Metabolomics and stoichiometry adapted to the study of environmental impacts on plants

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PhD Thesis

Chemistry Doctoral Program

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June 2015

Metabolomics and stoichiometry adapted to the study of environmental impacts on plants

Tesi doctoral per optar al Grau de Doctor per Albert Gargallo Garriga

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Bellaterra, 23/06/2015

Gargallo Garriga, Albert (2015)

Metabolomics and stoichiometry adapted to the study of environmental impacts on plants

Keywords: metabolomics, ecometabolomics, stoichiometry, climate change, wounding, phyllosphere, NMR spectroscopy, LC-MS.

This PhD thesis has been carried out between the Centre for Ecological Research and Forestry Applications (CREAF) and the Nuclear Magnetic Resonance Service (SeRMN) of the Universitat Autònoma de Barcelona (UAB).

This PhD thesis has been funded by the following grants: a PIF (chapter IV) grant of the UAB; the Spanish Government grants CGL2013 and CTQ2012-32436; the Catalan Government grant SGR 2009-458 and the European Research Council Synergy grant ERC-2013-SyG-610028 IMBALANCE-P.

Metabolòmica i estequiometria adaptades a l'estudi d'impacte ambiental a les plantes

Paraules clau: metabolòmica, ecometabolòmica, estequiometria, canvi climàtic, ferida, filosfera, espectroscòpia de RMN, LC-MS.

Aquesta tesi doctoral s'ha portat a terme entre el Centre de Recerca Ecològica i Aplicacions Forestals (CREAF) i el Servei de Ressonància Magnètica Nuclear (SeRMN) pertanyents a la Universitat Autònoma de Barcelona (UAB).

Aquesta tesi doctoral s'ha dut a terme gràcies als aports financers següents: beca PIF (capítol IV) de la UAB; projecte CGL2013 de l'Agencia Española de Cooperación y Desarrollo' (AECID); projecte CTQ2012-32436 del 'Ministerio de Economía y Competitividad'; projecte SGR 2009-458 concedit per la Generalitat de Catalunya; projecte europeu ERC-2013-SyG-610028 IMBALANCE-P concedit pel 'European Research Council Synergy'.

Certifiquen que:

Aquesta tesi duta a terme per Albert Gargallo Garriga al departament de Química, entre el Servei de Ressonància Magnètica Nuclear i el Centre de Recerca Ecològica i Aplicacions Forestals, titulada **Metabolomics and stoichiometry adapted to the study of environmental impacts on plants** ha estat realitzada sota la seva direcció de la Dra. Míriam Pérez-Trujillo, el Dr. Jordi Sardans Galobart, el Prof. Josep Peñuelas Reixach i sota la tutoria del Dr. Teodor Parella Coll.

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Universitat Autònoma de Barcelona
2015

*"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado.
Un esfuerzo total es una victoria completa"*

Mahatma Gandhi

*"Sería posible describir todo científicamente, pero no tendría ningún sentido; carecería de significado el que usted describiera a la sinfonía de Beethoven
como una variación de la presión de la onda auditiva."*

Albert Einstein

"No arrepentirse de nada es el principio de toda ciencia"

Ludwig Borne

"Un árbol necesita dos cosas: sustancia bajo tierra y belleza exterior. Son criaturas concretas, pero impulsadas por una fuerza de elegancia. La belleza que necesitan es viento, luz, grillos, hormigas y una miríada de estrellas hacia las que dirigir la fórmula de sus ramas."

Erri de Luca

"La verdadera ciencia enseña, sobre todo, a dudar y a ser ignorante."

Ernest Rutherford

"La ciencia es el arte de crear ilusiones adecuadas que el loco cree o rebate, pero de cuya belleza o inventiva disfruta el sabio."

Karl Gustav Jung

"Toda la historia del progreso humano se puede reducir a la lucha de la ciencia contra la superstición"

Gregorio Marañón

La ciencia se compone de errores, que a su vez, son los pasos hacia la verdad.

Julio Verne

A la meva família,

A l'àvia i l'avi

A la teta, el pare i en especial a la Mare

A la Nena i a la Pollito

Acknowledgments

Vull agrair sincerament el suport a totes aquelles persones que han confiat en mi al llarg d'aquests anys i que m'han donat un cop de mà quan el necessitava, sense ells aquesta tesi no hagués estat possible.

En primer lloc vull donar les gràcies els directors de la tesi, he tingut molta sort de tenir-vos com a directors i agraeixo tot el temps que heu estat invertint en la meva formació científica. Personalment, crec que científicament són un reflex de la seva personalitat i crec que em porto una combinació de tots ells, gràcies per ajudar-me a créixer tant a nivell científic com en lo personal. Gràcies per l'oportunitat d'involucrar-me en el projecte de metabolòmica.

Agrair en Teo per donar-me la oportunitat de fer la tesi, li agraeixo tot el que he après durant els anys de tesi i m'emporto bons records, fent que dinars de grup siguin molt amens amb veritats absolutes relacionades amb les dones i amb la política.

Agrair a la Míriam per la seva rigorositat, la seva persistència i a la seva paciència durant tota la tesi. Les seves explicacions de RMN han sigut l'eina per començar en el camp de la metabolòmica. Gràcies per la teva personalitat que m'ha ajudat a créixer com a persona.

Agrair en Jordi, el despatx on es desxifren els grans secrets dels ecosistemes i de les seves respostes al canvi global a través dels canvis en l'estructura molecular i elemental del medi i dels organismes, només cal entrar per adonar-se de la magnitud de la seva feina. En tot desordre existeix un odre, la primera vegada que vai entrar en el seu despatx no em vaig poder assentar de la quantitat de manuscrits que hi havien, m'adono dia darrere dia que ell només és capaç d'entendre tot aquest escampat, i és que en Jordi a part de ser el "encarregat de producció" tal com s'esmenta a ell mateix és un reflex d'una persona treballadora i honesta. M'ha ajudat molt en el desenvolupament de la tesi, en la meva formació en ecologia i en l'estadística.

Agrair en Josep Peñuelas, el "jefe" o el peñu, on no només ha fet com a director de tesi sinó que fa que la distancia entre director i doctorant desaparegui. Gràcies per la oportunitat de fer la tesi i t'agraeixo tot el que he après durant els anys de la tesi. Des del principi te n'adones, ho vas esbrinant, un exemple tant a nivell professional com a nivell personal degut a la teva personalitat. Tot i que mai té temps, sempre hi ha un espai per parlar amb molta proximitat.

M'he sentit molt afortunat de caure en dos grups de recerca liderats possiblement per la gent més experta en el seu àmbit.

No em vull deixar els companys del SeRMN, agrair en Miquel on la seva figura es indispensable al servei ja que dóna un cop de mà a tothom amb qualsevol contratemps que pugui sorgir. A la Sílvia, l'Eva, la Núria i l' Andre per fer més amè el dia a dia al SeRMN. A la Laura, amb la que hem compartit màster i alguna cerveseta. I en especial en Josep i el Pau, crec que m'emporto a dos amics... El Josep pel seu caràcter i per tots els moments que hem passat junts (fent escacs, el rival a batre). En Pau company que hagués pogut també ser director de tesi gràcies als seus consells amb cervesa en mà.

Agrair el centre d'excel·lència el CREAF, tot i que els primers anys de tesi no vaig anar gaire per allà mica en mica he estat més involucrat i m'he adonat que hi ha molta qualitat en el personal tant becaris com treballadors. A la Magda i a la Marta pel seu esforç diari i no en vull oblidar del Vicenç, l'Adrià, l' Ander, el David, el Gerard, l'Albert, el Guillem, la Rebeca, la Lucia, la Ifi, la Tere, en Josep, el Marcos, la Marta, la Sara, la Clara, el Sergio, la Mar, el Jofre i el Joan que

m’han donat forces quan les necessitava. Així donar gràcies també els que no he anomenat perquè no en se el nom o m’he oblidat d’ells.
En especial m’agradaria donar les gràcies a las del SUD, a la Rosa i a la Helena, per tots els riures que hem arribat a fer i espero seguir gaudint en un futur. M’emporto a dues amigues.
Agrair també a la gent de química, en especial a la Cristina, la Laura, al Joseju, la Katia, l’Alba, la Silvia, la Carme i en Marc entre d’altres, per tots els moment que hem compartit.
Guardo un gran récord pels locos dels triatletas (Txema, Lluís, Carles, Neus, Xavi i l’Andreu) els quals m’han ajudat també a créixer com a persona veient que amb esforç tot es pot aconseguir i que l’edat no importa, tot i què influeix...
Agrair a l‘Abbie, per ajudar-me a millorar l’anglès, acollir-me a casa seva en els Estats Units i en algunes correccions d’alguns manuscrits, una altre amiga pel llarg camí....
Els companys de bàsquet que m’han ensenyat a gaudir de la vida fent esport el Barbas, el Tetilla, el Bananas entre d’altres.
Agrair el meu cercle d’amic més pròxim, a la Claudia, el Dito, el Ivan, la Chunga, la Vane, el Ferran, la Sandra amb els que hem demostrat que tot i no veure’ns tots els caps de setmana tenim una gran família.
Agrair a la meva família pel suport incondicional, als meus pares que sempre han cregut en mi i que tot el que sóc és gràcies en gran part a vosaltres. A la meva Teta que m’ha ensenyat moltes coses sobre la vida.
Finalment, a l’altra part de la família, la Boira tot i que mai ho podrà llegir... però que amb una mirada hi ha prou per dir-li les coses. També per compartir gran part d’hores d’aquest últim any en el despatx.
Agrair sobretot a la pollito, a la meva parella, a la mare dels meus fills, ets increïble, la millor persona que conec del món. M’has fet veure les coses des d’ una altra perspectiva i has tingut molta paciència i el més important sempre has estat al meu costat tot i el que hem passat.

Moltes gràcies.

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// Summary

Metabolomics has allowed significant advances in biological sciences. An increasing number of ecological studies have applied a metabolomic approach to answer ecological questions (ecometabolomics) during the last few years. The work developed throughout this PhD thesis means a further step in the field of ecometabolomics.

The first chapter of results of this thesis is dedicated to the adaptation of a previously described protocol to conduct metabolomics in ecological studies. It describes in detail the procedures to follow, in the field and in the laboratory, to perform metabolomic analyses using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) coupled to stoichiometric analysis. This improvement allowed to combine the study of ecological stoichiometry and metabolomics in a single protocol and obtain a wider overview of what is happing to the organism.

In the second and third chapters, the described protocol is applied to study how some climate change factors (drought, warming and irrigation) affect different organs of the plant (shoots and roots of *Holcus lanatus* and *Alopecurus pratensis*) at two different seasons. The results show a relationship between the metabolic variation observed in roots and the observed in shoots. The simultaneous metabolomic analysis of both organs of these grasses provide a complete view of the entire plant; including the response of different organs to environmental changes, the global phenotypic response and the metabolic mechanisms underlying these responses.

The fourth chapter is dedicated to the study of the simulated attack to *Quercus Ilex* leaves by a herbivore. The results show differences in the ^{1}H NMR metabolic profiles of the leaf before and after the wound. A marked increase in C-rich secondary metabolites, quinic acid and quercitol, which are related to the shikimic metabolic pathway, was observed. The results also confirm that quinic acid and quercitol are present in great content in the genus *Quercus* and they are involved in mechanisms against biotic stress.

In the fifth chapter a study of the relationship between the epiphytic microorganisms and the plant host is described. This study highlights the large complexity of the episphere, the existence of internal microbial communities and the strong relationships between the structure and function of the internal and external plant metabolomes. These results warrant further study of the specific relationships between plants and the microbial communities living on and in them.

Resum

La metabolòmica ha permès importants avanços en les ciències biològiques. Un nombre creixent d'estudis ecològics han aplicat un enfoc metabolòmic per respondre a preguntes ecològiques (ecometabolòmica) durant els últims anys. El treball desenvolupat al llarg d'aquesta tesi doctoral significa un pas més en el camp de la ecometabolòmica.

En el primer capítol d'aquesta tesi doctoral, hem fet l'adaptació del protocol per dur a terme la metabolòmica en estudis ecològics. En ell es descriuen en detall els procediments per al camp i de laboratori per realitzar anàlisis de metabolòmica mitjançant la Ressonància Magnètica Nuclear (RMN) especroscòpica i cromatografia líquida amb espectrometria de masses (HPLC-MS), juntament amb l'anàlisi de l'estequiometria. Aquesta millora ens ha permès combinar l'estudi d'estequiometria ecològica i metabolòmica en un sol protocol i donar una visió general del que està passant amb els organismes.

En el segon i tercer capítols s’ha aplicat el protocol sobre la part aèria i arrels en l’estudi dels efectes de factors relacionats amb el canvi climàtic (sequera, escalfament, els factors de risc). Els resultats confirmen la variació metabolòmica entre la part aèria i les arrels, en les diferents estacions de l’any i en diferents tractaments d'aigua. L'anàlisi ecometabolomic simultània entre la part aèria i les arrels ha proporcionat una visió completa de tota la planta, incloent la resposta dels diferents òrgans als canvis ambientals, la resposta global fenotípica i els mecanismes metabòlics subjacents a aquestes respostes.

En el quart capítol es descriu la metabolòmica de fulles d’alzina (*Quercus ilex*) simulant un atac dels herbívors. El perfils de RMN de les fulles del Quercus ilex canvien després de la ferida. Es va observar, un marcat augment en els metabòlits secundaris rics en carboni, del quercitol i del àcid quínic, que estan relacionats amb la via metabòlica de l'àcid shiquímic. Els resultats també confirmen que l'àcid quínic i el quercitol són presents en gran contingut en el gènere Quercus i estan involucrats en els mecanismes contra factors biòtics.

Finalment, el cinquè capítol estudia les relacions entre els microorganismes epífitics i la planta. L’estudi mostra que els microorganismes que viuen a la filosfera de la fulla juguen un paper important, existeixen microorganismes dins dels òrgans de la planta i les relacions que hi ha entre l’estructura i les funcions del metabolisme intern i extern de la planta. Aquests resultats aporten un major coneixement de les relacions específiques entre les plantes i les comunitats microbianes que viuen sobre i dins d'ells.

LIST OF ACRONYMS

NMR	**N**uclear **M**agnetic **R**esonance
LC-MS	**L**iquid **C**hromatography–**M**ass **S**pectrometry
GC-MS	**G**as **C**hromatography–**M**ass **S**pectrometry
ICP/OES	**I**nductively **C**oupled **P**lasma **O**ptical **E**mission **S**pectrometry
S/N	**S**ignal-to-**N**oise ratio
COSY	**CO**rrelation **S**pectroscop**Y**
TOCSY	**TO**tal **C**orrelation **S**pectroscop**Y**
HSQC	**H**eteronuclear **S**ingle **Q**uantum **C**oherence
^{1}H *J*-RES	**^{1}H *J*-RES**olved experiment
m/z	**M**ass-to-**C**harge ratio
ESI	**E**lectro**S**pray **I**onization
APCI	**A**tmospheric **P**ressure **C**hemical **I**onization
UPLC	**U**ltra-**P**erformance **L**iquid **C**hromatography
RT	**R**etention **T**ime
TIC	**T**otal **I**on **C**urrent
MOA	**M**ultivariate **O**rdination **A**nalysis
PCA	**P**rincipal **C**omponents **A**nalysis
PLS-DA	**P**artial **L**east **S**quares **D**iscriminant **A**nalysis
PERMANOVAs	**PER**mutational ANOVAs

THESIS OUTLINE

This PhD thesis has been organized in the following sections.

I. INTRODUCTION AND OBJECTIVES

This section starts with an introduction, which contains some general concepts that are needed to understand this research work. Specifically, the concept of metabolomics and ecometabolomics, the general procedure followed in ecometabolomic studies, the chemical analytical techniques used and an introduction to ecological questions are described. The presented work is contextualized and the state of the art is explained. Next, the main goals posed for this thesis are detailed. General goals and concrete objectives are described (Chapter 2).

II. RESULTS AND DISCUSSION

This part of the thesis starts with an initial chapter describing the adapted procedure to perform NMR- and LC-MS-based ecometabolomic and stoichiometric studies in plants (Chapter 3). This protocol is followed in the studies described next.

The following chapters of this section correspond to each ecometabolomic and stoichiometric study performed, where questions related to plant responses to abiotic and biotic environmental factors are addressed (Chapter 4 to 8). Each chapter starts with an abstract of the study and contains the following sections: an introduction of the specific study, methods and materials, results and discussion.

III. CONCLUSIONS

A summary of the conclusions extracted from this thesis are described.

IV. REFERENCES

V. APPENDIXES

In this section, supplementary material - like additional figures and tables - of the different studies are gathered.

I. INTRODUCTION AND OBJECTIVES

Chapter 1

Introduction

1.1 Introduction to ecometabolomics

1.1.1 Towards understanding ecosystem structure and function

Ecosystem ecology is most simply defined as the study of biotic (living) and abiotic (non-living) components of an ecosystem and their interactions within a complex framework. A given ecosystem is comprised of populations of organisms which may interact within communities and contribute to the cycling of water, nutrients and carbon, and energy flowing through the system. In addition to this complexity, explaining general patterns of structure, function, and evolution at the level of an ecosystem is complicated by the large number of interacting factors and inherent variability that exists among the components that comprise a particular ecosystem. Individuals within the same species, for example, present large differences in elemental composition, metabolism, phenology, which present many challenges with regard to sample size and statistical analysis in order to appropriately capture such variability in space and time.

Individual components of an ecosystem may be influenced by global environmental changes, especially those associated with anthropogenic-driven climate change and pollution (Fig. 1.1). Such global changes may be abiotic or biotic in nature. Abiotic components comprise physical conditions and non-living resources that affect living organisms; while biotic factors include any living component requiring energy to do work and food for proper growth, which may interact and thus affect other organisms.

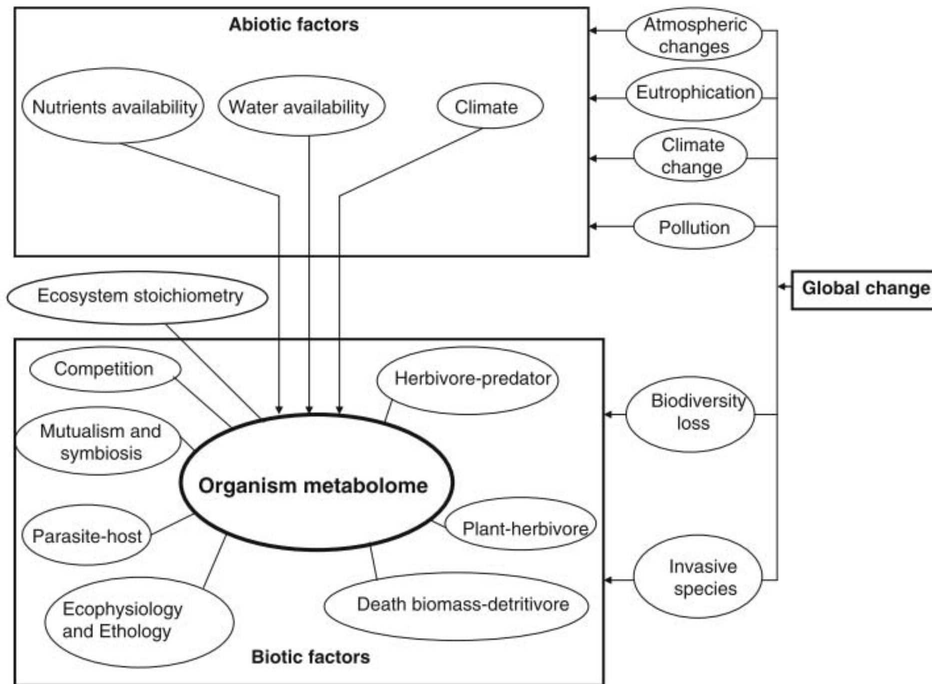

Figure 1.1. Ecological topics.

1.1.2 Ecological and environmental stoichiometry

An important research area in ecology is called ecological stoichiometry. The objective of ecological stoichiometry is to explain the structure and function of the ecosystem in relation to concentrations, allocation and ratios of carbon (C), nitrogen (N) and phosphorus (P) of organisms and of environmental factors (soil, water). C, N and P are the principal elements that constitute the living systems, being essential for all organisms. Thus, the analysis of the C:N:P biomass ratios of an organism are considered to be the elemental phenotype of an organism they function in several critical ecological processes, such as decomposition of litter (D'Annunzio *et al.*, 2008; Güsewell & Gessner, 2009), plant-herbivore-predator relationships (Ngai & Jefferies, 2004; Tibbets & Molles, 2005; Kagata & Ohgushi, 2006), ecosystem-specific composition and diversity (Roem & Berendse, 2000; Güsewell *et al.*, 2005) and the capacity of an organism to adapt to environmental stress (Sardans and Peñuelas 2007; Sardans, Rivas-Ubach, and Peñuelas 2012; Sardans et al. 2012).

1.1.3 The ecometabolomic approach

Several thousands of small molecules (metabolites) are involved in the life processes of organisms. Although some of these substances are externally acquired, most are the products and intermediates of metabolic reactions, including sugars, organic acids, amino acids,

terpenes, lipids, phenols, etc... The metabolome, as the entirety of molecules present in an organism, is the final expression of an organism's genotype at a particular moment (Fiehn, 2002; Peñuelas & Sardans, 2009a,b). As a result, the metabolome can also be thought of as a molecular snapshot of biological diversity because each living species has its own metabolic profile (Gromova & Roby, 2010). The metabolome of an organism is known to be highly responsive to internal and external stressors (Fiehn et al. 2000; Gargallo-Garriga et al. 2014; Leiss et al. 2011; Peñuelas and Sardans 2009; Sardans et al., 2011).

The term "metabolome" is a moderately recent concept and consequently the analysis of the whole metabolome is a recent tool called metabolomics. Thus, metabolomics is the study of the complete collection of metabolites present in a cell or tissue under a particular set of conditions generating a biochemical profile. Comprehensive analysis of the metabolome is crucial to the understanding of cellular function.

This large-scale analysis of metabolites is an important addition to extensive studies of DNA sequences (genome) and proteins (proteome). For the analysis of the metabolome information, technology has generated "data driven" or "-omics" biosciences, in which large amounts of data can be collected in a comprehensive manner and consolidated for analysis using computers. In general, such top-down approaches have provided in-depth view of chemical changes in cells, tissues organs or organisms evoked by cellular processes in response to genetic and environmental causes. Such an approach has become an integral part of systems biology and has provided a direct link between an external stimulus and the phenotype or physiology responses of a biological system (Gygi *et al.*, 1999; Sumner *et al.*, 2003).

