Pichereau, V., Rolland, J.P., Thomas, D., Bernard, T., Blanco, C. 1998. Sucrose is a non-accumulated osmoprotectant in *Sinorhizobium meliloti*. Journal of Bacteriology 180, 5044-5051.

Groat, R.G., Vance, C.P. 1981. Root Nodule Enzymes of Ammonia Assimilation in Alfalfa (*Medicago-Sativa* L) - Developmental Patterns and Response to Applied Nitrogen. Plant Physiology 67, 1198-1203.

Hare, P.D., Cress, W.A., Van Staden J. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell and Environment 21, 535-553.

Hendershot, K.L., Volenec, J.J. 1993. Taproot nitrogen accumulation and use in overwintering alfalfa (*Medicago sativa* L.). Journal of Plant Physiology 141, 68-74.

Hoagland, D.R., Arnon, D.I. 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347, 1–32.

Irigoyen, J.J., Emerich, D.W., Sanchez-Diaz, M. 1992. Alfalfa Leaf Senescence Induced by Drought Stress - Photosynthesis, Hydrogen-Peroxide Metabolism, Lipid-Peroxidation and Ethylene Evolution. Physiologia Plantarum 84, 67-72.

Kim, T.H., Ourry, A., Boucaud, J., Lemaire, G. 1991. Changes in the source-sink relationship for nitrogen during regrowth of lucerne (*Medicago sativa* L.) following removal of shoots. Australian Journal of Plant Physiology 18, 593-602.

Kim, T.H., Ourry, A., Boucaud, J., Lemaire, G. 1993. Partitioning of nitrogen derived from N_2 fixation and reserves in nodulated *Medicago sativa* L. during regrowth. Journal of experimental Botany 44, 555-562.

King, C.A., Purcell, L.C. 2005. Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiology 137, 1389-1396.

Kohl, D.H., Straub, P.F., Shearer, G. 1994. Does Proline Play A Special Role in Bacteroid Metabolism? Plant Cell and Environment 17, 1257-1262.

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., Ladrera, R., Arrese-Igor, C., Weckwerth, W., Gonzalez, E.M. 2009. Carbon Metabolism and Bacteroid Functioning Are Involved in the Regulation of Nitrogen Fixation in *Medicago truncatula* Under Drought and Recovery. Molecular Plant-Microbe Interactions 22, 1565-1576.

Lawlor, D.W. 2002. Limitation to photosynthesis in water-stressed leaves, Stomata vs. metabolism and the role of ATP. Annals of Botany 89, 871-885.

Lodwig, E., Poole, P. 2003. Metabolism of Rhizobium bacteroids. Critical Reviews in Plant Sciences 22, 37-78.

Lodwig, E.M., Hosie, A.H.F., Bordes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J.A., Poole, P.S. 2003. Amino-acid cycling drives nitrogen fixation in the legume - Rhizobium symbiosis. Nature 422, 722-726.

Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., Gonzalez, E.M. 2007. Nitrogen fixation control under drought stress. Localized or systemic? Plant Physiology 143, 1968-1974.

Martinelli, T., Whittaker, A., Masclaux-Daubresse C., Farrant, J.M., Brilli, F., Loreto, F., Vazzana, C. 2007. Evidence for the presence of photorespiration in desiccation-sensitive leaves of the C-4 'resurrection' plant *Sporobolus stapfianus* during dehydration stress. Journal of Experimental Botany 58, 3929-3939.

Medrano, H., Escalona, J.M., Bota, J., Gulias, J., Flexas, J. 2002. Regulation of photosynthesis of C-3 plants in response to progressive drought, Stomatal conductance as a reference parameter. Annals of Botany 89, 895-905.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell and Environment 33, 453–467.

Molero, G., Aranjuelo, I., Teixidor, P., Araus, J.L. Nogués, S. 2011. Measurement of ^{13}C and ^{15}N isotope labeling by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry to study amino acid fluxes in a symbiotic plant - microbe association. Rapid Communications in Mass Spectrometry 25, 599-607.

Nanni, V., Paolini, M., Innocenti, A., Mulè, P., Vargiu, M. 2014. Abstract Evaluation of dormancy and winter hardiness of alfalfa *(Medicago sativa* L.) experimental lines, obtained by crossing parental lines characterized within the framework of the PERMED project. *In Quantitative traits breeding for multifunctional grasslands and turf.* (eds D. Sokolović, C. Huyghe & J. Radović), Springer, Dordrecht, the Netherlands.

Naya, L., Ladrera, R., Ramos, J., Gonzalez, E.M., Arrese-Igor, C., Minchin, F.R., Becana, M. 2007. The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiology 144, 1104-1114.

Neo, H.H., Layzell, D.B. 1997. Phloem glutamine and the regulation of O-2 diffusion in legume nodules. Plant Physiology 113, 259-267.

Nogués, S., Baker N.R. 2000. Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation. Journal of Experimental Botany 51, 1309- 1317.

Novitskaya, L., Trevanion, S.J., Driscoll, S., Foyer, C.H., Noctor, G. 2002. How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. Plant Cell and Environment 25, 821-835.

Ourry, A., Kim, T.H., Boucaud, J. 1994. Nitrogen reserve mobilization during regrowth of *Medicago sativa* L., relationships between their availability and regrowth yield. Plant Physiology 105, 831-837.

Ourry, A., MacDuff, J., Volenec, J.J., Gaudillère, J.P. 2001. Nitrogen traffic during plant growth and development. In, Lea PJ, Morot-Gaudry JF. (Eds), Plant Nitrogen. Springer Verlag. pp 255-273.

Prell, J., Poole, P. 2006. Metabolic changes of rhizobia in legume nodules. Trends in Microbiology 14, 161-168.