The metabolome analysis is applicable to various fields of biotechnology as well as in biomedicine (Nicholson *et al.*, 1999; Wishart *et al.*, 2007; Urban *et al.*, 2010), toxicology (Robertson, 2005; Alam *et al.*, 2010; Hasegawa *et al.*, 2010), plant biology (Fiehn *et al.*, 2000; Hirai *et al.*, 2004; Weckwerth *et al.*, 2004; Scott *et al.*, 2010), and ecological and ecophysiological studies (Peñuelas & Sardans, 2009a,b)(Peñuelas & Sardans, 2009a,b; Gargallo-Garriga *et al.*, 2014, 2015).

The concept of top-down omics approaches allows one to begin at the highest conceptual level and works down to the details. In this way, metabolomics provides a better analysis of the different response capacities conferred by the phenotypic plasticity of each species, allowing for elucidation of metabolic pathways that might be involved in a specific phenotypic response. Metabolomics can also be used as a preliminary screening study of the metabolome response. This does not exclude the simultaneous or subsequent use of targeted chemical analyses.

The research described in this thesis is centered on the emerging field of ecometabolomics, consisting on metabonomic studies applied in the field of ecology. Ecological metabolomics can thus serve as a powerful indication of clues for defining organism lifestyle and global functional strategies of organisms to respond to environmental changes at short- and long-term. Furthermore, we may now be able to achieve a dynamic, holistic view of the metabolism and health not only of an organism, but also of a population or an ecosystem, and in this fashion ecometabolomics opens the door to exciting new insights in ecology. Current studies of this nature have demonstrated that ecometabolomic techniques have great sensitivity in detecting the phenotypic mechanisms and key molecules underlying organism responses to abiotic environmental changes and to biotic interactions, such studies are often limited to the direct effects of a single abiotic factor or of biotic interactions between two trophic levels under controlled conditions. More generally, there is a considerable interest in using metabolic phenotypes as the basis for discriminating between plants of different genotypes, or between plants subjected to different treatments (Roessner *et al.*, 2001, 2002). Moreover, it is also a powerful tool to monitor the phenotypic variability of one genotype in response to environmental changes in drought (Fumagalli *et al.*, 2009), nutrient availability (Hirai *et al.*, 2004), pollutants (Jones *et al.*, 2008a; Bundy *et al.*, 2009), salinity (Fumagalli *et al.*, 2009), temperature (Michaud & Denlinger, 2007) and biotic interactions (Choi *et al.*, 2006), among other ecological factors. These studies are especially useful for plants because metabolomic studies enable the simultaneous analysis of primary metabolites together with secondary metabolites, which may have defensive and protective functions.

There are two basic different approaches to metabolomics: *Fingerprinting* and *Profiling* (Allwood & Goodacre, 2010). *Fingerprinting* is the most commonly used method in metabolomics and it is based upon the multivariate analysis of a dataset consisting on a large amount of sample spectra or chromatograms. It is not required or possible to identify every metabolite, it is often sufficient to rapidly classify samples according to their origin or their ecological or eco-physiological relevance. Each spectrum can be considered a *fingerprint* of unassigned signals arising from low molecular weight analytes. This method enables an unbiased exploration and examination of the sample molecular biochemistry and it can be used to study plant responses to environmental changes (Gidman *et al.*, 2005, 2006). *Profiling* is a more challenging but ultimately more meaningful approach for analyses. It is based on the analysis of an array of metabolites known to be involved in a given biochemical pathway or identifying and quantifying as many metabolites as possible. It does not allow for fast and high throughput automated measurements since considerable manual post-processing steps are

needed to guide the identification and quantification of metabolites, especially when dealing with complex mixtures of metabolites such as it occurs in plant tissue.

The availability of computer hardware and software to interpret and visualize large data sets has led to many improvements in the throughput of these analytical methods (Gehlenborg *et al.*, 2010). Such improvements have also allowed to rapidly and simultaneously identify and quantify an increasing number of compounds (e.g., carbohydrates, amino acids and peptides, lipids, phenolic compounds and terpenoids). These advances will enable us not only to take 'static pictures' or snapshots of the metabolome, but also to capture dynamics of the metabolome.

1.1.4 Relationship between stoichiometry and ecometabolomics

Parallel to ecometabolomics, complementary simultaneous analogue stoichiometric analyses have been performed in some studies (Rivas-ubach *et al.*, 2012, 2014). While stoichiometry is considered the baseline of the metabolome, it fails to provide global information about which processes are taking place at a metabolic level in the plant. A relationship between metabolomic and stoichiometric analyses, i.e. between the metabolome of an organism and its elementary composition, in response to abiotic or biotic factors was demonstrated in a previous work of the group (Rivas-Ubach et al. 2012). In this study, it was found that the N:P ratio of *Erica multiflora* leaves decreased in the metabolically active growing season, coinciding with an increase in the content of primary metabolites and illustrating that the elemental composition correlates mostly as parts of molecular compounds or metabolites. Shifts in the elemental stoichiometry of organisms in response to their ontogeny and to changing environmental conditions should be related to metabolomic changes because elements are integrated in the metabolome of the organism.

1.2 Chemical analysis techniques

Analytical chemistry is at the heart of metabolomics. Chemical analysis techniques, such as nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography and gas chromatography hyphenated to mass spectrometry (LC-MS and GC-MS) among others, allow the detection and the identification of metabolites of biological samples and they are the basis of they metabolomic studies. Metabolomics is a transversal tool which requires the combination of chemical analysis with other fields such as experimental design, data processing and statistics. The recent progress made in data processing and statistics has allowed the advance in metabolomics research. Advanced methodology is needed to measure large numbers of metabolites over a short time period and there are many technical challenges. In the past, studies have therefore tended to pinpoint measurements of the levels of individual preselected substances only.

1.1.5 Elemental analysis (stoichiometry)

Elemental analysis is a powerful tool for the determination of carbon (C), hydrogen (H), nitrogen (N), sulfur (S) and oxygen (O) concentrations by combustion coupled to gas chromatography. Samples are packed into lightweight containers of oxidizable metal, and dropped at preset times into a vertical quartz tube, heated to 970°C, through which a constant flow of helium stream is maintained. When the sample is introduced, the helium stream is temporary enriched with pure oxygen. Flash combustion takes place, enhanced by the oxidation of the container. Quantitative combustion is then achieved by passing the combustion products over several catalysts and specialized oxidation reagents producing carbon dioxide (CO_2), water (H_2O) and nitrogen (N_2) from the elemental carbon, hydrogen and nitrogen respectively. Gases are captured in the mixing chamber of the gas control zone. Here, gases are rapidly mixed and precisely maintained at controlled conditions of pressure, temperature and volume. By controlling the product gases from combustion/pyrolysis to the same exact conditions (pressure, volume and temperature) for every run, outside influences (barometric pressure changes, altitude) are eliminated. The combustion process is separated from the column and detector which gives the flexibility of varying combustion conditions in the same series of runs without influencing separation and detection and the gases are mechanically homogenized therefore providing precision and accuracy. The amount of CO_2, H_2O and N_2 produced is determined using frontal gas chromatography.

Inductively coupled plasma/optical emission spectrometry (ICP/OES) is a powerful tool for the determination of metals in a variety of different sample matrices. With this technique, the sample solutions are converted to an aerosol and are injected into a radiofrequency (RF)-induced argon plasma using one of a variety of nebulizers. At its core, the inductively coupled plasma (ICP) sustains a temperature of approximately 10000 K, so the aerosol is quickly vaporized. Analyte elements are liberated as free atoms in the gaseous state. Further collisional excitation within the plasma imparts additional energy to the atoms, promoting them to excited states. Sufficient energy is often available to convert the atoms to ions and subsequently promote the ions to excited states. Both the atomic and ionic excited state species may then relax to the ground state via the emission of a photon. These photons have characteristic energies that are determined by the quantized energy level structure for the atoms or ions. Thus the wavelength of the photons can be used to identify the elements from which they originated. The total number of photons is directly proportional to the concentration of the originating element in the sample. The instrumentation associated with an ICP/OES system is relatively simple. A portion of the photons emitted by the ICP is collected with a lens or a concave mirror. This focusing optic forms an image of the ICP on the entrance aperture of a wavelength selection device such as a monochromator. The particular wavelength exiting the monochromator is converted to an electrical signal by a photodetector. The signal is amplified and processed by the detector electronics, then displayed and stored by a personal computer.

1.1.6 Analytical techniques for ecometabolomics

Metabolomics requires the simultaneous measurement of a large number of metabolites, NMR spectroscopy and LC-MS play a leading role in this field. Currently, no single analytical method or combination of methods (i.e., chromatography coupled with mass spectrometry) can detect all metabolites (estimated to be between 100,000 and 200,000 in the plant kingdom) within a given biological sample. NMR and MS techniques show the best capacity to detect, identify and quantify molecules, being both techniques amenable to undirected profiling of a broad range of metabolites and generate quantitative measurements. However, each technique has inherent advantages and drawbacks.

1.1.6.1 NMR spectroscopy

NMR spectroscopy has the advantage of providing an unbiased overview of all the small molecules in the solution, with minimal sample preparation, non-destructively and relatively quick. It is effective for targeted and untargeted studies (Krishnan *et al.*, 2005) and suitable for the analysis of a wide set of molecules with different polarities, polar, semipolar and nonpolar (Rivas-ubach *et al.*, 2012). ^{1}H NMR signals are directly and linearly correlated to metabolite abundance (Lewis *et al.*, 2007), leading to a straightforward quantification and avoiding calibration curves of standards (Kim *et al.*, 2010). It is a highly reproducible, robust technique and the results obtained are comparable independently of the NMR spectrometer used and of its magnetic field (Keun *et al.*, 2002b). This turns out very convenient for the identification of metabolites; since the NMR data of a new study can be compared with all prior data reported, as long as the solvent, temperature and pH of the sample are the same. Most of the reported data are compiled in useful open access NMR spectral databases, which allow the identification of most of the peaks of the spectrum. Because of the non-destructive nature of the technique, one can return to a sample and perform other NMR experiments, such as one-dimensional (1D) selective and two-dimensional (2D) NMR experiments which provide valuable structural and conformational information, and assign unknown resonances to its correspondent metabolite.. Other stable nuclei such as ^{15}N and ^{31}P have been successfully measured *in vivo* NMR metabolomics in ecophysiological studies (Kikuchi *et al.*, 2004; Lundberg & Lundquist, 2004). All this makes NMR spectroscopy the key technique for the elucidation of unknown metabolites, making possible the identification of most of the metabolites detected and being even capable to differentiate between structural isomers, diastereoisomers (Pérez-Trujillo *et al.*, 2010; Ellis *et al.*, 2012), and even between enantiomeric molecules (chiral metabonomics)(Pérez-Trujillo *et al.*, 2012).

For ^{1}H NMR, the concentration threshold for routine detection of a metabolite in an extract using a modern high field spectrometer is roughly 10 µM, corresponding to a quantity of 6 nmol in the typical sample volume of 600 µl. In practice, the achievable sensitivity is strongly dependent on the field strength of the magnet, and on the design of the probe head that allows the signals to be detected. NMR spectrometers are available with field strengths up to 23.5 Tesla, corresponding to a ^{1}H NMR frequency of 1000 MHz, but most metabolic analyses are conducted on the more commonly available instruments that operate in the range 400–600 MHz. Compared to MS-based techniques, NMR–based metabolomics has a main drawback which is its inherent low sensitivity - typically a few tens of metabolites can be detected and identified within a sample. The availability of high magnetic field spectrometers as well as the

use of cryogenically cooled probes (which can enhance the signal to noise ratio, S/N, up to a factor of four by decreasing the thermal noise), has significantly improved the sensitivity of this technique, permitting even the routine analysis of low abundance nuclei, as ^{13}C, for metabolomic studies (Keun *et al.*, 2002a).

The 1D ^{1}H spectrum is the most common NMR-based fingerprinting tool, due to the high sensitivity of the experiment and to the linear correlation between the signal of the metabolite and its abundance in the sample. The main drawback of the experiment is the overlapping (often severe) of some of the proton signals. This overlap can be reduced by working with a high magnetic field spectrometer (since spectral resolution is increased). If necessary, fractionation of the sample by polarity through chromatographic methods or extraction methods prior to the analysis may help avoiding excessive signal overlap and, therefore, aid in the identification of known and unknown molecules. In this way, the use of the hyphenated on-flow LC-NMR technique could be of help (Lindon *et al.*, 1996). Another alternative is to analyze ^{13}C instead of ^{1}H nuclei; with an inherent more disperse spectrum (Keun *et al.*, 2002a; Palomino-Schätzlein *et al.*, 2011; Clendinen *et al.*, 2014). However, this could result in long experimental times. Other fingerprinting tools include the 2D NMR experiments ^{1}H *J*-resolved (^{1}H *J*-RES) (Vliegenthart *et al.*, 1983; Fonville *et al.*, 2010), ^{1}H-^{1}H correlation spectroscopy (COSY) (Marion & Wüthrich, 1983; Xi *et al.*, 2006), ^{1}H-^{1}H total correlation spectroscopy (TOCSY) (Bax & Donald, 1969; Sandusky & Raftery, 2005) and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) (Vuister & Bax, 1992; Lewis *et al.*, 2007). The ^{1}H *J*-RES experiment yields a spectrum that separates the chemical shift and spin-spin coupling data onto different axes, being the projection of the 2D dataset onto the chemical shift axis (termed p-*J*RES) considerably less congested than the 1D ^{1}H spectrum. COSY and TOCSY correlations provide information regarding ^{1}H,^{1}H scalar couplings (i.e. connectivities between atoms) and the HSQC experiment provide information regarding ^{1}H,^{13}C scalar couplings (^{1}H -^{13}C one bond connectivities). 2D experiments and particularly heteronuclear correlations are of great interest for overcoming overlapping problems due to the wide chemical shift range of the indirect (^{13}C) dimension. Besides, 2D NMR spectroscopy enables the detection of connectivity between signals and hence helping the identification of metabolites. When a metabolomic study aims to elucidate chemical structures that probably are unknowns, NMR-based metabolomics is the most adequate tool (see a recent review on this topic by Leiss et al. 2011).

1.1.6.2 LC-MS and GC-MS

Liquid chromatography and gas chromatography are both used as standard methods; when combined with MS these techniques are known as LC-MS and GC-MS, respectively. A mass spectrometer detects the mass-to-charge ratio (m/z) and abundance of the various analytes generated during the ionization of a sample extract or chromatographic fraction. Ionization is a key step since ions are far more easily manipulated than neutral molecules. Unfortunately, differing analytes vary in their ionization efficiencies, i.e. the proportion of metabolite in solution converted to ions in the gas phase. Once ionized, the mass analyzer detects the ion abundance and m/z, which can be related back to the analytes absolute molecular weight. A data system (run on a personal computer) is responsible for the storage of the paired m/z and abundance values as well as their processing and display in a mass spectral format. The three principal components found in all varieties of MS are an ionization source, a mass analyzer and a detector; all three components are maintained under vacuum to optimize the transmission of ions to the analyzer and detector (Niessen, 1998; Gross, 2004). MS has the disadvantage of destroying the sample as it is run, and requires a prior chromatographic separation, such as gas chromatography and high- or ultra-performance liquid chromatography (GC, HPLC, UPLC) to simplify the mixture and separate molecules as they enter the mass spectrometer. The separation prevents unpredictable interactions between molecules affecting the levels of ionization, that may in turn bias quantification, and some molecules may be resistant to ionization entirely.

Prior to ionization, the sample must first be introduced into the ionization source. A probe is commonly employed in both LC- and GC-MS analyses. The sample is introduced either by manual direct infusion (DI) or via HPLC or UHPLC followed by DI, following which the sample is delivered to the probe via a capillary; the probe functions to translocate the liquid sample from the capillary to the needle of the ion source. GC-MS, LC-MS are the procedures with the best capacity to detect the widest ranging sets of metabolites (from tens to hundreds of metabolites). GC-MS has proven to be a robust tool for the study of volatile organic compounds (Degen *et al.*, 2004; Llusià *et al.*, 2010); however, GC-MS analyses of extracts containing other analytes such as organic acids, sugars, amino acids and steroids is complicated. Many metabolites are non-volatile and must be derivatized prior to GC-MS analysis (Gullberg *et al.*, 2004). Using GC-MS, thermolabile compounds may be lost. In addition, it is difficult to elucidate the unknown structures of metabolites by using GC-MS alone. LC-MS is of particular importance to study a great number of metabolic pathways at once like in plant metabolism, which embodies a huge range of semi-polar compounds

including many key groups of secondary metabolites (Allwood & Goodacre, 2010). Thus, while GC-MS is best suited for compound classes appearing mainly in primary metabolism (frequently after derivation), i.e., amino acids, fatty acids and sugars or volatile compounds, LC-MS is more adequate to determine the overall biochemical richness of plants including several semi-polar groups of secondary metabolites.

Two ionization sources are generally employed in LC-MS based metabolomics: electrospray ionization (ESI) is the most commonly employed (Tolstikov *et al.*, 2003; Jander *et al.*, 2004; Hanhineva *et al.*, 2008) and is particularly well suited to the ionization of a wide range of metabolites including, drug compounds, amino and organic acids, sugars and sugar alcohols, sterols, steroids, phospholipids and fatty acids; however, atmospheric pressure chemical ionization (APCI) is also an appropriate method, especially for the ionization of non-polar metabolite species such as phospholipids, fatty acids, sterols, steroids, certain esters and essential oils, one classic example in plant studies being the analysis of lipid soluble carotenoids (Dachtler *et al.*, 2001; Schweiggert *et al.*, 2005).

Essentially, the hardware requirements for APCI and ESI are very similar; the needles for sample introduction the are nearly identical with the inner needle having a central channel for translocation of sample and outer channels for the delivery of nebulization and desolvation gases. The arrangement of sample and extraction cones is also nearly identical. The core difference between each ionization method is that in ESI the sample is ionized by application of a high electric charge to the sample needle, whereas in APCI the sample is ionized by application of a high charge to a corona pin after the sample has exited (been sprayed) from the sample needle.

ESI can be applied in both positive and negative ionization polarities, depending on the capillary voltage that is applied (typically ∼+3 kV and ∼−2 kV, respectively). In ESI+ mode, common adducts include protonated [M + H]+, sodiated [M +Na]+, and potasiated [M + K]+. In ESI− mode the most common ions are deprotonated [M − H]−, although when chlorine is present within the sample metabolites, negatively charged chlorine adducts may be also observed. Many further complex and diverse adduct species can be observed in both ionization modes.

In comparison to alternative ionization techniques, ESI has several key advantages, including ionization across a large mass range appropriate for proteome and metabolome analysis, good sensitivity, soft ionization (i.e. does not ‘over’ fragment target analytes) and high adaptability (Gaskell, 1997). It is largely the mass range that can be covered by ESI that has made it one of the most widespread ionization techniques in use, since it is applicable to both metabolite and peptide/protein analysis. However, ESI also has several disadvantages,

including adduct formation, quenching, low tolerance towards salts and suppression of the ionization of one metabolite species due to the presence of a high concentration of a different species (i.e. matrix effects), both leading to ionization suppression (King *et al.*, 2000) and failure to detect certain metabolite classes/species.

Analyzers can be grouped into classes on the basis of many properties, including ion beam versus ion-trapping types, continuous versus pulsed analysis, operation using low versus high translational energy ions, and on the basis of the time scale of the analysis and the pressure required for optimum performance. The Orbitrap mass analyzer, the analyzer used in this PhD thesis, have a similarity to an earlier ion storage device, the Kingdon trap, as well as to two types of ion-trapping mass analyzers, the Paul trap (quadrupole ion trap), and the Fourier transform ion cyclotron resonance instrument. The Orbitrap is a powerful mass spectrometer that can be used to examine a variety of types of chemical systems. It provides high resolution, high-mass accuracy, and good dynamic range. The instrument has been applied to a wide range of analytes, for this reason it is convinient to be used in metabolomics.

1.1.6.3 Comparison of analytical techniques

The next question to consider is the extent to which NMR spectroscopyor MS measures up to the metabolomic ideal of a high-throughput, system-wide analytical technique and, in particular, to consider the advantages and disadvantages of NMR relative to MS. Sensitivity is an important requirement for metabolomics, since high sensitivity favors the rapid analysis of a greater fraction of the metabolome. The main advantage of MS detection is its high sensitivity. ^{1}H NMR spectroscopy, with a detection threshold of ca. 6 nmol, is several orders of magnitude less sensitive than MS, which has a detection threshold of 10^{-3} nmol (Sumner *et al.*, 2003), making possible the detection of hundreds of metabolites within a sample. However, the identification of detected metabolites is difficult and often not as condusive as NMR spectroscopy. This is due to: (i) the chemical information obtained by MS, and also MSn, is not as rich as that obtained by NMR spectroscopy for instance, the distinction between structural isomers, diastereoisomers and enantiomers is difficult and most times impossible by solely MS; (ii) the technique is not as reproducible when compared across platforms as NMR spectroscopy, making the identification of metabolites by comparison to prior described data not as reliable though the availability of MS libraries (Chemspider (Pence & Williams, 2010), HMDB (Wishart *et al.*, 2007), METLIN (Smith *et al.*, 2005; Wishart *et al.*, 2007), MZedDB (Draper *et al.*, 2009) or KEGG (Ogata *et al.*, 1999)). Researchers, however, can build up their

own spectral libraries of standards, using the same specific method and equipment used in their metabolomic study, though that could mean a long time consuming and expensive work. When dealing with the identification of an unknown metabolite, and also with the unequivocal identification between possible structural isomers, knowing the exact mass, the chromatographic retention time and also the MS/MS fragmentation pattern, is in most cases not conclusive to confirm its structure. The unequivocal and complete characterization of an unknown metabolite demands on almost always the use of NMR spectroscopy (Sumner et al. 2003; Verpoorte et al. 2008).

GC-MS has proven to be a robust tool for the study of volatile organic compounds (Hu *et al.*, 2005), but it is not efficient enough to analyze other less volatile metabolites such as organic acids, sugars, amino acids and steroids as they must be derivatized prior to GC-MS analysis (Gullberg *et al.*, 2004). Besides, utilizing GC-MS, thermolabile compounds may be lost. These facts reduce its fields of application.