Ramos, M.L.G., Gordon, A.J., Minchin, F.R., Sprent, J.I., Parsons, R. 1999. Effect of water stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris* L.). Annals of Botany 83, 57-63.

Saeed, A., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J. 2003. TM4, A free, open-source system for microarray data management and analysis. BioTechniques 34, 374-378.

Salon, C., Avice, J.C., Colombie, S., Dieuaide-Noubhani, M., Gallardo, K., Jeudy, C., Ourry, A., Prudent, M, Voisinm A., Rolin, D. 2017. Fluxomics links cellular functional analyses to whole-plant phenotyping. Journal of Experimental Botany 68, 2083-2098.

Schobert, B., Tschesche, H. 1978. Unusual Solution Properties of Proline and Its Interaction with Proteins. Biochimica et Biophysica Acta 541, 270-277.

Schubert, S., Serraj, R., Pliesbalzer, E., Mengel, K. 1995. Effect of Drought Stress on Growth, Sugar Concentrations and Amino-Acid Accumulation in N-2-Fixing Alfalfa (*Medicago Sativa*). Journal of Plant Physiology 146, 541-546.

Serraj, R., Sinclair, T.R., Purcell, L.C. 1999a. Symbiotic N2 fixation response to drought. Journal of Experimental Botany 50, 143–155.

Serraj, R., Vadez, V., Denison, R.F., Sinclair, T.R. 1999b. Involvement of ureides in nitrogen fixation inhibition in soybean. Plant Physiology 119, 289-296.

Serraj, R., Vadez, V., Sinclair, T.R. 2001. Feedback regulation of symbiotic N-2 fixation under drought stress. Agronomie 21, 621-626.

Sheaffer, C. C., Tanner, C. B. & Kirkham, M. B. 1988. Alfalfa water relations and irrigation. In: Hanson, A.A., Barnes, D.K. & Hill, R.R., Jr. eds. Alfalfa and alfalfa improvement. Madison, Wisconsin: American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Vol. 29, 373-409.

Steunou, A.S., Jensen, S.I., Brecht, E., Becraft, E.D., Bateson, M.M., Kilian, O., Bhaya, D., Ward, D.M., Peters, J.W., Grossman, A.R., Kuhl, M. 2008. Regulation of nif gene expression and the energetics of N-2 fixation over the diel cycle in a hot spring microbial mat. Isme Journal 2, 364-378.

Streeter, J.G. 1987. Effect of Nitrate on the Organic-Acid and Amino-Acid-Composition of Legume Nodules. Plant Physiology 85, 774-779.

Streeter, J.G., Lohnes, D.G., Fioritto, R.J. 2001. Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. Plant Cell and Environment 24, 429-438.

Ta, T.C., MacDowall, F.D.H., Frais, M.A. 1990. Utilization of carbon and nitrogen reserves of alfalfa roots in supporting N_2-fixation and shoot regrowth. Plant Soil. 127, 231-236.

Tardieu, F., Cabrera-Bosquet, L., Pridmore, T., Bennett, M. 2017 Plant Phenomics, From Sensors to Knowledge. Current Biology 27, R770-R783.

Temple, S.J., Vance, C.P., Gantt, J.S. 1998. Glutamate synthase and nitrogen assimilation. Trends in Plant Science 3, 51-56.

The World Bank. 2007. World development report 2008. Agriculture for development. Washington DC. http,//siteresources.worldbank.org/ INTWDR2008/Resources/ WDR_00_book.pdf. 364 pp

Thomas, Robertson, M.J, Fukai, S., Peoples, M.B. 2004. The effect of timing and severity of water deficit on growth, development, yield accumulation and nitrogen fixation of mungbean. Field Crops Research 86, 67-80.

Udvardi, M.K., Day, D.A. 1997. Metabolite transport across symbiotic membranes of legume nodules. Annual Review of Plant Physiology and Plant Molecular Biology 48, 493-523.

Vance, C.P., Gregerson, R.G., Robinson, D.L., Miller, S.S., Gantt, J.S. 1994. Primary Assimilation of Nitrogen in Alfalfa Nodules - Molecular-Features of the Enzymes Involved. Plant Science 101, 51-64.

Volenec, J.J., Ourry, A., Joern, B.C. 1996. A role for nitrogen reserves in forage regrowth and stress tolerance. PhysiolOgia Plantarum 97, 185-193.

Von Caemmerer, S., Farquhar, G.D. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of *Phaseolus vulgaris* Cultivar Hawkesbury-Wonder Leaves. Planta (Heidelberg) 153, 376-387.

Walsh, K.B. 1995. Physiology of the Legume Nodule and Its Response to Stress. Soil Biology & Biochemistry 27, 637-655.

Weatherley, P. 1950. Studies in the water relations of the cotton plant. 1. The field measurement of water deficits in leaves. New Phytologist 49, 81-97.

Wingler, A., Quick, W.P., Bungard, R.A., Bailey, K.J., Lea, P.J., Leegood, R.C. 1999. The role of photorespiration during drought stress, an analysis utilizing barley mutants with reduced activities of photorespiratory enzymes. Plant Cell and Environment 22, 361-373.

Woo, K.L., Chang, D.K. 1993. Determination of 22 Protein Amino-Acids As N(O)-Tert-Butyldimethylsilyl Derivatives by Gas-Chromatography. Journal of Chromatography 638, 97-107.

Woo, K.L., Lee, D.S. 1995. Capillary Gas-Chromatographic Determination of Proteins and Biological Amino-Acids As N(O)-Tert-Butyldimethylsilyl Derivatives. Journal of Chromatography B-Biomedical Applications 665, 15-25.

Zahran, H.H. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews 63, 968-989.