Currently, LC-MS is gaining prominence in metabolomic studies (Want *et al.*, 2010). It takes particular importance in the study of a great number of metabolic pathways at once. Plant metabolism embodies a huge range of semi-polar compounds, including many key groups of secondary metabolites, which can be detected through LC-MS (Allwood & Goodacre, 2010). The identification of these metabolites is mainly done by GC-MS and LC-MS spectral libraries, which is quick. However, sometimes the level of certainty associated to some compound identification is less than 50%. For example, Kluender et al. 2009 using GC-MS detected 283 metabolites but it was only possible to identify 39. On the other hand, Jones et al. 2008 used both techniques to analyze the same samples and identify 32 molecules by NMR and detected 51 by GS-MS but only identify 42 (Sardans *et al.*, 2011).

In fact, a simple calculation suggests that both NMR and MS should be capable of detecting signals from the whole metabolome, provided the extraction procedure is scaled correctly. Given that the Michaelis constants of most enzymes are in the range 1 µM to 10 mM, it is likely that 1 µM is the lower limit for the concentration of most intracellular metabolites. Assuming that a metabolite with this concentration is restricted to just 10% of the tissue volume, the tissue content would be 0.1 nmol g^{-1} fresh weight. Thus the extraction of 50 g of tissue should permit the detection of the whole metabolome by ^{1}H NMR, and just 10 mg of tissue should be sufficient for MS. If it is further assumed that a typical cell might contain 5000 metabolites, then it appears that MS identifies considerably less than 5% of the metabolome, even though the whole metabolome is potentially detectable. This argument ignores any bias against particular classes of compound arising from the extraction method, but it serves to emphasize that sensitivity is not the only issue for metabolomic techniques, and that the difficulty of

detecting minor components in the presence of much larger signals may be the most serious obstacle to a complete analysis (Krishnan *et al.*, 2005).

Overlapping signals, and the dynamic range problem associated with a potential concentration range of some five orders of magnitude, limit the scope of both MS and NMR for metabolomic analysis. For example, minor signals in MS can be difficult to identify in the isotopomeric noise surrounding the mass ions of major components. However, the precision and range of the mass measurements is an advantage in unravelling the mass spectrum, as is the routine use of GC to fractionate the extract. Similarly, the restricted chemical shift range in a one-dimensional ^{1}H NMR spectrum is a major hindrance to metabolite identification at even the highest magnetic fields, but the problem is alleviated by using two-dimensional NMR techniques or by fractionating the sample with LC.

NMR also has a slight advantage for quantitative analysis, since the high stability of modern spectrometers makes this task straightforward, by contrast with MS where frequent calibration and variable retention times can complicate quantitative analysis. Both techniques usually generate multiple signals, which is an advantage for metabolite identification and a disadvantage in terms of spectral complexity. However, in MS, some of this multiplicity comes from the fragmentation of the mass ion, complicating quantitative analyses; whereas in NMR, multiple signals arise directly from the same molecule and thus provide a cross-check on metabolite quantitation.

Considering the features described above, in this thesis we have used modern NMR spectroscopy (Rivas-ubach *et al.*, 2012) combined with LC-MS techniques, which yields a comprehensive picture of an organism’s metabolome, and allows the detection, quantification and identification of compounds of interest. Simultaneously combining these tools with elemental analysis (stoichiometry), we are able to gain knowledge on the biogeochemical and metabolomic basis of the structure and function of the ecosystem in relation to the environment and interpret changes within the metabolome in relation to elemental stoichiometry.

1.3 General method for NMR-based ecometabolomics

An optimized protocol for ecometabolomic studies was described recently by our research group (Rivas-Ubach *et al.*, 2013). The method provides an unbiased overview of the metabolome of an organism, including polar and nonpolar metabolites. This protocol was tested and optimized by using two field plant species (*Erica multiflora* and *Quercus ilex*) sampled once per season (Rivas-ubach *et al.*, 2012, 2014).

The method provides an overall analysis of the main metabolites in field samples, including secondary and nonpolar compounds. In addition to NMR fingerprinting, it identifies metabolites for generating metabolic profiles, mainly through the analysis of complex mixtures by NMR experiments, applying strategies of elucidation of small molecules typically used in natural product research, and allowing the identification of secondary and unknown metabolites.

^{1}H NMR signals are directly and linearly correlated to metabolite abundance, leading to a straightforward quantification that avoids the need for calibration curves of standards.

The described protocol is reproducible and amenable to robust statistical analyzes and covers sample storage and preparation, the acquisition of NMR fingerprint data of all samples, the identification of metabolites for providing a metabolic profile (based on the analysis of complex mixtures by NMR spectroscopy and the statistical analyses of the NMR data (Fig. 1.2). The protocol offers a detailed description of the raw data coming from the ^{1}H-NMR spectra to perform the statistical analyses such as PCAs, PLS-DAs or PERMANOVAS.

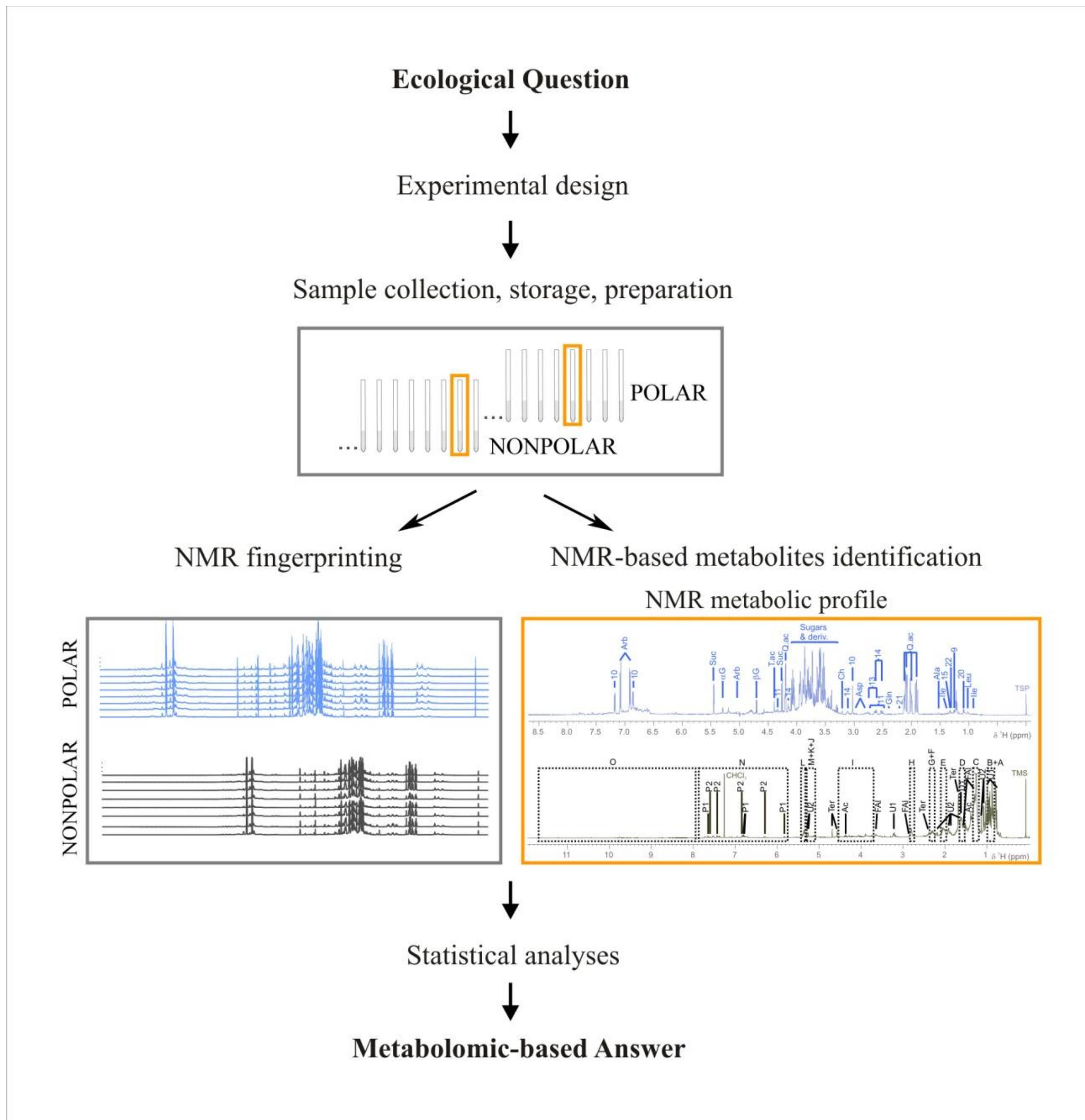

Figure. 1.2 General NMR-based procedure for an ecometabolomic study.

1.4 Ecological questions

Metabolomic analysis is a measure of the phenotypic expression and in the last two decades it has been increasingly recognized by ecologists as a good potential tool to capture global organism's functional shift in response to environmental changes. Metabolomics supposes an extra advantage due to the possibility to be applied to a wide array of organisms given the universal nature of metabolites throughout the biological taxonomy spectrum (Bundy *et al.*, 2009). However, until the last few years studies were still scarce and in most cases they were limited to the direct effects of a unique abiotic factor or of biotic interactions between two trophic levels under controlled conditions and, moreover, limited to a specific organism organ (Sardans *et al.*, 2011). There is a lack of studies conducted in more natural conditions and also involving more than two trophic levels, or combining the effects of abiotic gradients and also combining metabolomics with the study of other important ecological variables such as growth, nutrient use, composition and stoichiometry. These studies should allow a more robust assessment of the functional shifts of the global organism, in order to better understanding the relationships between other variables changes with the functional shifts of the overall organism; and finally, be able to relate to environmental changes with several organism traits that further implied feed-backs with ecosystem. As an example, to discern the functional implications of the organisms stoichiometry composition, nutrient uptake and use changes linked to environmental shift would be an step forward in ecological studies by improving the knowledge of the multiple feed-backs and mutual dependences between sources availability and cycles with global ecosystem structure and function.

Given the simplified perspective of previous work in this field, this thesis aims to make and step forward in coupling metabolomics analysis with ecological studies by exploring the following issues.

The metabolome of the whole plant including shoots and roots and therefore root-shoot metabolomic differences and relationships and the capacity of an organism to adapt to environmental stress (Chapter 4 and 5),

Discern whether mechanical wound injury in leaves changes the metabolome of a widespread Mediterranean tree, *Quercus ilex* (holm oak) (Chapter 6)

Discern how the metabolic profile of the plant surfaces changes when epiphytic microorganisms are suppressed (Chapter 7)

Chapter 2

Objectives

The objectives of this PhD thesis are divided into: objectives related to the methodology, specific objectives of each ecometabolomic study and general objectives.

The objectives related to the methodology consist of:

To modify the protocol described previously in the group to perform NMR-based ecometabolomics (A.Rivas-Ubach et al. MEE), in order to extend it to the performance of combined NMR- and LC-MS-based ecometabolomics and stoichiometrical analysis; with special interest in the adaptation of the sample preparation procedure to the multiple analyses. Results regarding these objectives are described in Chapter 3.

The specific objectives of the ecometabolomic studies are the following:

Study the metabolism and stoichiometry of plant shoots and roots in response to - drought and warming - in order to better understand each organ role in the acclimation to stress. Study if there is any relationship (or correlation) between the metabolic response and the stoichiometric response of the overall plant and of each organ -shoot and root. To perform this study two species of grasses were chosen: *Holcus lanatus* and *Alopecuris pratensis* .This study is addressed in Chapter 4.

To extend the previous study to the survey of the impact of the factorial combination of simultaneous warming and drought (water availability) on plant shoots and roots along different seasons. To test the hypothesis that warming differentially influences the effects of drought on the stoichiometry and metabolome of shoots and roots. The results and discussion of these objectives are described in Chapter 5.

To discern whether mechanical wound injury in leaves changes the metabolome of the widespread Mediterranean tree *Quercus ilex* L. (holm oak), that is one of the most abundant species in Mediterranean forests. This species has, been reported to suffer from episodic outbreaks of herbivores like *Lymantria dispar* L. (Staudt & Lhoutellier, 2007) which could potentially lead to changes in its metabolism by producing inducible defenses with posterior effects at different levels, such as signal molecules for neighbor plants or for other organs of the same individual plant. This study is addressed in Chapter 6.

To understand better the relationship between the epiphytic microorganisms and the plant by: (i) determining the changes in the metabolic profile of the plant surface when epispheric microorganisms are suppressed, (ii) determing the changes in the metabolic profile inside the plant organs when epispheric microorganisms are suppressed, and (iii) studying the

similarities and differences between the internal and epispheric metabolomes. The objectives also allow (iv) to investigate the synergies and antagonisms between the metabolic functions of the plants and the epispheric microorganisms. The species *Sambucus nigra* was chosen to perform the study (Chapter 7).

The general objectives of this PhD thesis are the following ones:

To consolidate the protocol and procedures for the performance of NMR-based and LC-MS-based ecometabolomics combined with stoichiometrical analysis.

To have a better understanding of the relationship between the information obtained from metabolome analysis and from stoichiometric analysis.

To have a deeper knowledge of the effects of environmental changes on the metabolome and stoichiometry of terrestrial organisms and on their organs.

II. RESULTS AND DISCUSSION

Chapter 3

Adaptation of the procedure to perform NMR- and LC-MS-based ecometabolomics and stoichiometrical studies

3.1 General procedure for ecometabolomics combined with ecological stoichiometrical studies

This section describes the general procedure followed to perform ecometabolomic and stoichiometric studies, which encompasses seven main points: 1) the formulation of the ecological question; 2) the experimental design; 3) sample collection, storage, extraction and preparation; 4) measurement (for fingerprinting and metabolites identification and for stoichiometry analysis) supported by different analytical platforms (NMR spectroscopy, LC-MS, ICP-OES and elemental analysis); 5) data processing 6) statistical analysis (chemometrics) and 7) interpretation of the results (obtention of a metabolomics- and stoichiometric-based answer).

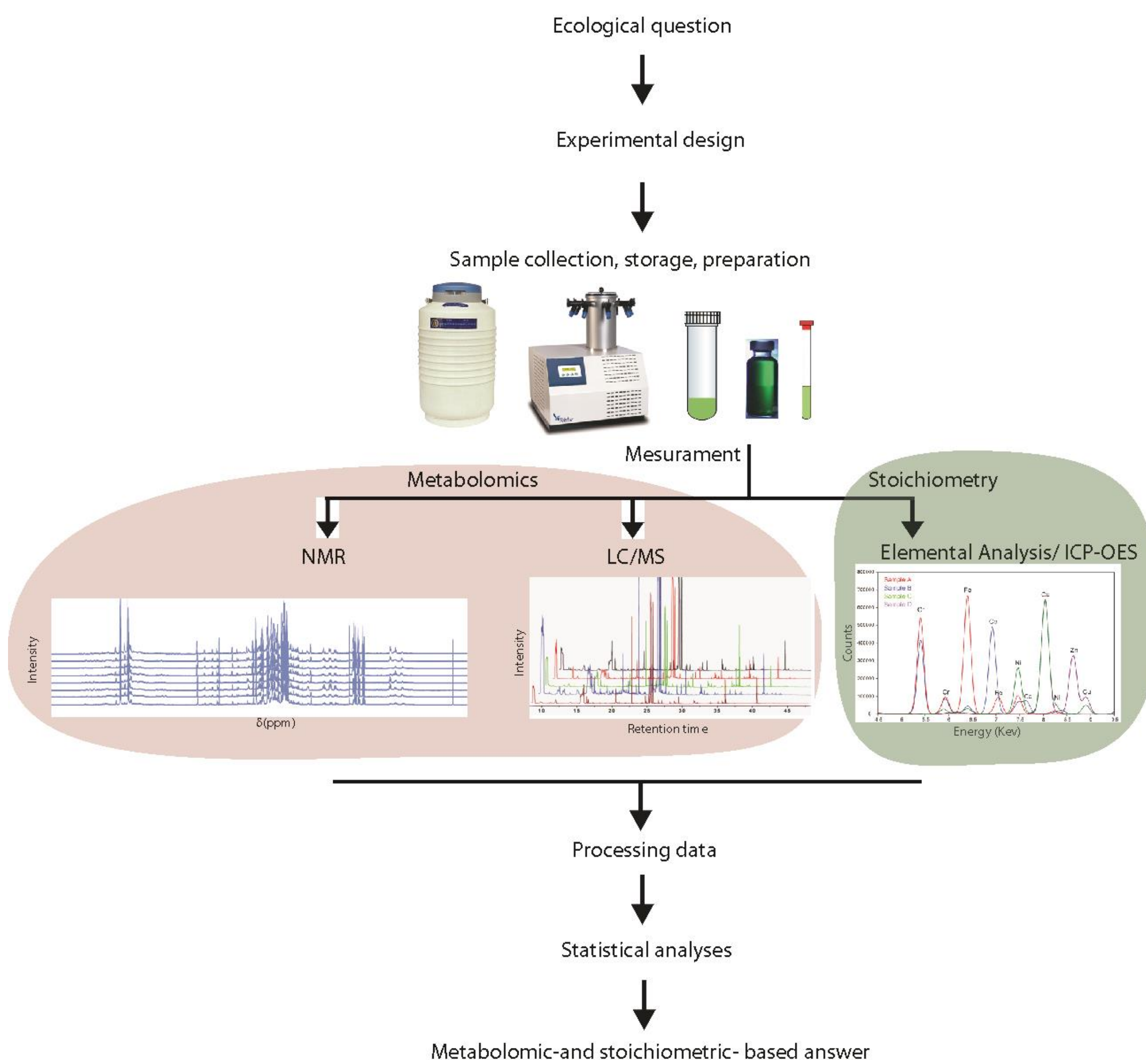

Figure 3.1. General procedure for ecometabolomic- and stoichiometric- studies.

3.1.1 Ecological questions

As in any scientific research, the question that wants to be tackled must be clear and well definied at the beginning.

The specific ecological questions tackled in this PhD thesis are described in the objectives section and, more in detail, in the following chapters.

3.1.2 Experimental design

Once the ecological question has been formulated the experiment is designed. In this part, the different treatments or conditions that are going to be studied and in which species are considered. Other important considerations are:the selection of the samples (e.g. which time and timing of sample collection, which kind of sample), the number of replicates, the analytical platforms that are most convenient for the study, the timing of the whole process, etc.

The experiments described in this PhD thesis explore the effect of a wide variety of abiotic and biotic factors on plant metabolism. The experimental designs are detailed in the following chapters.

3.1.3 Sample collection, storage, extraction and preparation

During the processes of sample collection, storage and preparation prior to the analysis, it is very important to avoid the degradation of the sample, i.e. to ensure that the metabolome keeps unaltered. To prevent post-sampling degradation of metabolites and alteration of the organism's metabolome, samples must be immediately frozen (commonly by introducing them immediately into liquid N_2) and then lyophilized until complete dryness (Kim *et al.*, 2010). As no single solvent allows all metabolites to be extracted, combinations of several different solvents can be used. Kim et al. 2010 have tested different extraction methods in plant metabolomics. The use of a two-phase solvent system (polar and non-polar), composed of a mixture of chloroform, methanol and water (2:1:1, v/v/v), has proven to yield good results (Choi *et al.*, 2006).

A protocol for NMR-based ecometabolomics, covering from the sample collection, storage and preparation to the statistical analysis of the NMR data, was described by our research group (Rivas-Ubach *et al.*, 2013). For the objectives of this PhD thesis, that protocol has been extended, covering now the combined analysis of the sample by LC-MS, NMR spectroscopy and elemental analysis. Accordingly, the sample preparation procedure has been modified to cover now the three analytical techniques; meaning that for each individual three samples are

prepared and analyzed. A scheme showing the modified protocol from sample storage to sample analyses is presented in Figure 3.3.

Several protocols to explore the metabolome in humans and animals (Beckonert *et al.*, 2007; Jukarainen *et al.*, 2008; Le Guennec *et al.*, 2012) and in microorganisms (Smart *et al.*, 2010; Roberts *et al.*, 2012) have recently been published. Plants have received less attention, but some methods for conducting metabolomic analyses of laboratory subjects based on NMR spectroscopy have been described (Kruger *et al.*, 2008; Kim & Verpoorte, 2010; Kim *et al.*, 2010) and also for those based on GC-MS and LC-MS (Wilson *et al.*, 2005). The field of ecology currently lacks a standard protocol for the analysis of the metabolome of wild plants, which are sampled from the field under very heterogeneous environmental conditions. A protocol is thus needed to provide an overall analysis of the main metabolites in field samples, including secondary and nonpolar compounds, and which allows the detection and identification of those metabolites that play a key role in an organism's response to environmental change. The protocol must also be reproducible and amenable to robust statistical analyses.

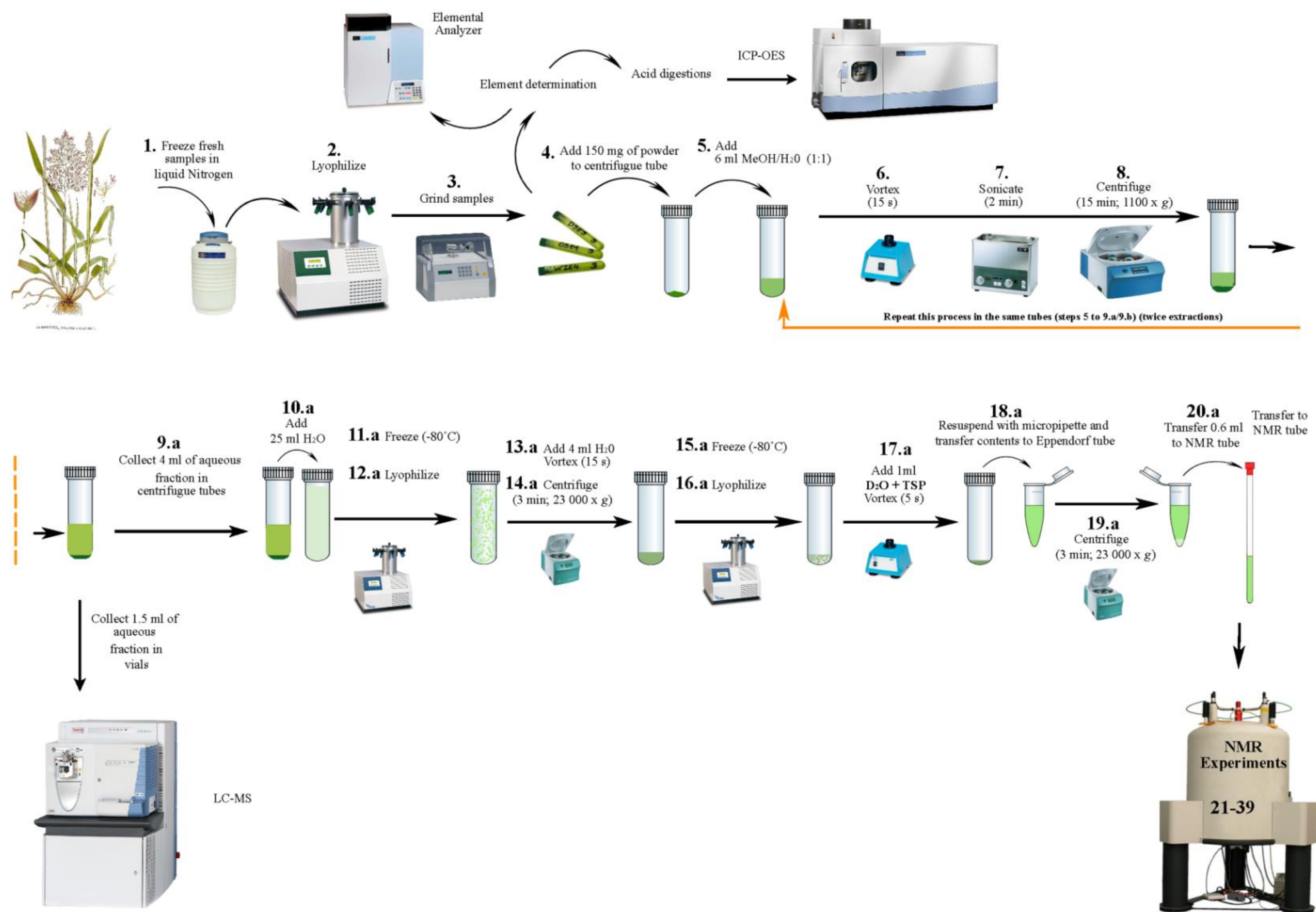

Figure 3. 3. Experimental procedure for the preparation of extracts of plant tissue for its posterior NMR, LC-MS and elemental analyses.

3.1.3.1 Sample collection and storage

Generally, fresh plant material is collected and rapidly packed, labelled, and frozen in liquid N_2 to prevent the degradation of metabolites (Kim & Verpoorte, 2010). Plant physiological processes vary throughout the day, so individual subjects are typically sampled within a narrow range of time and under a constant environment. The frozen plant material is lyophilized and kept in plastic cans in desiccators or frozen. A rapid freeze-drying is a crucial step in ecometabolomics, since lyophilization avoids the hydrolysis of metabolites and maintains inactive any enzymatic reaction. Following lyophilization, samples are ground with a ball miller. Grounding time may vary depending on the plant species and tissue type (steps 1 to 3 of Fig. 3.3)

3.1.3.2 Metabolite extraction and sample preparation for ecometabolomics

The general process of extraction of metabolites of powdered samples is depicted in steps 4 to 8 of Figure 3.3. Briefly, an exact amount of the sample is added to a centrifuge tube and extracted with a water/methanol mixture, followed by vortex, sonication and centrifuge processes, which are repeated twice. At this point, an aliquot of the supernatant consists in the sample for the LC-MS analysis. The rest of the supernatant is enriched in H_2O and lyophilized until completely dry. Finally, the solid is dissolved in a phosphate buffered D_2O solution containing 0.01 % of 3-(trimethylsilyl)propanoic acid (TSP), that acts as an internal reference in the NMR analysis (steps 9 to 20).

The detailed description and conditions for metabolite extraction and sample preparation used in each ecometabolomic study performed in this thesis are explained in chapters dedicated to each specific study (Chapters 4 to 7).

3.1.3.3 Sample preparation for stoichiometric analysis

The powdered sample obtained after grounding is directly used for the elemental analysis.

3.1.4 Data acquisition

3.1.4.1 Stoichiometric data

C and N concentrations are determined from the powdered sample by combustion coupled to gas chromatography using a CHNS-O elemental analyzer. P, K, Fe, Mn, Mg, Ca, and S concentrations are determined by means of extraction by acid digestion in a microwave reaction system under high pressure and temperature. The elemental concentrations are determined by ICP-OES (more details of the technique in section 1.2).

3.1.4.2 Metabolic fingerprinting

At this point a NMR- and a LC-MS-based metabolic fingerprint of each sample are acquired. Like in the previous step (sample collection, storage and preparation), the analyses of all samples must be performed exactly under the same conditions to guarantee the quality of the data and, therefore, the quality of the final results.

3.1.4.2.1 NMR-based fingerprinting

The NMR-based fingerprint of the metabolome of a biological sample consists of a quantitative NMR spectrum. The spectrum presents unassigned signals that correspond to the different metabolites in the sample, and the signal intensities directly correlate to metabolite concentrations (Ludwig & Viant, 2010). The spectra must be obtained under specific defined conditions.

Experiments are acquired using an autosampler and following a defined method for the acquisition and basic processing of the spectra. All experiments are acquired at a constant known temperature. For this, the temperature of the equipment is calibrated (using a standard methanol sample) and maintained constant at 298.0 K. To ensure that, once the tube is in the core of the magnet and before starting the acquisition, a temperature equilibration delay (2-4 min) is defined in the method.

Processes such as insertion/ejection of the sample into the magnet, temperature equilibration delay, automatic locking onto the signal of the deuterated solvent, homogenization of the magnetic field, adjustment of the receiver gain, and the execution of the experiment (parameter set configured previously), which includes the acquisition of the FID, Fourier transform, and the pre-processing of the spectrum are automatized.

For ^{1}H NMR metabolic fingerprinting, samples are analyzed using a standard quantitative 90° pulse-acquisition ^{1}H NMR experiment with solvent suppression (Zheng & Price, 2010). The standard water pre-saturation experiment, a conventional composite 90° ^{1}H pulse sequence with suppression of the residual water signal (Bax, 1985), or a 90° ^{1}H NOE enhanced pulse sequence commonly termed 1D NOESY-presat (Nicholson *et al.*, 1995) are adequate (Tab. 3.1).

Before starting the analysis of one set of samples, the correct 90° ^{1}H pulse length must be determined on a representative sample of the set; for polar samples, the offset of the water signal must also be determined for its optimal suppression. These values must be saved and used for the complete set of experiments. Samples are then analyzed separately running automatically the set-up experiments. All spectra must then be visually inspected, and those that are poorly phased or baseline corrected must be corrected manually. Finally, the spectra must be calibrated using the signal of the internal calibrating reference added to the sample.

3.1.4.2.2 LC-MS-based fingerprinting

The LC-MS fingerprint of the metabolome of a biological sample consists of the MS signal-based chromatogram. Each chromatographic peak corresponds to a metabolite eluting at a specific retention time and with a concrete MS spectrum (sometimes a peak can correspond to more than one metabolite eluting together) and the peak area correlates to the metabolite concentration in the sample. Thus, each chromatographic signal (retention time and signal intensity) can be assigned to a metabolite. Chromatograms are obtained under specific optimized conditions.

The LC-MS-based ecometabolomic studies performed throughout this PhD thesis followed the method described in Rivas-ubach *et al.*, (2014). A reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3-µ particle size) at 30 °C was used. The mobile phase consists of a mixture of acetonitrile (A) and water (0.1% acetic acid) (B) solutions. Both solutions are filtered and degased for 10 min in an ultrasonic bath prior to use. The elution gradient, at a flow rate of 0.3 mL per minute, begins at 10% A (90% B) and is maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10% A and 90% B) is gradually recovered over the next 5 min, and the column is then washed and stabilized for 5 min before the next sample is injected. The injection volume of the samples is 5 µL. HESI is used for MS detection. All samples are injected twice, once with the ESI operating in negative ionization mode (-H) and once in positive ionization mode (+H). The Orbitrap mass spectrometer used was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60 000). The resolution and sensitivity of the spectrometer are

monitored by injecting a standard of caffeine after every 10 samples, and the resolution is further monitored with lock masses (phthalates). Blank samples are also analyzed during the sequence.

3.1.4.3 NMR metabolic profiling

The NMR metabolic profile of a sample is obtained when each peak of the NMR spectrum is assigned to its corresponding metabolite. The profile gives the NMR signals a biomolecular meaning (Ludwig & Viant, 2010). This analysis is usually performed on a single representative sample, which could be any of the NMR samples of the fingerprinting procedure or one originating from different samples of the study and prepared following the same described procedure. The differences observed among samples are mainly due to differences in metabolite concentrations. Qualitative differences, however, may occur. The assignment of a specific peak is sometimes not possible (for example, when peaks overlap or when signals have a low intensity). In these cases, the analysis of another sample can be of help. A visual inspection of all fingerprinting spectra can help to find a better sample for the elucidation of a specific signal.

The assignment of ^{1}H NMR signals is conducted following two approaches. Firstly, by comparison of the resonance frequencies (chemical shifts, δ) and line shapes (multiplicity and coupling constants, *J*) of the spectrum to bibliographical data (Breitmaier *et al.*, 1979; Gunstone, 1995; Fan, 1996; Fan & Lane, 2011) and NMR spectral databases (BMRB) (Ulrich & Zhulin, 2007), MDL (Lundberg *et al.*, 2005), HMDB (Wishart *et al.*, 2009), MMCD (Cui *et al.*, 2008). Secondly, by the structural elucidation of the mixture (sample) through the performance of a suite of 2D NMR experiments (Tab. 3.1) and the concerted analyses of the data obtained. Basic NMR strategies followed for the structural characterization of natural products are applied for the elucidation of complex mixtures of small biological molecules (metabolites), instead of to isolated molecules as in the case of natural products research (Robinette *et al.*, 2012). Briefly, protons connected by three to five chemical bonds are identified using 2D ^{1}H-NMR homonuclear COSY and TOCSY correlations. ^{1}H-^{1}H NOESY experiments determine connections between different parts of a same molecule, and heteronuclear ^{1}H-^{13}C HSQC and HMBC methods identify the carbon skeleton of a molecule. This approach, the structural elucidation of the mixture by NMR spectroscopy, is particularly helpful for the identification of secondary metabolites, since less published NMR data of them is available. These experiments are time-consuming, but they only need to be performed once. 1D-selective ^{1}H experiments can be complementary to the 2D experiments, depending on the

problem requiring elucidation. They are less time-consuming than the 2D correlations but retain maximum resolution and are used to get specific information of a chosen NMR signal of the 1D ^{1}H spectrum. The spectrum is much simpler to analyze and only shows the correlation information for the selected peak. 1D-selective ^{1}H experiments are valuable tools for elucidating and confirming problematic molecules (Ellis *et al.*, 2012). NMR experiments are performed at the same experimental temperature used for the NMR fingerprint spectra (298.0 K). However, to use the same NMR spectrometer is not necessary, since NMR data are fully comparable independently of the spectrometer used. The version of the experiment with water-signal pre-saturation is recommended for samples containing H_2O. For the 1D ^{1}H-selective experiments, the offset frequency should be adjusted according to the signal of interest to saturate. The acquisition of an initial 1D ^{1}H spectrum helps to adjust some parameters and also provides information about the concentration of the sample, which is useful for the adjustment of the number of transients.

Each specific assignment problem will require the performance of some or others NMR experiments. The most commonly used NMR experiments providing structural information are indicated in Table 3.1. Many other NMR experiments are available in spectrometer libraries that can be useful for specific problems of elucidation.

Table 3.1. Most common NMR experiments for identification of metabolites. Standard versions and brief description of their application for structural elucidation problems.

Experiment	Version (pulse sequence[a])	Description[b]
1D ^{1}H	· Conventional pulse-acquisition (zg) · With solvent presaturation (zgpr or zgcppr), also 1D NOESY with presaturation (noesypr1d)	Standard experiment routinely used for fingerprinting, identification of metabolites, and determining chemical shifts (d) and coupling constant (*J*) values. Also used for quantification.
2D ^{1}H-^{1}H COSY	· Gradient selection (cosygpqf) (Aue, Bartholdi & Ernst 1976; Nagayama *et al.* 1980) · With solvent presaturation (cosypgqfpr)	Homonuclear Correlation Spectroscopy based on ^{1}H-^{1}H scalar coupling. Routinely used for the identification of metabolites, it correlates spin systems separated through chemical bonds.
2D ^{1}H-^{1}H TOCSY	· Conventional (mlevph)(Braunschweiler & Ernst 1983; Bax 1985) · With solvent presaturation (mlevphpr) · Selective 1D mode (selmlgp.2)(Bax 1985; Kessler *et al.* 1986; Stonehouse *et al.* 1994; Stott *et al.* 1995)	Total Correlation Spectroscopy. Based on homonuclear ^{1}H-^{1}H scalar coupling. It correlates spin subsystems within the same molecule.
2D ^{1}H-^{1}H NOESY	· Conventional (noesygpph) (Jeener *et al.* 1979; Wagner & Berger 1996) · With solvent presaturation (noesygpphpr) · Selective 1D mode (selnogp) (Kessler *et al.* 1986; Stonehouse *et al.* 1994; Stott *et al.* 1995)	Nuclear Overhauser Effect Spectroscopy. Based on homonuclear ^{1}H-^{1}H through-space interactions. Routinely used for the identification of metabolites, it provides information about which protons are close together in space (≤ 4Å).
2D ^{1}H-^{13}C HSQC	· Conventional using adiabatic ^{13}C pulses (hsqcetgpsisp) (Palmer *et al.* 1991; Kay, Keifer & Saarinen 1992; Schleucher *et al.* 1994)	Heteronuclear Single Quantum Correlation. Based on heteronuclear one-bond ^{1}H-^{13}C scalar coupling. Routinely used for the identification of metabolites, it correlates protons to their directly bonded carbon atom.
2D ^{1}H-^{13}C HMBC	· Conventional using low-pass *J*-filter (hmbcgplpndqf) (Bax & Summers 1986; Bax & Marion 1988)	Heteronuclear Multiple Bond Correlation. Based on heteronuclear long-range ^{1}H-^{13}C scalar coupling. Routinely used for the identification of metabolites, it correlates protons to carbon atoms separated by multiple (usually 2,3) bonds.

[a] According to Bruker nomenclature.

[b] Extensive and updated description of the vast library of NMR experiments and their different versions is collected in the NMR Guide and Enciclopedia of Bruker.
[c] Experiments for suppression of the signal of the residual water.

3.1.4.4 LC-MS metabolic profiling

Total ion current (TIC) chromatographic peaks are assigned to metabolites by comparison of the retention time and the MS spectrum of each peak to LC-MS profiles of standards of a homemade library. For data being comparable, the standards were analyzed using the same method and equipment.

3.1.5 Data processing

3.1.5.1 Processing of NMR data

The NMR data from fingerprint spectra are processed prior to statistical analyses. The bucketing process consists of obtaining the integral numeric value of the selected regions of the spectra (buckets) directly correlated with the molar concentration by its relationship to the initial concentration of the internal standard (TSP). For ecometabolomics, variable-size bucketing is highly recommended over regular-size bucketing (Kim *et al.*, 2010) for reducing the number of variables for statistical analyses. First, a pattern for the spectrum is created. The pattern is determined by identifying exactly where an NMR signal (peak) begins and ends for all peaks in the spectrum, and then the bucketing process can be executed based on this pattern. All empty areas (without peaks) of spectra can also be introduced into the pattern to detect any qualitative differences between samples. In this thesis, we have used the variable-size bucketing option, scaling the buckets relative to the internal standard (TSP), although other options of processing can be chosen. The output is a data set containing the integral values for each ^{1}H-NMR spectral peak accounted for in the described pattern.

The bucketed data sets from the NMR fingerprint spectra can be directly analyzed (without a previous assignment of the metabolites), when a rapid classification of samples according to their origin or their ecological or ecophysiological relevance is sufficient (Sardans *et al.*, 2011). This approach does not attempt to identify the metabolites but provide the metabolomic signature of the organism allowing the detection of any shift or anomaly in its metabolism.

3.1.5.2 Processing of LC-MS data

The LC-MS raw data files are processed using MZMINE 2.10 open access software (Pluskal *et al.*, 2010) using the processing parameters shown in Table 3.2. Before the numerical database is exported in “csv” format, the chromatograms are baseline-corrected, deconvoluted, aligned and filtered. The LC-MS data for the statistical analyses corresponds to the absolute peak area at each retention time (RT). The area of a peak is proportional to the concentration of its corresponding (assigned) metabolite in the sample. Thus, when samples are prepared and analyses performed exactly the same, a change in the area of a peak will correspond to a change in the concentration of the assigned metabolite.

Table 3.2. Processing parameters of LC-MS chromatograms using MzMine 2.10 (Pluskal *et al.*, 2010). Chromatograms presented correspond to the total ion current (TIC).

		(+H) Chromatograms		(-H) Chromatograms
1	**Baseline correction**			
	Chromatogram type	TIC		TIC
	MS level	1		1
	Smoothing	10E6		10E6
	Asymmetry	0.001		0.001
2	**Mass detection** (Exact Mass)			
	Noise level	4.5×10^5		4.5×10^5
3	**Chromatogram builder**			
	Min time span	0.05		0.05
	Min height	25000		25000
	m/z tolerance	0.002		0.002
4	**Smoothing**			
	Filter width	5		5
5	**Chromatogram deconvolution (Local minimum search)**			
	Chromatographic threshold	70%		70%
	Search minimum in RT range (min)	0.1		0.1
	Minimum relative height	7.0%		7.0%
	Minimum absolute height	30000		30000
	Min ratio of peak top/edge	2		2
	Peak duration range	0.0-2.0		0.0-2.0
6	**Chromatogram alignment (join alignment)**			
	m/z tolerance	0.001		0.001
	weight for m/z	80		80
	RT tolerance	0.3		0.3
	Weight for RT	20		20
7	**Gap filling (Peak Finder)**			
	Intensity tolerance	20%		20%
	m/z tolerance	0.001		0.001
	Retention time tolerance	0.1		0.1
	RT correction	marked		marked
8	**Filtering**			
	Minimum peaks in a row	25		25
	Ions excluded from database	<75 Between 0.0 and 1 min Between 28.5 and 30 min		<85 Between 0.0 and 1,1 min Between 27.0 and 30 min

3.1.6 Statistical data analysis

Statistical analyses of metabolic profiles (where the assignments of the NMR signals and the chromatogram peaks are considered) can be performed following two main ways when the ^{1}H-NMR spectra have been treated by variable-size bucketing. (i) All ^{1}H-NMR spectral peaks are used as individual variables. Here the result is a data set where the number of variables is equivalent to the number of buckets. (ii) The peaks (buckets) corresponding to the same molecular compound are added up. In this last case, the final number of variables in the data set is highly reduced, and the statistical results are easier to interpret (Samuelsson *et al.*, 2006; Ludwig & Viant, 2010; Robinette *et al.*, 2012). In the studies described in the following chapters we have used the second option.

Once processed, data from the different analyses (LC-MS and NMR-based fingerprinting and stoichiometric data) are analyzed by univariate and multivariate statistical analyses.

Multivariate ordination analysis (MOA) is one of the most common statistical analyses for metabolomic studies to describe significant sources of variation in complex datasets. Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are commonly used in metabolomics studies (Ramadan *et al.*, 2006). PLS-DA uses a linear model to discriminate the different tested groups and to project the variables and cases onto new axes presenting the maximum discrimination of those groups. In comparison, PCA depicts variable cases without taking into account the different groups tested. PCA and PLS-DA are constructed from the analysis of HPLC-MS and NMR data and stoichiometric data. Metabolic profiles and elemental concentrations are included as variables to enable the identification of clusters, groups, and outliers. MOA do not provide any measure of significance and it is limited to show the relation of cases with the used variables. PCs loadings of cases provided by MOA can be used to detect any significant difference of the investigated treatments by statistical inference such as t-student test or ANOVAS.

When the data for metabolites are non-normal or when a better accommodation of random effects and interaction terms is needed, all metabolites combined are analyzed using permutational MANOVAs (PERMANOVAs) (Anderson *et al.*, 2008). ANOVA/MANOVA assumes normal distributions and, implicitly, Euclidean distance, but PERMANOVA works with any distance measure appropriate to the data and uses permutations to make it distribution free.

3.1.7 Metabolomic- and stoichiometric- based answer (relationships between stoichiometry and metabolomics)

The interpretation of the results yielded by the statistical analysis led to the metabolomic- and stoichiometric-based answer to the ecological question formulated.

Plant C:N:P stoichiometric ratios are conditioned by several factors like soil conditions, canopy development, decomposition rate, source and quantity of water supply, phylogenetic affiliation, climatic condition, microbial communities (Daufresne & Loreau, 2001; Sterner *et al.*, 2002; Güsewell & Gessner, 2009). This variance in terrestrial environments can cause mismatches between supply and demand at ecological interfaces (plant vs. inorganic resources, herbivore vs. plant, detritivore vs. detritus), affecting nutrient recycling, and consequently the elemental stoichiometry in all the ecosystem(Fujita et al. 2014; Moe et al. 2005; Peñuelas and Sardans 2009b; Sardans et al. 2012; Sterner et al. 2002). Shifts in growth rate under different climate conditions have been successful related to changes in N:P ratios within and between species (Ågren, 2004, 2008; Wright *et al.*, 2004; Niklas *et al.*, 2005; Reich *et al.*, 2010). Furthermore, elemental stoichiometry shifts within and between species is linked

with changes in photosynthesis rates and/or efficiency, transpiration changes or other physiological traits under different environment conditions like water, nutrient or light availability (Wright *et al.*, 2004; Cernusak *et al.*, 2010; Reich *et al.*, 2010; Wang *et al.*, 2011a; Rivas-ubach *et al.*, 2012, 2014).

In a step forward, the “biogeochemical niche” hypothesis (Peñuelas et al. 2008; Sardans et al. 2015) proposes that plant species occupy a particular region in the multivariate space generated by the contents and ratios of macro- and micronutrients in plant tissues. Broaden the spectrum of elements (than only C, N and P) analyzed and examined all together (N, P, K, Mg or Ca) can provide more complete information of the global functioning of plants; and finally to have a more accurate view of the ecosystem stoichiometry and the balance between demand and supply (Peñuelas & Sardans, 2009a).

Several studies have investigated the responses of some metabolic pathways in organisms to changes in abiotic factors such as climate (temperature and water availability), nutrient availability, salinity or pollution. Changes in the composition of some metabolite groups have been described using analytical target methods in response to changes in several environmental factors, such as drought (Llusia and Penuelas et al. 1999; Llusia et al. 2008; Penuelas et al. 2009), temperature (Penuelas and Llusia 1999; Filella et al. 2007), pollutants (Penuelas et al. 1999), irradiance (Penuelas and Llusia 1999) or CO2 (Penuelas and Llusia 1997). Moreover, several studies have reported that the metabolites produced in response to abiotic or biotic environmental changes further interact with other abiotic and/or biotic ecosystem constituents, e.g., terpene emissions that affect the climatic and atmospheric conditions (Andreae and Crutzen 1997; Kavouras et al. 1998;Penuelas and Llusia 2003; Penuelas et al. 2009a; Penuelas and Staudt 2010). Now ecometabolomic studies provide the possibility to take a step forward in knowledge at the level of global organism responses to environmental changes. Some reports have already begun to explore the possibilities of the use of metabolomic approaches in ecological studies.

A serious challenge for ecometabolomic studies is to satisfy the need to disentangle the biologically relevant functions and response shifts under environmental changes, which implies determining and quantifying the maximum number of metabolites as possible. Furthermore, the metabolome changes continuously, an additional challenge that is accentuated when measuring the metabolomes of several individuals from a free-living population, which will necessarily include considerable metabolic variation. There will be high levels of variation in metabolite concentrations between individuals, owing to differences in individual genetics, gender, age, organs, health status, and spatial and temporal environmental changes.

The phenotypical responses in all basic organism's functions (growth, reproduction, defense, anti-stress mechanisms, storage, etc.) should be considered altogether. Thus, as different elements and metabolites participate asymmetrically in different plant functions, by coupling ecological stoichiometrical studies with metabolomics studies we are able to assess the relationships of C/N/P ratios with an organism's metabolome and lifestyle and finally with the structure and function, linking plant elemental composition with plant function and with plant-ecosystem relationships (Peñuelas and Sardans 2009c; Rivas-ubach et al. 2012). Thus, in this Thesis we have coupled wide stoichiometrical with metabolomic analysis in varios of the experimental studies. Our goal was to consider the first step of such relationships, i.e. to link stoichiometry to the metabolome and thereafter the sign of this relationship with other plant ecophysiological level responses.

Chapter 4

Study of metabolic responses of plant shoot and root to drought

This study has been published in the journal *Scientific Reports* as

Opposite metabolic responses of shoots and roots to drought.

Albert Gargallo-Garriga*, Jordi Sardans, Míriam Pérez-Trujillo, Albert Rivas-Ubach, Michal Oravec, Kristyna Vecerova, Otmar Urban, Anke Jentsch, Juergen Kreyling, Carl Beierkuhnlein, Teodor Parella, Josep Peñuelas, *Scientific Reports*, **2014**, 4, 6829-6834.

DOI: 10.1038/srep06829

4.1 Abstract

Shoots and roots are autotrophic and heterotrophic organs of plants with different physiological functions. Do they have different metabolomes? Do their metabolisms respond differently to environmental changes such as drought? We used ecometabolomics and elemental analyses to answer these questions. First, we show that shoots and roots have different metabolomes and nutrient and elemental stoichiometries. Second, we show that the shoot metabolome is much more variable among species and seasons than is the root metabolome. Third, we show that the metabolic response of shoots to drought contrasts with that of roots; shoots decrease their growth metabolism (lower concentrations of sugars, amino acids, nucleosides, N, P, and K), and roots increase it in a mirrored response. Shoots are metabolically deactivated during drought to reduce the consumption of water and nutrients, whereas roots are metabolically activated to enhance the uptake of water and nutrients, together buffering the effects of drought, at least at the short term.

4.2 Introduction

Plants have complex and intricate regulatory machinery that coordinates the demands of physiological activity, growth, and development. Plants regulate their shoot/root ratios of biomass in response to the availability of substrates and to environmental changes (Hibberd & Quick, 2002). Shoots and roots have different functions: shoots essentially have a photosynthetic function, whereas roots take up water and nutrients. Shoots and roots may thus compete for the resources that a plant acquires (Brouwer, 1962; Zerihun *et al.*, 1998)(Zerihun *et al.*, 1998). Plants under different resource availabilities differentially allocate the available resources to shoots and roots to optimize the efficiency of their use (Peñuelas *et al.*, 1993; Mao *et al.*, 2012; Sims *et al.*, 2012). Plants generally allocate relatively fewer resources to their roots when light is low and the availabilities of water and nutrients are high, consistent with the resource optimization hypothesis (Agren & Franklin, 2003). For example, the up-regulation of root growth under reduced supplies of nitrogen was confirmed by a meta-analysis of published data (Poorter & Nagel, 2000). Moreover, differential allocation to root and leaves has been observed as a response to biotic factors under various competitive conditions (Berendse & Möller, 2008) or to various soil physicochemical traits (Albaugh *et al.*, 2005). Several models based on carbon balance have been developed to explain the mechanisms behind the shoot/root allocation of carbon (Wilson, 1988; Cannell & Dewar, 1994; Agren & Franklin, 2003). Plants, however, are likely to respond to perturbations in the growth environment not only by altering their allocation of biomass to shoots and roots, but also by changing the metabolic activities of these organs. We hypothesized that shoots and roots would present contrasting metabolisms in response to changing environmental conditions given their different physiological functions and their different roles in the acclimation to stress. We subjected two common grass species (*Holcus lanatus* and *Alopecurus pratensis*) to drought conditions in the field (a simulated 1000-year recurrence of drought in a long-term experiment of rainfall manipulation in a semi-natural grassland at Bayreuth, Germany) and used metabolomics to test this hypothesis. We thus analysed metabolomes, which can be defined as the totality of thousands of compounds of low molecular weight (metabolites) required for maintenance, growth, and cellular functions of an organism at a given time. We also conducted elemental analyses to simultaneously assess the metabolic and stoichiometric responses of shoots and roots to drought (Peñuelas and Sardans 2009c; Sardans et al. 2011; Shulaev et al. 2008).

4.3 Materials and methods

4.1.1 Field experiment and sampling

Instant metabolomic and stoichiometric responses to a simulated 1000-year recurrence of drought (complete exclusion of rainfall for 42 days prior to the first sampling) and its subsequent effects to the end of the growing season three months later, and after irrigation with the deficit amount of rainfall, were assessed for the common C3 grasses *A. pratensis* and *H. lanatus* in a long-term experiment of rainfall manipulation in a semi-natural grassland at Bayreuth, Germany (EVENT II experiment(Walter *et al.*, 2013)). Samples from the cases (2 species × 2 organs (leaf blades and fine roots) × 2 sampling dates × 3 precipitation manipulations × 15 plots) were collected in the field, immediately frozen, and further prepared following the method described in Chapter 3, based on the procedure described in Rivas-Ubach et al. (2013) (Rivas-Ubach *et al.*, 2013). More details about the field experiment are gathered in the Appendix section (Appendix 4).

4.1.2 Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex, Waltham, Massachusetts, USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with an HESI II (heated electrospray ionization) source. Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3 µm particle size; Thermo Scientific, Waltham, Massachusetts, USA) at 30 °C. The mobile phases consisted of water (0.1 % acetic acid) (A) and acetonitrile (B). More details about the method are gathered in the Appendix section (Appendix 4).

4.1.3 Nuclear magnetic resonance (NMR) analysis

NMR experiments were performed using a Bruker AVANCE 600 spectrometer equipped with an automatic sample changer and a multinuclear triple resonance TBI probe (BrukerBiospin, Rheinstetten, Germany) at a field strength of 14.1 T (600.13 MHz ^{1}H frequency) and at 298.0 K. Following the introduction to the probe, the samples were allowed to equilibrate (2 min) prior to shimming to ensure good homogeneity of the magnetic field. All spectra were referenced to trimethylsilyl propionate (TSP) (^{1}H and ^{13}C at δ 0.00 ppm). All handling of liquid samples, automation, and acquisition were controlled using TopSpin 3.1 software (BrukerBiospin, Rheinstetten, Germany). More details are gathered in the Appendix section (Appendix 4).

4.1.4 Statistical analyses

The changes in the stoichiometries and metabolomes of *H. lanatus* and *A. pratensis* with the factors studied (shoots and roots, season, species, and drought treatment) were analyzed by principal component analysis (PCA) and PERMANOVA with Euclidean distances. The PCAs were performed with the *pca* function of the *mixOmics* package of R (R Development Core Team 2008). The PERMANOVA as well as PLS (partial least squares) analysis, and CIM (clustered image maps) conducted with R (R Development Core Team 2008). A Kolmogorov-Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally distributed was removed from the data set. Statistica v8.0 (StatSoft) was used to perform the ANOVAs, post-hoc tests, and KS tests. More details about statistical analysis are gathered in the Appendix section (Appendix 4).

4.3 Results and discussion

The responses of plants to drought are crucial because drought is one of the most important environmental stressors for plants, and changes in the shoot/root ratios of biomass have been frequently observed in response to drought (Carrow, 1996; Lloret *et al.*, 1999). Moreover, the frequency and length of droughts are projected to increase under global climate change (Trenberth *et al.*, 2003; IPCC, 2007), and more regions are expected to be affected by severe droughts (IPCC, 2007; Beniston *et al.*, 2007; Li *et al.*, 2009; Wang *et al.*, 2011b). We have previously reported a significant impact of drought on the metabolomes of plant species at the foliar level and have observed that these changes are strongly linked to changes in the elemental C/N/P/K stoichiometry (Rivas-ubach *et al.*, 2012). The simultaneous analysis of the metabolism and the nutrient stoichiometries of both shoots and roots that we now conducted assessed the allocation of metabolites and nutrients for various plant functions, such as growth, defense, reproduction, mechanisms of health, and avoidance of stress at the level of whole plants, thereby assessing the likely contrasting responses of shoots and roots to experimental drought and to seasonally changing conditions.

In addition to the expected differences between the two species and between the two seasons of sampling, July and September, we observed clear differences between shoots and roots at both the metabolic and elemental concentration levels (Fig. 4.1).

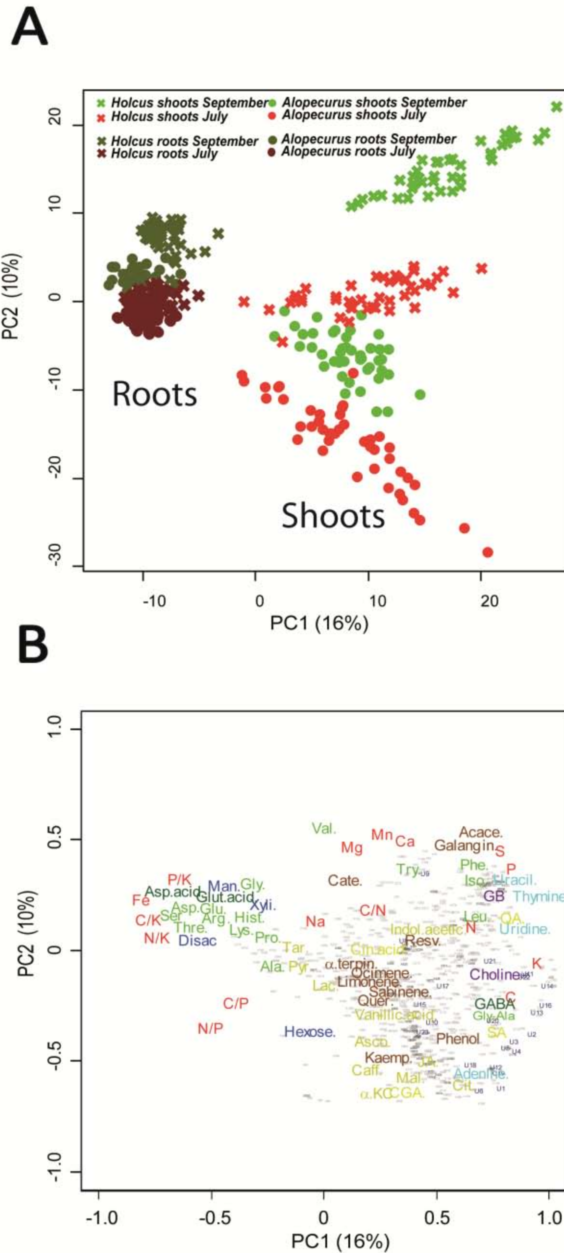

Figure 4.1. Plots of cases and variables in the PCA conducted with the elemental, stoichiometric and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* using PC1 versus PC2. (A) The cases are categorized by season and organ. Seasons are indicated by different colors (green, September; red, July). The two species are indicated by geometric figures (circles, *A. pratensis*; crosses, *H. lanatus*). Dark green and red colors are for roots, and light green and red colours are for shoots. (B) Loadings of the various elemental stoichiometric and metabolomic variables in PC1 and PC2. C, N, P, and K concentrations and ratios and Fe, Mn, Mg, Ca, and S concentrations are shown in red. The various metabolomic families are represented by colors: dark blue, sugars; green, amino acids; dark green, amino-acid derivatives; yellow, related compounds to the metabolism of amino acids and sugars; cyan, nucleotides; and brown, terpenes and phenolics. Metabolites: glycine-alanine (Gly-Ala), valine (Val), tryptophan (Try), threonine (Thr), serine (Ser), lysine (Lys), leucine (Leu), proline (Pro), phenylalanine (Phe), histidine (His), glycine (Gly), glutamine (Gln), asparagine (Asn), isoleucine (Ile), arginine (Arg), alanine (Ala), glutamic acid (Glu), aspartic acid (Asp), gamma-aminobutyric acid (GABA), glycine betaine (GB), choline (Choline), tartaric acid (Tar), pyruvate (Pyr), malic acid (Mal), jasmonic acid (JA), indol acetic acid (IAA), caffeic acid (Caff), ascorbic acid (Asco), vanillic acid (Vanillic.acid), citric acid (Cit), α-ketoglutaric acid (αKC), lactic acid (Lac), shikimic acid (SA), quinic acid (QA), chlorogenic acid (CGA), chinic acid (Cin.acid;), xylose (Xyl), hexoses (Hex), mannose (Man), disaccharide (Dis), adenine (Adenine), uracil (Uracil), thymine (Thymine), uridine (Uridine), acacetin (Acace), catechin (Cate), α-terpinene (αTerpin,), sabinene (Sabinene), resveratrol (Resv), quercetin (Quer), ocimene (Ocimene), limonene (Limonene), galangin (Galangin), kaempferol (Kaemp), phenolic group (Phenol). Unassigned metabolites are represented by small grey points.

Principal component analyses (PCAs) of both metabolomic and stoichiometric data showed that different species and seasons had different scores along the second PC axis (Fig. 4.1), but the most significant changes in metabolomic structure were between shoots and roots, which were separated along the first PC axis (Fig. 4.1). These results were confirmed by PERMANOVA analysis (species: pseudo-F = 51.2, P < 0.001; season: pseudo-F = 43.4, P < 0.001; shoot/root: pseudo-F = 154.4, P < 0.001). Shoots had higher concentrations of nucleotides, compounds related to the metabolism of amino acids and sugars, osmolytes, and secondary metabolites such as terpenes and phenols, while the roots had higher concentrations of amino acids and sugars irrespective of season and species (Fig. 4.2). The concentrations of C, N, P, and K and C/P ratio were higher in shoots than in roots, whereas the C/N, C/K, N/K, P/K, and N/P ratios and the concentration of Fe were lower (Apendix 1, Table S1). Roots had lower concentrations of nitrogenous bases and higher concentrations of most soluble sugars than did shoots (Fig. 4.2 and 4.3), likely due to the need of energy for the assimilation of soil resources, such as nutrients, and for root growth (Keys, A., J. *et al.*, 1978; Nunes-Nesi *et al.*, 2010)(Keys, A., J. *et al.*, 1978). Most of the compounds that participated in these functions, such as compounds related to the metabolism of amino acids and sugars, should be synthesized in leaves but allocated and used in large amounts in roots.

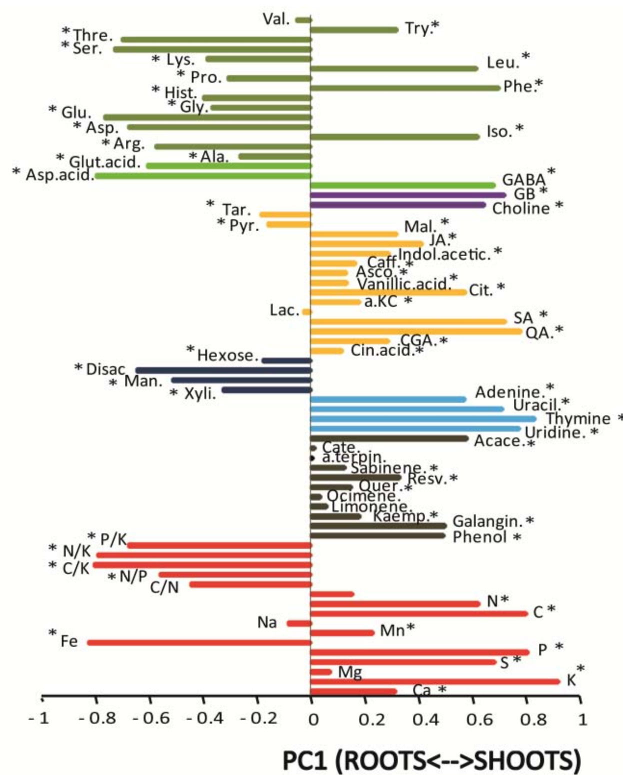

Figure 4.2. Loading of elemental stoichiometric and metabolomic variables in PC1 separating shoots and roots (Fig. 4.1). Variables are colored and labeled as described in the caption for Fig. 4.1. Asterisks showed statistical significance (P < 0.05) in one-way ANOVAs.

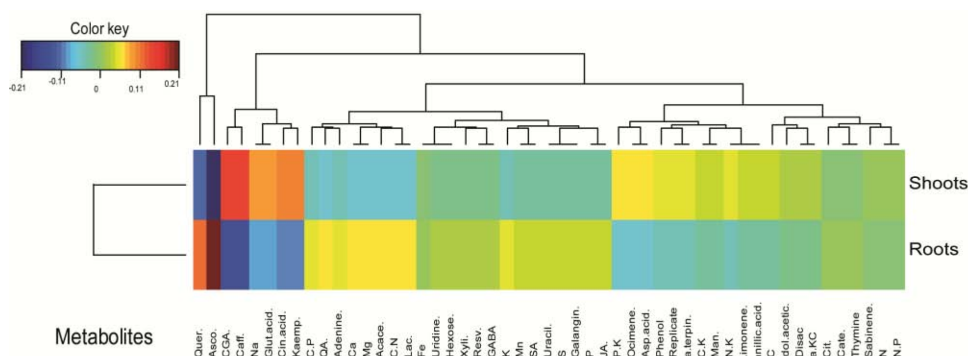

Figure 4.3. Clustered image maps of the metabolites in roots and shoots based on the data of the PLS analysis. The red and blue colors indicate positive and negative correlations respectively.

The observed variability of the metabolome was lower in the root samples than in the shoot samples (Fig. 4.1). The shifts in the metabolome of roots among different individual plants, species, and seasons were much less significant and smaller than those of the shoots. The coefficient of variation of the PC2 scores was 15 % for roots and 57 % for shoots. The metabolism of roots thus appears to be much more conservative and homeostatic than that of shoots.

Our results notably showed that the metabolome and stoichiometry of shoots and roots of both plant species responded to drought in opposite ways (Fig. 4.4 and 4.5). The results of the PCA were confirmed by the PERMANOVA analysis (treatment × shoot/root interaction; pseudo-F = 3.0; P < 0.001). The concentrations of choline and glycine betaine, which are involved in osmotic protection (McNeil *et al.*, 2001), and of gamma-aminobutyric acid and primary metabolites decreased in the shoots, and terpenes and metabolites related to anti-stress mechanisms increased in shoots under drought (Figs. 4.4A). Contrasting changes were observed in the metabolome of roots and shoots for both species and for both seasons (Appendix 1, Fig. S4.1 and S4.2 and Tab. S4.1 to S4.4). The concentration of gamma-aminobutyric acid increased in roots, suggesting that roots remain more active than shoots under drought stress (Kinnersleya & Turanob, 2010). Moreover, the concentrations of N, P, and K decreased in roots but increased in shoots (Fig. 4.2). The concentrations of primary metabolites thus increased in roots. A decrease in primary metabolism combined with an increase in some secondary metabolites in shoots in response to drought is consistent with the decrease in the shoot/root ratio usually observed under drought (Huang, B. Gaob, 2000; Álvarez & Sánchez-Blanco, 2013). The results are also consistent with the hypothesis that

plants allocate more carbon to antistress mechanisms under drought (Peñuelas & Estiarte, 1998), and with the previous results in this experimental site: reduced leaf water potential, leaf gas exchange, leaf protein content and efficiency of photosynthetic light conversion, and increased leaf carbon isotope signature and leaf carbohydrate content in response to drought (Jentsch *et al.*, 2011). Terpenes were the secondary metabolites that presented highest responsiveness to drought (Appendix 1, Fig. S4.3 and Tab. S4.1 to S4.4). The function of terpenes are still controversial, but they seem to protect the plants against abiotic and biotic stresses (Peñuelas & Staudt, 2010).

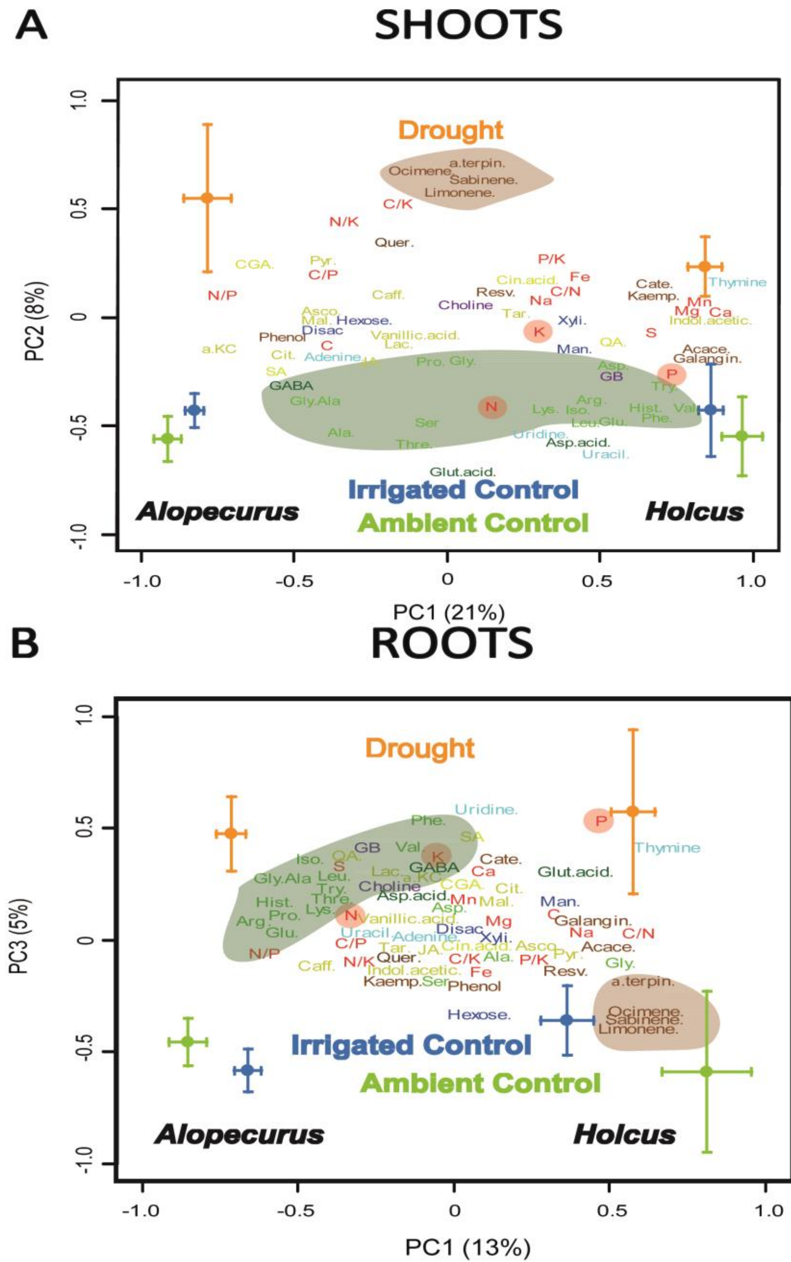

Figure 4.4. Plots of cases and variables in the PCAs conducted with the elemental, stoichiometric, and metabolomic variables in plants sampled in September. (A) Plot of cases and variables for shoots. (B) Plot of cases and variables for roots. C/N/P/K ratios are shown in red. The various metabolomic families are represented by colors: blue, sugars; green, amino acids; dark green, amino-acid derivatives; yellow, related compounds to the amino acids and sugars metabolism; cyan, nucleotides; violet, osmolytes; and brown, terpenes and phenols. Variables are colored and labeled as described in the caption for Fig. 4.1. The means of the cases are indicated by color: blue, irrigated control; green, ambient control; and orange, drought. *Holcus lanatus* is indicated as *Holcus* and *Alopecurus pratensis* as *Alopecurus*.

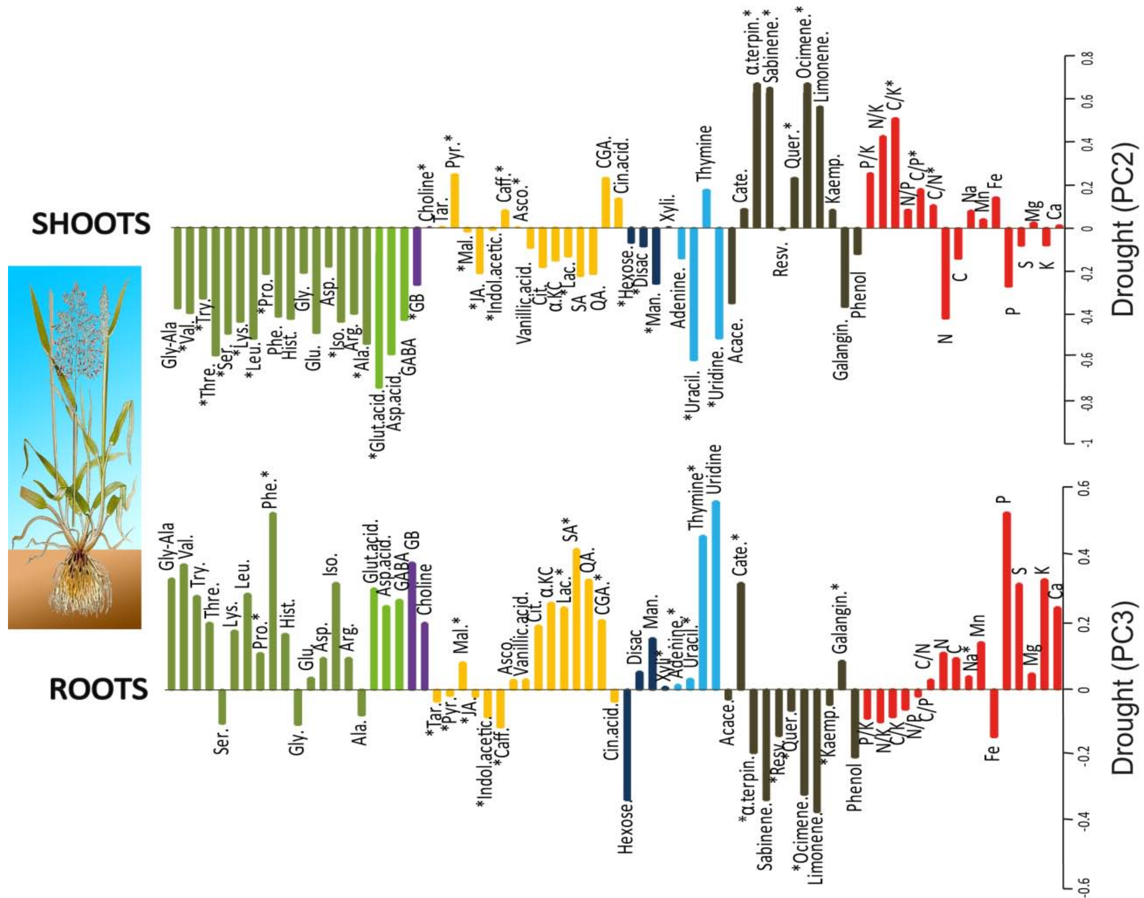

Figure 4.5. Loadings of elemental stoichiometric and metabolomic variables in the PC axes separating drought treatments (Fig.4. 3). Variables are colored and labeled as described in the caption for Fig. 4.1. Asterisks showed statistical significance (P < 0.05) in one-way ANOVAs. The drawing is a reproduction of the painting by C. A. M. Lindman.

The results provide clear evidence that plants have a high capacity to modulate and vary the allocation of nutrients and the relative activities of different metabolic pathways for producing biomass in both shoots and roots. Primary metabolites in plants, such as sugars, amino acids, and fatty acids, are synthesized mainly in the photosynthetic tissues of shoots, where the concentrations of these metabolites are higher than in roots. Under drought conditions, however, the plasticity of the plants allow a shift to increased synthesis or allocation of several primary metabolites to roots while decreasing allocation to shoots. These results have important implications for ecological studies. First, they provide clear evidence of the complexity of the stoichiometric shifts in terrestrial plants in response to environmental gradients and changes. The N/P ratio decreases in roots under drought conditions, coinciding with the investment in root growth, whereas this ratio increases in shoots, which is consistent with the growth rate hypothesis (Sterner *et al.*, 2002). The results, however, also indicate that the study of N/P ratios only in the leaves of terrestrial plants is unable to provide a general view of the relationships of N/P ratios with ecological traits and gradients. Second, the shift in

the allocation of metabolites and nutrients from shoots to roots in response to drought demonstrates the high capacity of plant metabolomes to respond modularly to stressors. When water is a limiting factor, metabolites involved with energy production and growth (especially sugars and amino acids) are shifted from shoots to roots. The simultaneous ecometabolomic analysis of roots and shoots can provide a complete view of the entire plant, including the response of different organs to environmental changes, the global phenotypic response, and the metabolic mechanisms underlying these responses. Such a simultaneous analysis has shown that shoots and roots have different metabolomes and nutrient concentrations, the shoot metabolome is much more variable than the root metabolome, and roots and shoots respond to drought with opposite metabolic changes. When metabolism is activated in roots, metabolism is deactivated in shoots. These opposite metabolic responses may account for the frequent lack of large reductions in productivity in drought experiments, at least for short term (Peñuelas *et al.*, 2007).

4.4 Supporting Information

Supporting information and the following figures are enclosed in Appendix 1 of the manuscript. Examples of the ^{1}H NMR and chromatogram metabolic profiles of the extract sample of *Holcus Lanatus* and *Alopecurus Pratensis* and the tables in the attached CD.

Figure. S4.1 PC1 versus PC2 of a September PCA of *Holcus lanatus* and *Alopecurus pratensis* shoots and roots.

Figure S4.2. Loading of variables in the PC axes separating drought treatments.

Figure S4.3. Clustered image maps of the metabolites in shoots treatments .

Figure S4.4. Clustered image maps of the metabolites in roots treatments.

Figure A4.1 Examples of the ^{1}H NMR metabolic profiles of the extract sample of *Holcus Lanatus* and *Alopecurus Pratensis*.

Figure A4.2 Examples of the chromatogram metabolic profiles of the extract sample of *Holcus Lanatus* and *Alopecurus Pratensis* in positive and negative mode.

Table S4.1 One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots and roots in September in drought treatment.

Table S4.2 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in July in September in drought treatment.

Table S4.3 One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots and roots in July in drought treatment.

Table S4.4 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in July in in drought treatment.

Chapter 5

Study of the influence of warming on the effects of drought on stoichiometry and metabolomics in shoots and roots

This study has been published in the journal *New Phytologist* as

Warming differentially influences the effects of drought on stoichiometry and metabolomics in shoots and roots.

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DOI:10.1111/nph.13377

5.1 Abstract

Plants in natural environments are increasingly subjected to a combination of abiotic stresses such as drought and warming in many regions. The effects of each stress and the combination of stresses on shoots and roots functioning have been studied extensively, but little is known about the simultaneous metabolome responses of the different organs of the plant to different stresses acting at once. We studied the shift in metabolism and elemental composition of shoots and roots of two perennial grasses, *Holcus lanatus* and *Alopecurus pratensis*, in response to simultaneous drought and warming.These species responded differently to individual and to simultaneous stresses. These responses were even opposite in roots and shoots. In plants exposed to simultaneous drought and warming, terpenes, catechin, and indole acetic acid accumulated in shoots, while amino acids, quinic acid, nitrogenous bases, the osmoprotectants choline and glycine-betaine, and elements involved in growth (N, P, and K) accumulated in roots. Under drought, warming further increased the allocation of primary metabolic activity to roots and changed the composition of secondary metabolites in shoots.These results highlight the plasticity of plant metabolomes and stoichiometry and the different complementary responses of shoots and roots to complex environmental conditions.

5.2 Introduction

Predictions of climate change project that different stresses will occur simultaneously in many regions. The combination of drought and warming and its high impact on aridity are of great concern (Mittler *et al.*, 2001; Moffat, 2002; Rizhsky *et al.*, 2004). Regions such as the Mediterranean Basin or the Sahel are already affected and may become increasingly affected (IPCC, 2012). In other regions such as central Europe, extreme events such as winter warming and summer drought are likely to occur together with higher frequency (Jentsch *et al*., 2011; IPCC, 2012). Some experimental approaches, simulating the impact of extremes in precipitation (periods of drought or heavy rain) and warming, have been tested in various ecosystems (Beier *et al.*, 2004; Peñuelas *et al.*, 2004, 2007, 2013b; Fay *et al.*, 2008; Smith, 2011). A combination of stresses from drought and warming alters the physiological status of grasses and other plants, inhibiting photosynthesis and accumulating products of lipid peroxidation (Jianga & Huang; Perdomo *et al.*, 1996; Jagtap *et al.*, 1998). Some of these studies suggest a molecular response of the plants to the combined effects of drought and warming different from those caused by the single stresses (Rizhsky *et al.*, 2002), but we know little of the metabolomic response at a whole-plant level and of the relationships between the overall use of nutrients by plants, and the elemental stoichiometric composition, and shifts in metabolites.

Also Central Europe is affected by extreme events, such as the 2003 heat wave (Schär & Jendritzky, 2004 Jentsch *et al*., 2007). Numerous studies have observed changes in extremes, for example centennial increases in frequency of heavy precipitation (10-30%) in Switzerland (Schmidli & Frei, 2005) and increases in duration of both extremely wet conditions in winter (Schonwiese *et al.*, 2003) and of unusually dry periods in summer in whole Europe (Beck *et al.*, 2001). The heat wave of 2003 has convincingly been associated with anthropogenically forced global warming (Schär & Jendritzky, 2004). Both drought and warming can thus increase their frequency and intensity (IPCC, 2013), which can affect plants in different seasons, e.g. extreme heat waves in winter and summer (during the growing season) and droughts in summer (Jentsch *et al.*, 2007). These climatic events are expected to have a large impact on plants and ecosystems, to the point of surpassing the thresholds of resistance of ecosystems (Gutschick & BassiriRad, 2003; Schär & Jendritzky, 2004; Reusch *et al.*, 2005; Knapp *et al.*, 2008; Jentsch & Beierkuhnlein, 2008; Jentsch *et al.*, 2011; Smith, 2011). Improving tolerance to these events will be a target for ongoing and future agricultural and nature-conservation programmes. Species of fundamental importance in nature conservation, such as the grasses *Holcus lanatus*

and *Alopecurus pratensis* (Beierkuhnlein *et al.*, 2011), can be affected by these extreme events. These species are interesting subjects for studying the impacts of droughts and warming on the metabolomic and stoichiometric shifts in roots and shoots.

Metabolomics is a powerful tool for improving our understanding of the changes in metabolism and biochemical composition of organisms, i.e. the ultimate phenotypic response to environmental changes (Fiehn *et al.*, 2000; Weckwerth *et al.*, 2004; Peñuelas & Sardans, 2009b; Sardans *et al.*, 2011). It is increasingly applied to ecological studies in what has been called ecometabolomics (Peñuelas & Sardans, 2009; Bundy *et al*., 2009; Sardans *et al.*, 2011; Rivas-ubach *et al.*, 2014). Ecometabolomics can explore the effects of the ecological organism-environment interaction by detecting the final phenotypic response of the organism and by detecting the metabolic pathways that are up- and down-regulated in response to environmental changes.

Ecometabolomics has recently been used to monitor the phenotypic changes of a particular genotype in response to the drivers of global change, particularly shifts in temperature (Pinheiro *et al.*, 2004; Michaud & Denlinger, 2007; Michaud *et al.*, 2008; Charlton *et al.*, 2008; Lugan *et al.*, 2009; Fumagalli *et al.*, 2009; Sardans *et al.*, 2011; Rivas-ubach *et al.*, 2012, 2014). The effects of drought and warming on metabolomes have been widely studied separately (Cramer *et al.*, 2007; Michaud *et al.*, 2008; Lugan *et al.*, 2009; Fumagalli *et al.*, 2009; Sardans *et al.*, 2011; Rivas-ubach *et al.*, 2012, 2014), but less is known about their combined effect in plants (Rizhsky *et al.*, 2002, 2004). The majority of studies have focused on a single stress treatment applied to plants under controlled conditions, mostly only the effect on photosynthetic tissues. Real field conditions, however, involve different stresses occurring simultaneously, and various plant organs can respond differently to these changes. Roots and shoots can respond asymmetrically, as has been observed at the morphological level, e.g. shifts in the shoot/root biomass and growth-rate ratios occur when the availability of soil water changes (Jefferies, 1993; Guenni & Mar, 2002; García *et al*., 2007; Cordoba-Rodriguez, 2011).

Exposure to drought has led to the accumulation of fructans, several amino acids, and GABA (Alvarez *et al*., 2008; Charlton *et al*., 2008; Fumagalli *et al*., 2009; Rizhsky *et al*., 2004; Sardans *et al*., 2011). Gargallo-Garriga *et al*. (2014), in the first study of the metabolomic response to drought in whole plants (shots and roots), observed that the metabolomic response differed between and was nearly opposite in shoots and roots. Metabolomic studies of warming stress have observed increases in the concentrations of saturated fatty acids (Horváth *et al.*, 1989; Allakhverdiev *et al.*, 1999) in the thylakoid (Vigh *et al.*, 1989) and plasma

membranes (Vigh *et al.*, 1993). As observed for the drought conditions (Gargallo-Garriga *et al.*, 2014), plants respond differently to warming at the shoot and root levels. Under elevate temperature, the level of saturation of membrane lipids extracted from the leaves of creeping bentgrass increased, whereas no change in membrane lipids was observed in root tissues (Larkindale & Huang, 2004). Warming has also increased biomass production in several ecosystems (Rustad *et al.*, 2001). Warming can have a positive effect on growth and biological activity when water is not limited, but it can also negatively affect plant growth and primary productivity in other ecosystems, mostly due to lower water availability. Hence we investigated the effects of warming and drought on plant metabolomics in different organs (shoots and roots) simultaneously.

Metabolomic changes can imply shifts in the proportional use of various nutrients and the consequent changes in elemental composition and stoichiometry. Rivas-Ubach *et al*. (2012, 2014) have recently reported foliar metabolomic changes associated with changes in foliar elemental composition and stoichiometry in response to abiotic (climatic) and biotic (herbivory) factors. These elemental changes are of great ecological importance because they may lead to changes in the species composition of communities and in ecosystem function (Sterner & Elser, 2002; Sardans *et al.*, 2012b; Peñuelas *et al.*, 2013a). The relationships of the metabolomes and stoichiometries of whole plants (roots and shoots) in response to simultaneous conditions of drought and warming, however, have not received much attention.

We investigated the impact of water availability and warming in factorial combination on the elemental composition and stoichiometry and the metabolomic structure of above- and belowground organs (shoots and roots) of *H. lanatus* and *A. pratensis* in different seasons. We tested the hypothesis that warming differentially influences the effects of drought on stoichiometry and metabolomics in shoots and roots.

5.3 Materials and methods

5.1.1 Study site

The sampling was part of the EVENT II experiment, described in detail by Walter *et al.* (2013) and Gargallo-Garriga *et al.* (2014), where precipitation patterns have been experimentally modified in a semi-natural, extensively managed grassland in the Ecological-Botanical Garden of the University of Bayreuth, Germany (49°55'19''N, 11°34'55''E, 365 m a.s.l.). The climate is

temperate and moderately continental with a mean annual temperature of 8.2 °C and a mean annual precipitation of 724 mm (1971-2000, data from the German Weather Service).

5.1.2 Experimental design

The field experiment had a two-factorial design manipulating (1) drought (irrigated control, ambient control, and drought) and (2) warming (ambient, winter warming, and summer warming). The design consisted of 45 plots, each 1.5 × 1.5 m in size, with five replications of all factorial combinations (Figure S1). The warmed and unwarmed plots were blocked and randomly assigned within each manipulation of the precipitation. The treatments are described in detail in the supplementary material of *Chapter 4* (Appendix 1). Gargallo-Garriga *et al.* (2014).

5.1.3 Target species

Two C3 grasses were selected as the target species for this study: *A. pratensis* and *H. lanatus*. Both species were selected based on their high frequency in the experimental plots and their importance in semi-natural grasslands across Central Europe. *A. pratensis* is the dominant species at the experimental site, producing about 18% of the annual aboveground biomass. It is a tall (up to 110 cm) and productive crop of agricultural importance in moist and nutrient-rich meadows. *H. lanatus* is also common at the site but is less productive (3% of the annual aboveground biomass). It occurs in semi-natural grasslands throughout Europe, Asia, and North Africa and is invasive in North America and Australia. It tolerates a wide range of conditions but prefers moist conditions.

5.1.4 Collection and preparation of tissue samples

Samples were collected at the end of the drought manipulation before irrigation in July and again at the end of the growing season in September. Above- and belowground 360 tissue samples were collected (2 species × 2 organs (leaf blades and fine roots) × 2 sampling dates × 3 precipitation manipulations × 15 plots). The procedure for sample preparation is described in detail by Rivas-Ubach *et al*. (2013). Briefly, the frozen samples were lyophilized and stored in plastic cans at -80 °C. Soil contamination was removed from the root samples. Finally, the samples were ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for 4 min, producing a fine powder that was stored at -80 °C until the extraction of the metabolites. For details see the supplementary material of *Chapter 4* (Appendix 1). Gargallo-Garriga *et al.* (2014)

5.1.5 Elemental analysis

C and N concentrations were determined from 1.5 mg of each powdered sample by combustion coupled to gas chromatography using a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

P, K, Fe, Mn, Mg, Ca, and S concentrations were determined by extraction by acid digestion in a MARS Xpress microwave reaction system (CEM, Mattheus, USA) under high pressure and temperature. Briefly, 250 mg of dry sample powder were added to 5 mL of nitric acid and 2 mL of H_2O_2 in a Teflon tube. The digested material was transferred to 50-mL flasks and resuspended in Milli-Q water to a final volume of 50 mL. The elemental concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (Perkin-Elmer Corporation, Norwalk, USA).

5.1.6 Extraction of metabolites

Two sets of 50-mL centrifuge tubes were labelled/ set A for analysis by liquid chromatography-mass spectrometry (LC-MS) and set B for analysis by nuclear magnetic resonance (NMR). Each tube of set A received 150 mg of a powdered sample and 6 mL of water/methanol (1/1), and the samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All tubes were centrifuged at 1100 × g for 15 min. Next, 4 mL of each tube of set A were transferred to its corresponding tube of set B. This procedure was repeated for two extractions of the same sample. The resulting extracts were used for metabolomic analysis.

5.1.7 Preparation of extracts for LC-MS and NMR analyses

Two millilitres of the supernatants of each tube of set A were collected using crystal syringes, filtered through 0.22-µm microfilters, and transferred to a labelled set of LC vials. The vials were stored at -80 °C until the LC-MS analysis.

Eight millilitres of the extracts were resuspended in water to reduce the proportion of methanol (<15%). The solutions were lyophilised, and 4 mL of water were added to each tube, which was vortexed and centrifuged at 23 000 × g for 3 min. The samples were frozen at -80 °C and lyophilised again. Finally, 1 mL of KD_2PO_4-buffered D_2O solution containing 0.01% TSP (trimethylsilyl propionic acid sodium salt) (pH 6.0) was added to each dried fraction. TSP was used as the internal standard for the NMR experiments. The solutions were transferred to 2-mL centrifuge tubes with a micropipette and centrifuged at 23 000 × *g* for 3 min, and 0.6 mL of

the supernatants were transferred to the NMR sample tubes. The procedure for the extraction of the metabolites is described in detail in Rivas-Ubach *et al.* (2013).

5.1.8 LC-MS analysis

LC-MS chromatograms were obtained with a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex, Waltham USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an HESI II (heated electrospray ionisation) source. Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3-µ particle size; Thermo Scientific, Waltham, USA) at 30 °C. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B). Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use. The elution gradient, at a flow rate of 0.3 mL per minute, began at 10% A (90% B) and was maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10% A and 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilised for 5 min before the next sample was injected. The injection volume of the samples was 5 µL. HESI was used for MS detection. All samples were injected twice, once with the ESI operating in negative ionisation mode (-H) and once in positive ionisation mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60 000). The resolution and sensitivity of the spectrometer were monitored by injecting a standard of caffeine after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence. The assignment of the metabolites was based on the standards, with the retention time and mass of the assigned metabolites in both positive and negative ionisation modes (Appendix 2, Tab. S1).

5.1.9 NMR analysis

^{1}H NMR-based fingerprints were obtained for all samples. One-dimensional (1D) ^{1}H NMR spectra were acquired with suppression of the residual water resonance. The water-resonance signal was presaturated using a power level of 55 dB during a relaxation delay of 2 sec. Each spectrum acquired 32 k data points over a spectral width of 16 ppm as the sum of 128 transients and with an acquisition time of 1.7 sec. The total experimental time was ~8 min per sample. All ^{1}H NMR spectra were phased and baseline corrected and referenced to the resonance of the internal standard (TSP) at δ 0.00 ppm using TOPSPIN 3.1 software (Bruker

BioSpin, Rheinstetten, Germany).. The data were subsequently used for the statistical analysis. A variable-size bucketing, where buckets were scaled relative to the internal standard (TSP), was applied to all ^{1}H NMR spectra using AMIX software (Bruker BioSpin, Rheinstetten, Germany). The output was a data set containing the integral values for each assigned ^{1}H NMR spectral peak in the described pattern. The buckets corresponding to the same molecular compound were summed.

For the assignment of the fingerprint peaks (i.e. identification of the metabolites), 2D NMR experiments on selected representative samples were carried out using the NMR equipment and software previously described. The probe temperature was set to 298.0 K. 1D ^{1}H NMR, 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC), and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) were acquired using standard Bruker pulse sequences and routine conditions (see Appendix 2,Method for details) (Rivas-Ubach *et al.*, 2013).

5.1.10 Processing of LC-MS and NMR data

The LC-MS raw data files were processed using MZMINE 2.10 (Pluskal et al., 2010) (Appendix 2,Tab. S1). Before the numerical database was exported in “csv” format, the chromatograms were base-line-corrected, deconvoluted, aligned and filtered. Metabolites were assigned by comparison with the analyses of the standards (retention time and mass spectrometry) (Appendix 2, Tab. S2). Assigned variables corresponding to the same molecular compounds were summed. The LC-MS data for the statistical analyses corresponds to the absolute peak area at each retention time (RT). The area of a peak is directly proportional to the concentration (i.e. µg/mL) of its corresponding (assigned) metabolite in the sample. Thus, a change in the area of a peak will mean a change in the concentration of its assigned metabolite.

The procedure followed for the processing of the ^{1}H NMR spectra and for the assignment of the NMR peaks to their corresponding metabolite is detailed in Rivas-Ubach et al. (2013). Briefly, for the statistical analysis, before the exportation of the ^{1}H NMR numerical databases, all spectra were phased, baseline-corrected and referenced to the resonance of the internal standard TSP (trimethylsilyl propionic acid sodium salt) at δ 0.00 ppm with TOPSPIN 3.1 (Bruker Biospin). A variable-size bucketing was thus applied to all the ^{1}H NMR spectra using AMIX software (Bruker Biospin) and the buckets were scaled relative to the internal standard (TSP). The output was a data set containing the integral values for each assigned ^{1}H NMR spectral peak in the described pattern. The buckets corresponding to the same molecular compound were summed.

5.1.11 Statistical analyses

HPLC-MS and NMR-based fingerprinting and stoichiometric data were analysed by univariate and multivariate statistical analyses. We conducted permutational multivariate analyses of variance (PERMANOVAs) (Anderson *et al*., 2008) using the Euclidean distance, with season (July and September), water-availability (control, drought, and irrigation), warming treatment (control, winter warming, and summer warming), and plant organ (shoots and roots) as fixed factors and individuals as random factors. Multivariate ordination principal component analyses (PCAs) (based on correlations) and partial least squares discriminant analyses (PLS-DAs) were also performed to detect patterns of sample ordination in the metabolomic and stoichiometric variables. The PCAs were initially constructed from the HPLC-MS analysis and the NMR data and included as variables the metabolic profiles and elemental concentrations and ratios of shoots and roots in the different seasons to enable the identification of clusters, groups, and outliers (Sandasi *et al*., 2011) (Fig. 5.1). The profiles of shoots and roots from July and September were additionally submitted to separate PCAs (Fig. 5.1). The PC scores of the cases were subjected to one-way ANOVAs to determine the statistical differences among groups with different levels of the categorical independent variables studied (season, species, plant organ, and climatic treatment). The PERMANOVAs, PCAs, PLS analyses, and clustered image maps were conducted by the *mixOmics* package of R software (R Development Core Team 2008). The Kolmogorov-Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally distributed was removed from the data set. Statistica v8.0 was used to perform the ANOVAs, post hoc tests, and KS tests.

5.4 Results

5.1.12 General results

Plant shoots and roots had different overall metabolisms (PERMANOVA pseudo-F = 162; P < 0.001). The overall metabolisms and elemental concentrations and stoichiometries were also significantly affected by species (pseudo-F = 53.7; P < 0.001), season (pseudo-F = 45.5; P < 0.001), drought (pseudo-F = 5.29; P < 0.001), and warming (pseudo-F = 5.53; P < 0.001). Some two-level interactions between factors were also significant season with organ (pseudo-F = 13.4; P < 0.001), season with species (pseudo-F = 12.02; P < 0.001), season with drought (pseudo-F = 2.44; P < 0.05), species with drought (pseudo-F = 2.56; P < 0.05), species with warming (pseudo-F = 2.07; P < 0.05), organ with drought (pseudo-F = 3.23; P < 0.001), organ with warming (pseudo-F = 2.50; P < 0.01), and drought with warming (pseudo-F = 1.98; P < 0.05). The interaction of season with warming was not significant (pseudo-F = 1.26; P > 0.05). More metabolites were found in the shoots than in the roots. In total 850 metabolic variables were detected, 729 were found in the shoots and 577 in the roots. Shoots and roots shared 456 metabolites, 273 compounds were detected in shoots but not in roots, and 121 metabolites in roots but not in shoots.

5.1.13 Elemental, stoichiometric, and metabolomic shifts across shoots and roots, species, and seasons

When all cases were analysed together, PC1 accounted for the differences between roots and shoots, whereas PC2 separated species and seasons (Fig. 5.1). PCs 1 and 2 explained 26 % of variance in the PCA conducted with the shoot samples (including seasons, species, and treatments). Post hoc analysis of the scores indicated that overall shoot metabolome and stoichiometry differed significantly depending on species (PC1, P < 0.001) and season (PC2, P < 0.05). Species was thus the primary factor and seasonality the secondary factor for plant shoots. N, P, and K concentrations and C/N, C/K, N/K, and K/P ratios also differed depending on season (Appendix2, Fig. S5.2 and Table S5.1). The highest P, N, and K concentration ratios were found in the growing season (September sampling) (Appendix2, Table S5.3 to S5.10), while the concentration of C and the C/P and N/P ratios were lower in September.

The shoot concentrations of amino acids, some related compounds of amino-acid and sugar metabolism (RCAAS), and some sugars such as xylose and mannose were higher in September than in July in both *H. lanatus* and *A. pratensis* (Appendix2, Fig. S5.2 and Table S5.4).

The PCA conducted with all root samples (including species, seasons, and treatments) and the elemental, stoichiometric, and metabolomic data indicated that 18 % of the variance was explained by the first and second PCs. A post hoc analysis of the score coordinates showed that the stoichiometries and metabolomes for the seasons were differentiated in PC1 ($P < 0.001$) and those for the species in PC2 ($P < 0.05$). These results differed from those for shoots, where the highest variance was explained first by species and secondly by season. Also, the overall metabolism/stoichiometry of the roots did not differ between the two species in July but did in September, when the plants were growing. The roots had the highest C, P, N, and K concentrations in the growing season (September sampling) (Appendix2, Fig. S5.3 and Sardans *et al*., 2013b).

In September, the roots had higher concentrations of some amino acids, while in July (mature plants) the roots had higher concentrations of some RCAAS and some sugars such as pentoses and disaccharides, products directly related to growth (Appendix2, Tab. S5.3 to S5.18).The shift in metabolism/stoichiometry between seasons was very similar in the roots and shoots (Appendix2, Fig. S5.2 and S5.3).

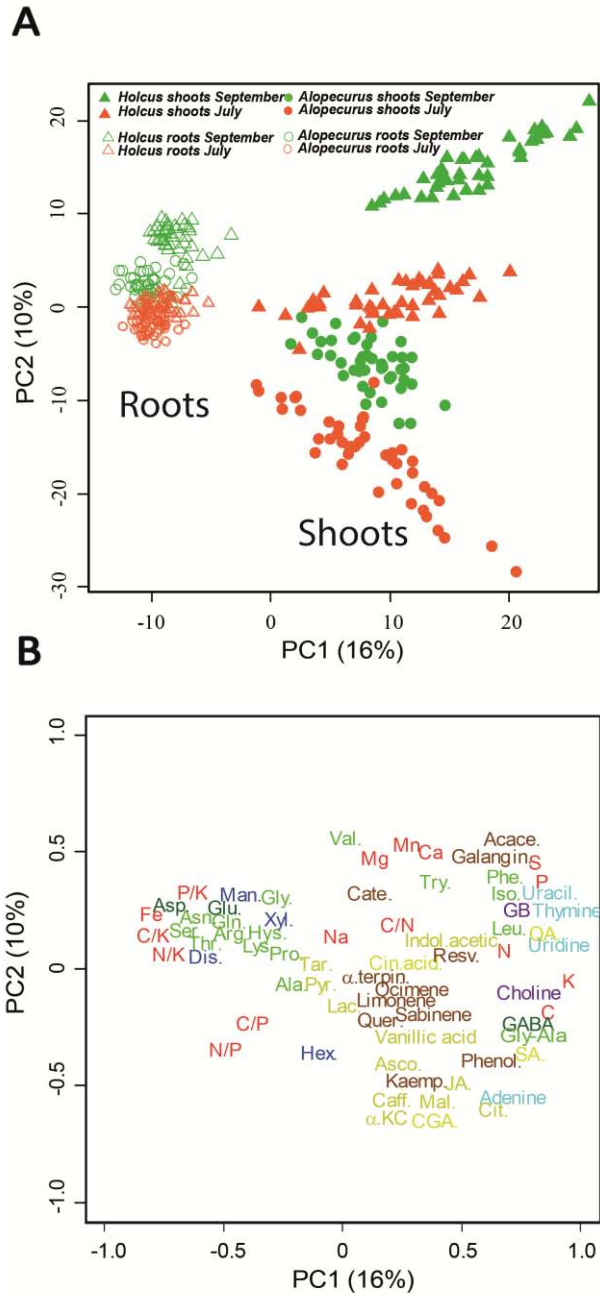


Figure 5.1. Plots of cases and variables in the PCA conducted with the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* using PC1 versus PC2. (A) The cases are categorized by season and organ. Seasons are indicated by different colours (green, September; red, July). The two species are indicated by geometric symbols (circles, *A. pratensis*; triangles, *H. lanatus*). Open symbols represent roots, and solid symbols represent shoots. (B) Loadings of the various elemental stoichiometric and metabolomic variables in PC1 and PC2. NMR variables are marked with inverted commas (‘) and LC-MS variables with asterisks (*). C, N, P, and K concentrations and ratios and Fe, Mn, Mg, Ca, and S concentrations are shown in red. The various metabolomic families are represented by colours; dark blue, sugars; green, amino acids; dark green, amino-acid derivats; yellow, compounds associated with the metabolism of amino acids and sugars; cyan, nucleotides; and brown, terpenes and phenolics. Metabolites; glycine-alanine‘ (Gly-Ala), valine* (Val.), tryptophan* (Try.), threonine* (Thr.), serine*‘ (Ser.), lysine* (Lys.), leucine* (Leu.), proline* (Pro.), phenylalanine* (Phe.), histidine* (Hys.), glycine* (Gly.), glutamine* (Gln.), asparagine* (Asn.), isoleucine* (Ile.), arginine* (Arg.), alanine* (Ala.), glutamic acid* (Glu.), aspartic acid* (Asp.), gamma-aminobutyric acid‘ (GABA), glycine betaine‘ (GB), choline‘ (choline), tartaric acid* (Tar.), pyruvate* (Pyr.), malic acid* (Mal.), jasmonic acid* (JA), indole acetic acid* (Indole acetic), caffeic acid* (Caff.), ascorbic acid* (Asco.), vanillic acid* (Vanillic acid), citric acid* (Cit.), α-ketoglutaric acid‘* (αKC), lactic acid* (Lac.), shikimic acid‘ (SA), quinic acid’* (QA), chlorogenic acid* (CGA), chinic acid* (Cin. acid), xylose* (Xyli.), hexose* (Hexose), mannose* (Man.), disaccharide*‘ (Dis.), adenine* (Adenine), uracil* (Uracil), thymine‘ (Thymine), uridine* (Uridine), acacetin* (Acace.), catechin* (Cate.), α-terpinene* (αTerpin.), sabinene* (Sabinene), resveratrol* (Resv.), quercetin* (Quer.), ocimene* (Ocimene), limonene* (Limonene), galangin* (Galangin), kaempferol* (Kamp.), phenolic group‘ (Phenol.). Unassigned metabolites are represented by small grey points.

5.1.14 Effects of drought on elemental, stoichiometric, and metabolomic structure in shoots and roots

A PLS-DA indicated that both shoot and root samples corresponding to plants growing in both season under the control temperature were separated by their different levels of water availability (control, drought, and irrigation) across factor 1 (Fig. 5.2). The second cause of variability (separated across PLS-DA factor 2) was plant organ, i.e. the differences between shoots and roots. Species were also separated in the shoot samples. Secondary metabolites, such as ocimene, α-terpinene, limonene, sabinene, and quercetin, had higher concentrations in the drought treatment. The C/N, C/K, N/K, C/P, and N/P ratios were higher in the root samples of the drought treatment than in those in the control and irrigated treatments. The concentrations of C, N, P, K, and S in shoots were higher in the control and irrigated shoot samples than in the drought samples.

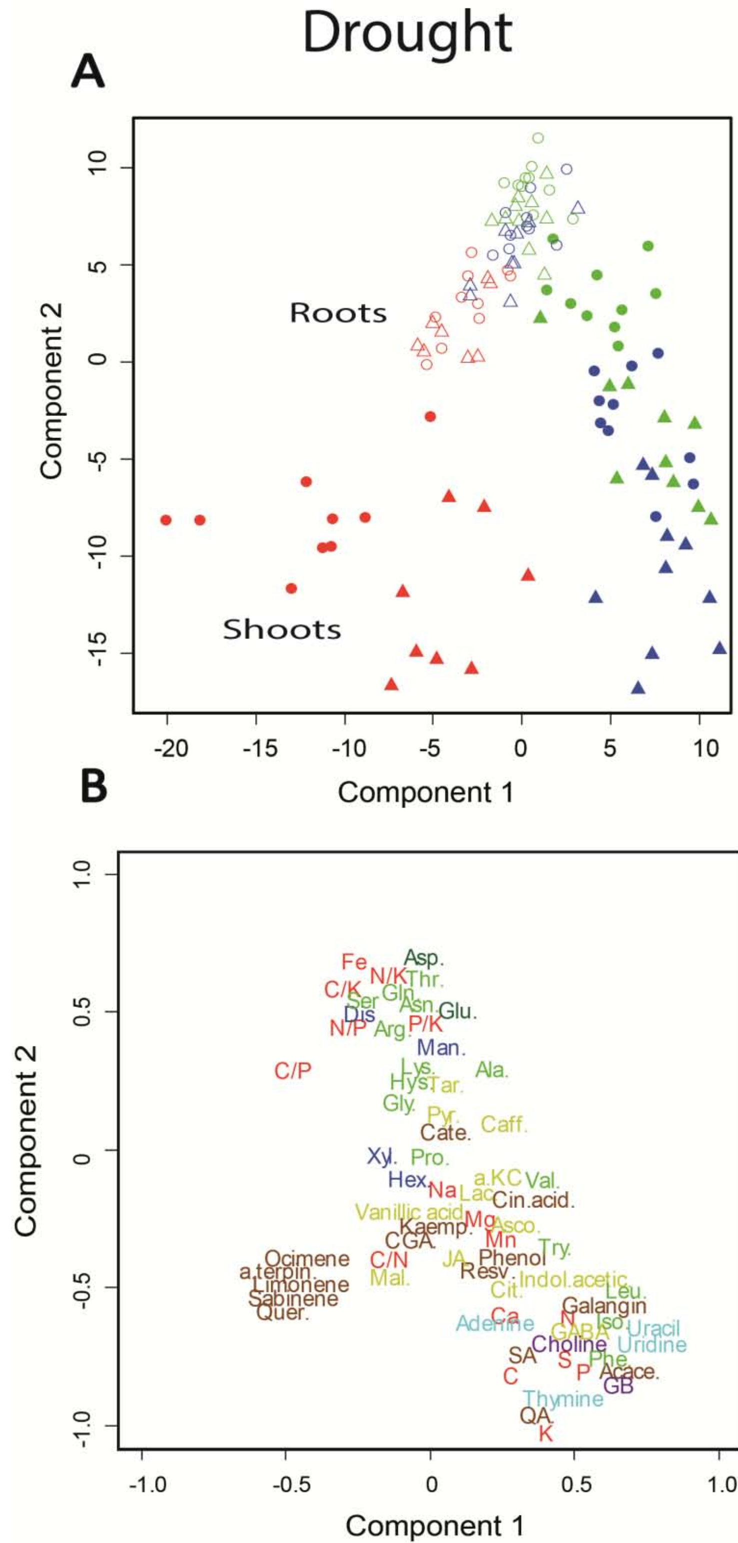


Figure 5.2. Component 1 *vs* component 2 of the partial least squares discriminant analysis (PLS-DA) of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised by shoots and roots in the drought treatment. Water availability is indicated by different colours (green, ambient control; red, drought; blue, irrigated). *Holcus lanatus* is represented by triangles and *Alopecurus pratensis* by circles. Shoots and roots are represented by solid and open symbols, respectively. (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Fig. 5.1.

5.1.15 Effects of warming on elemental, stoichiometric, and metabolomic composition in shoots and roots

The PLS-DA conducted only with samples of plants grown under control water conditions showed that shoots and roots growing under different warming stresses tended to be distributed in different directions with respect to axis 2 (Fig. 5.3 and Fig. S5.4 and S5.5 and Tab. S5.3 to S5.10 of Appendix 2), coinciding with the PERMANOVA results, and also that the metabolomes of shoots and roots differed. No differences among the three warming levels were observed throughout the different seasons, consistent with the lack of significance in the interaction of these two factors in the PERMANOVA. The PLS-DA indicated that the winter-warming and control treatments did not differ, but warming had a higher impact on metabolomic and stoichiometric composition under control water conditions when applied in summer than in winter. The main effects of warming were an increase in the concentrations of most amino acids (mainly in roots) and RCAAS (mainly in shoots) (Fig. 5.3).

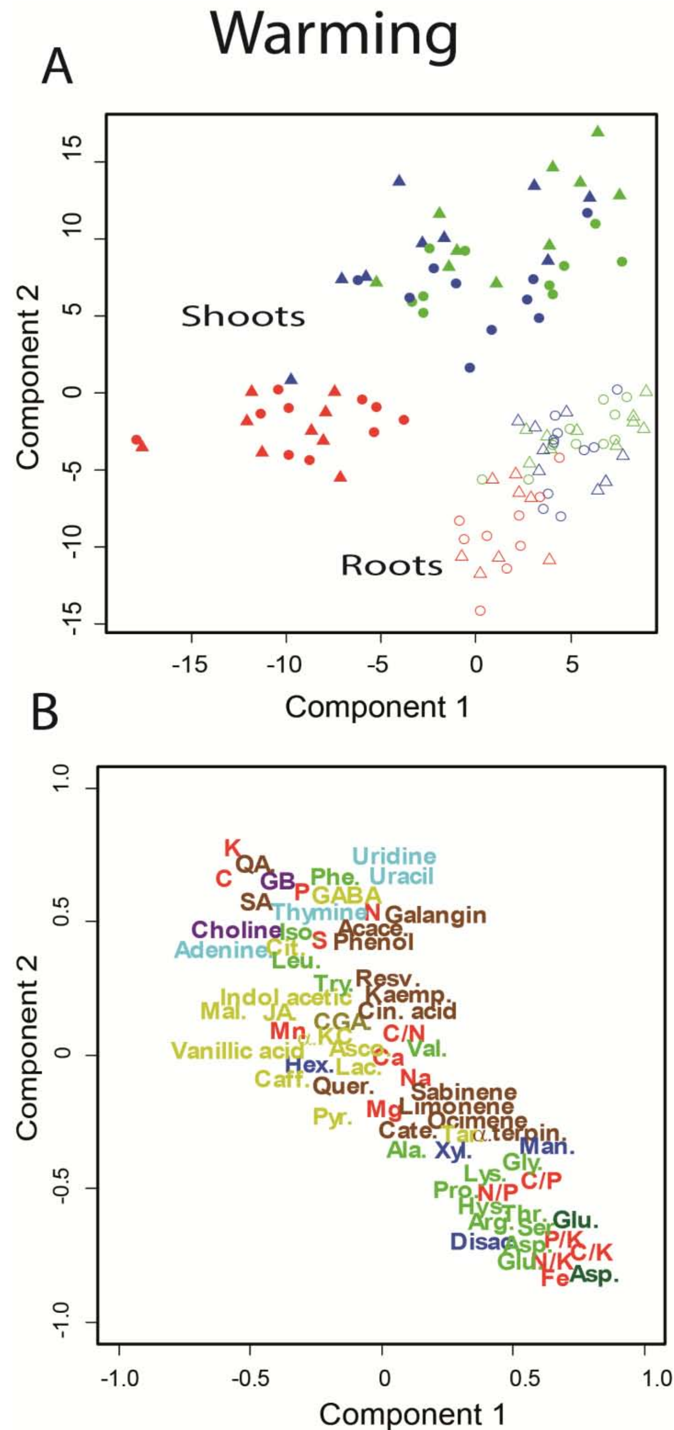


Figure 5.3. Component 1 *vs* component 2 of the partial least squares discriminant analysis (PLS-DA) of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised by shoots and roots in the warming treatment. Warming is indicated by different colours (green, ambient control; red, summer warming; blue, winter warming). *Holcus lanatus* is represented by triangles and *Alopecurus pratensis* by circles. Shoots and roots are represented by solid and open symbols, respectively. (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Fig. 5.1.

5.1.16 Effects of the interaction between drought and warming on elemental concentrations, stoichiometries, and metabolomes in shoots

Water availability separated shoot metabolomic-stoichiometric composition along factor 1 in the PLS-DA, whereas warming separated plant metabolomic-stoichiometric structure along factor 2 (Fig. 5.4 and in Appendix 2 Tab. S5.3, S5.5, S5.7and S5.9). The metabolites involved in plant growth had higher concentrations in the ambient control and irrigated samples in the water-manipulation treatments, and at these two levels of water availability, warming enhanced the concentrations of these metabolites very little. The PLS-DA thus indicated that warming produced different effects depending on water availability, which was consistent with the interaction between the drought and warming treatments identified by the PERMANOVA. The warming treatments along factor 1 identified different metabolomic-stoichiometric compositions only in the shoot samples of the drought-stressed plants. As previously indicated, secondary metabolites (sabinene, ocimene, α-terpinene, and limonene) generally had higher concentrations in the drought treatment. Catechin and indole acetic acid had higher concentrations when drought coincided with summer warming. Moreover, the warming treatment partially reduced the concentrations of some secondary metabolites, such as some terpenes, and also reduced the C/nutrient and N/P ratios (Fig. 5.4 and in Appendix 2 Fig.S5.4 and Tab. S5.11 to S5.14). Warming thus moderated the differences of the overall shoot metabolomic-stoichiometric composition of the drought-stressed plants relative to the control and irrigated plants (Fig. 5.3). Simultaneous drought and warming had different consequences on the metabolomic-stoichiometric composition in shoots than did drought and warming separately. Warming partially diminished the effect of drought but increased the concentrations of some secondary metabolites (catechin and acacetin) and elements (Ca, Mg, and Mn). These results were supported by the ANOVAs of the univariant analyses (Appendix 2, Tab. S5.11 to S5.18).

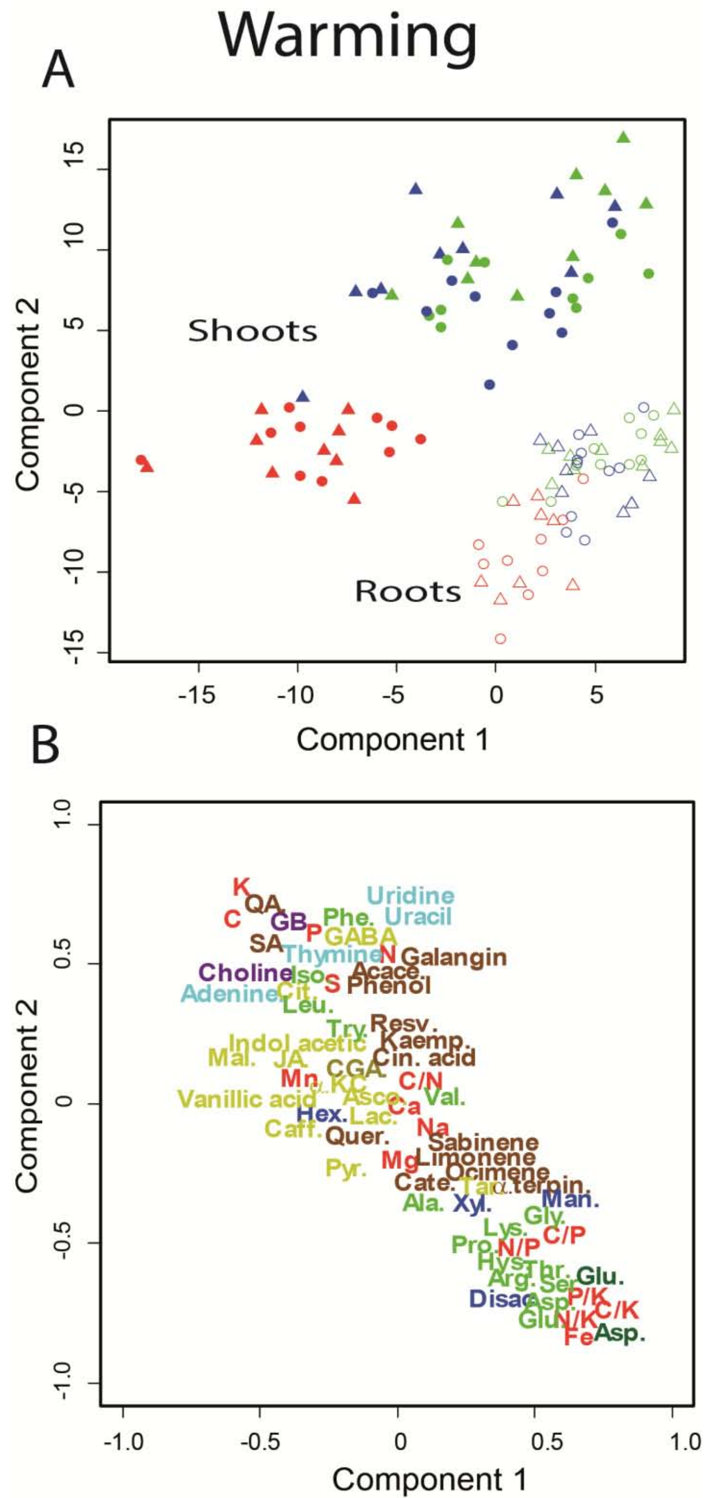


Figure 5.4. Component 1 *vs* component 2 of the partial least squares discriminant analysis of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised scores (mean ± S.E.) by shoots and roots in the warming plus drought (factorial) treatment. Drought is indicated by different colours (green, ambient control; red, drought; blue, irrigated) and letters (A, control ambient; D, drought; I, irrigated). Warming is indicated by letters (W_s, summer warming; W_w, winter warming). (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Fig. 5.1.

5.1.17 Effects of the interaction between drought and warming on elemental concentrations, stoichiometries, and metabolomes in roots

The PLS-DA identified the effect of the interaction of drought and warming on metabolomic-stoichiometric structure in roots (Fig. 5.5 and in Appendix 2 Fig. S5.6 and Tab. S5.15 to S5.18). Warming had different effects depending on the water availability of the plants, consistent with the PERMANOVA results.

Warming had opposite effects in drought-stressed and irrigated roots. The interactive effect in roots was completely different from that in shoots, where the warming treatment partially negated the effects of drought on the metabolomic-stoichiometric structure relative to the control and irrigated plants. Under drought, the warming treatments further increased the concentrations of some secondary metabolites, such as GABA, choline, and glycine betaine, and further decreased the C/nutrient ratio relative to the control and irrigated plants. The effects of the warming treatments were the opposite under irrigation, leading to a minor difference between the metabolomes and stoichiometries of the drought and control plants/roots (Fig. 5.5). The concentrations of choline and glycine betaine, which are involved in osmotic processes, were thus higher under drought and the combination of drought and warming treatments. In summary, the overall effects of the warming treatment on stoichiometry and metabolism in plant roots were dependent on water availability.

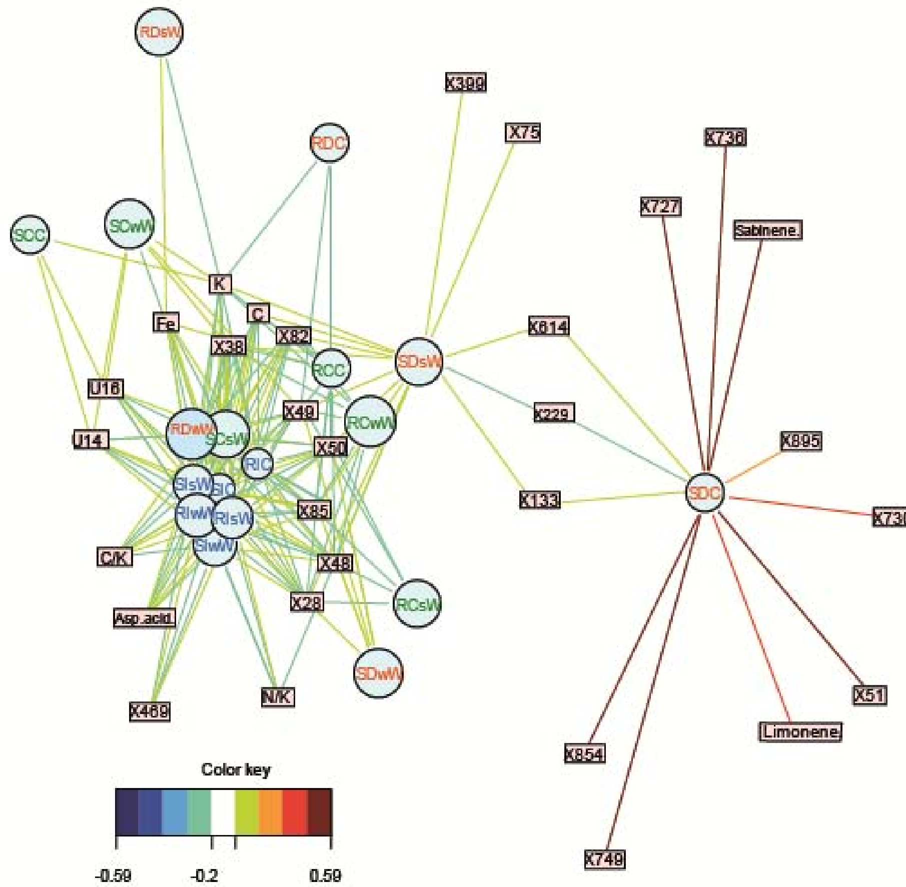


Figure 5.5. Relevance networks of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons for warming plus drought (factorial). Interaction networks between various environmental variables (root, shoots, drought, summer warming, and winter warming) and analysed metabolites. This plot was constructed after Sparse Partial Least Square (SPLS) analysis by differential metabolites among provenances. Green (blue) indicates a high positive (negative) correlation. Shoots and roots are represented by different letters (S, Shoots; R, Roots). Treatments are indicated by different letters colours (green, ambient control; red, drought; blue, irrigated), letters (C, control ambient; D, drought; I, irrigated) and circles. Warming is indicated by letters (sW, summer warming; wW, winter warming). Stoichiometric and metabolomic variables are represented by rectangle.

5.5 Discussion

5.1.18 Elemental, stoichiometric, and metabolomic shifts across shoots and roots, species, and seasons

The metabolome and elemental stoichiometry of shoots differed more between species than between seasons, whereas the opposite was observed for roots. Primary metabolic activity was higher in September (the growing season) than in July for both shoots and roots. The concentrations of amino acids directly linked to growth and pathways of energy metabolism were higher in September shoots, when the plants were growing. The concentrations of sugars were higher in shoots in July, when the plants were mature, likely due to their accumulation during spring and/or to an increase in cellular osmotic potentials. On the other hand, the shoots had the highest K/P ratio and the lowest N/K and C/K ratios in September, when the plants were growing. K is involved in the plant-water relationship (Babita *et al.*, 2010) through plant osmotic control (Sangakkara *et al.*, 2000; Babita *et al.*, 2010; Laus *et al.*, 2011) and improvement in stomatal function (Farhad *et al.*, 2011).

The shoots and roots had higher concentrations of amino acids in September than in July (Fig. 5.3b and in Appendix2 Table S5.3 to S5.10). The increase in the concentrations of primary metabolites coincided with an increase in N and P concentrations. The increase in P concentration was proportionally higher than the increase in N concentration, which led to lower N/P and C/P content ratios and also coincided with the decrease in the concentration of some C-rich secondary metabolites. These results are in agreement with the Growth Rate Hypothesis, which relates high growth with high concentrations of P and N and low N/P ratios (Sterner & Elser, 2002). High levels of these elements allow more synthesis of amino acids and proteins (more N), which in turn requires more synthesis of RNA (more N and especially more P). The decreasing N/P ratios during the growing season coupled to a shift towards primary metabolic pathways related to growth and energy enhancement have also been found in other terrestrial plants (Rivas-ubach *et al.*, 2012). Moreover, under these favourable conditions for growth, the assimilated C is allocated more to growth and energy supply (more primary metabolism) than to antistress or defensive mechanisms (less secondary metabolism). Higher levels of these elements and also the higher concentrations of nitrogenous bases allow more synthesis of amino acids and proteins (more N).

5.1.19 Effect of drought on elemental, stoichiometric, and metabolomic shifts in shoots and roots

The metabolomes of shoots and roots under drought conditions generally shift in opposite directions, although some metabolites change in the same direction (Gargallo-Garriga *et al*., 2014). Plants accumulate a variety of compounds that function as osmoprotectants in shoots. A moderate water stress may be accompanied by the accumulation of metabolites such as proline and glycine betaine, whereas a severe water stress may be accompanied by the accumulation of sugars such as sucrose (Bohnert, 2000; Hoekstra *et al.*, 2001). We observed that shoots under drought conditions also accumulated other metabolites such as related intermediate or derivative compounds of amino acids (RCAAS), osmoprotectants (glycine betaine and choline), and hexoses.

Roots accumulate disaccharides and amino acids when exposed to drought conditions. The accumulation of these metabolites has been described in leaves under different stresses (Rizhsky *et al.*, 2004; Pinheiro *et al.*, 2004; Charlton *et al.*, 2008; Rivas-ubach *et al.*, 2012, 2014) but have not been described in plant roots yet. The shoot samples of the drought-treated plants from September and July had higher concentrations than control plants of metabolites with an antioxidant function, such as some polyphenolic compounds, quinic acid, malic acid, jasmonic acid, and sugars such as those of the family of hexoses and xylose (Fig. 5.4). Quinic acid is a precursor in the shikimic acid pathway, a common metabolic pathway in the biosynthesis of aromatic amino acids such as tyrosine, tryptophan, and phenylalanine (Draths *et al.*, 1999) that are precursors of a large variety of secondary metabolites such as lignins, flavonoids, alkaloids, and phytodexins (Herrmann, 1995).

These metabolic differences were accompanied by an increase in the concentration of K, resulting in low C/K and N/K ratios and a high K/P ratio. The relationship between higher concentrations of osmoprotective secondary metabolites and K concentrations in response to drought has been also observed in the leaves of the Mediterranean shrub *Erica multiflora* (Rivas-Ubach *et al*., 2012) and is related to the improvement in the control of water use (Sangakkara *et al.*, 2000). In contrast, the production of other secondary metabolites related to osmotic protection, such as choline and glycine betaine (McNeil *et al*., 2001), has not been observed to be up-regulated in drought-stressed plants. These metabolic and stoichiometric changes in plants under drought conditions are consistent with the increase in oxidative stress.

5.1.20 Effect of warming on elemental, stoichiometric, and metabolomic shifts in shoots and roots

Warming under control conditions of water availability increased the concentrations of primary metabolites mainly related to energy metabolism (RCAAS) in shoots but increased the concentrations of amino acids in roots. Warming led to a general decrease in the concentrations of several secondary metabolites in both shoots and roots. Plants in the warming treatment did not have higher concentrations of metabolites related to the heat-shock response, such as sucrose and glucose, or a coordinated increase in the pool sizes of amino acids (asparagine, leucine, isoleucine, threonine, alanine, and valine), derivatives of oxalacetate, and pyruvate (Kaplan *et al.*, 2004). The warming treatment applied in this study, based on a realistic projection, thus apparently did not induce heat-shock metabolism. This moderate warming, however, was associated with an increase in some primary metabolites under the expected normal conditions of water availability.

5.1.21 Effects of the interaction between drought and warming on metabolomic and stoichiometric shifts across shoots and roots

Shoots and roots subjected to simultaneous drought and warming responded differently than when subjected to each treatment separately. Warming further increased the accumulation of proline in shoots under a severe water stress. In contrast, the concentrations of osmolytes and some compounds related to growth such as nitrogenous bases and some amino acids that help to protect the root under water stress increased in roots when drought was applied together with warming more than under drought alone. This increase was related to the higher concentrations of C, N, P, and K observed under drought plus warming than under drought alone, suggesting that the plants allocated more resources associated with growth and cellular activity under drought and warming than solely under drought. Thus, in contrast to shoots, roots had higher concentrations of metabolites linked to growth and energy in response to warming applied together with drought than when submitted only to drought.

The literature on transcripts involved in the defence of plants against abiotic conditions such as cold, drought, and salinity reports considerable common responses (e.g.,Kreps *et al.*, 2002; Oztur *et al.*, 2002; Seki *et al.*, 2002; Sardans *et al.*, 2013). Other studies have found that leaves respond to combined drought and warming stresses with a lower suppression of primary metabolism, the production of some terpenes, and increases in concentrations of other secondary metabolites such as catechin and indole acetic acid than do plants growing

under drought stress. Our results thus suggest that plants respond differently under simultaneous drought and warming depending on the tissue. Shoots suppressed their primary metabolism less and changed their anti-stress metabolic strategy less under combined drought and warming conditions than under drought alone. Warming under drought enhanced the concentrations of compounds in roots related to growth and energy metabolism more than solely under drought. The combination of both stresses in this case likely enhanced the effect of the drought by reducing the water availability due to the warming. Warming had a stronger effect alone in roots than when applied with drought.

Changes in soil temperature not only influence the growth and development of roots, but can also impact the root-shoot relationships. Gosselin & Trudel (1986) observed that increasing the temperature of the root zone from 12 to 36 °C tended to increase the shoot dry mass and the overall productivity of pepper (*Capsicum annum* L.). This higher activity of the plant and the increase in primary elemental sources related with the growth of roots can enhance the water-uptake capacity of plants. Other studies of *Arabidopsis* (Hellmann *et al.*, 2000) and *Nicotiana tabacum* (Rizhsky *et al.*, 2002) have suggested that this mode of defence response is conserved among different plants subjected to the combination of warming and drought.

The shift in metabolomic-stoichiometric composition in response to environmental changes has thus been demonstrated to be very different in above-and belowground tissues of the same plant. In response to drought, aboveground tissues had lower levels of metabolites associated with energy and growth metabolism (sugars, amino acids, and nucleosides), lower N, P, and K concentrations, and a higher C/N ratio. Belowground organs had the opposite pattern.

In summary, the stoichiometric and metabolomic responses of plants to warming strongly depend on water availability, and the response differs in shoots and roots. Warming under drought conditions stimulates root primary metabolic activity more than drought alone. Compared to drought alone, shoots under simultaneous warming and drought shifted their osmoprotective and antis-tress strategies by down- and up-regulating the synthesis of various secondary metabolites and by activating some primary metabolic pathways. Our results thus demonstrated different metabolomic expressions in different parts of the plant and a large plasticity in the responses to environmental changes.

5.6 Supporting Information

Supporting information and the following figures are enclosed in Appendix 2 of the manuscript. Examples of the ^{1}H NMR and chromatogram metabolic profiles of the extract sample of *Holcus Lanatus* and *Alopecurus Pratensis* and the tables in the attached CD.

Fig. S5.1 Experimental design.

Fig. S5.2 PC1 versus PC2 of a seasonal PCA of *Holcus lanatus* and *Alopecurus pratensis* shoots.

Fig. S5.3 PC1 versus PC2 of a seasonal PCA of *Holcus lanatus* and *Alopecurus pratensis* roots.

Fig. S5.4 Component 1 vs component 2 of the PLS-DA of *Holcus lanatus* and *Alopecurus pratensis* shoots in the warming plus drought (factorial) treatment.

Fig. S5.5 Component 1 vs component 2 of the PLS-DA of *Holcus lanatus* and *Alopecurus pratensis* roots in the warming plus drought (factorial) treatment.

Fig. S5.6 Component 1 vs component 2 of the PLS-DA of *Holcus lanatus* and *Alopecurus pratensis* shoots and roots in the warming plus drought (factorial) treatment.

Figure A5.1 Examples of the ^{1}H NMR metabolic profiles of the extract sample of *Holcus Lanatus* and *Alopecurus Pratensis*.

Figure A5.2 Examples of the chromatogram metabolic profiles of the extract sample of *Holcus Lanatus* and *Alopecurus Pratensis* in positive and negative mode.

Table S5.1 Analytical technique (LC-MS and/or NMR) used for the identification of the metabolites and their categorization in terms of biochemical group and metabolic pathway.

Table S5.2 Processing parameters of LC-MS chromatograms

Table S5.3 One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in July in different warming treatments.

Table S5.4 One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in July in different warming treatments.

Table S5.5 One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in September in different warming treatments.

Table S5.6 One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in September in different warming treatments.

Table S5.7 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in July in different warming treatments.

Table S5.8 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in July in different warming treatments.

Table S5.9 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in September in different warming treatments.

Table S5.10 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in September in different warming treatments.

Table S5.11 One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in July in different warming treatments within different levels of water availability.

Table S5.12 One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in September in different warming treatments within different levels of water availability.

Table S5.13 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in July in different warming treatments within different levels of water availability.

Table S5.14 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in September in different warming treatments within different levels of water availability.

Table S5.15 One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in July in different warming treatments within different levels of water availability.

Table S5.16 One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in September in different warming treatments within different levels of water availability.

Table S5.17 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in July in different warming treatments within different levels of water availability.

Table S5.18 One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in September in different warming treatments within different levels of water availability.

Methods S5.1 Details of NMR metabolite elucidation.

Chapter 6

Study of responses of *Quercus ilex* seedlings to wounding.

This study has been published in the journal *Plant Biology* as

Metabolic responses of Quercus ilex seedlings to wounding analyzed by nuclear magnetic resonance profiling.

Jordi Sardans, Albert Gargallo-Garriga, Miriam Pérez-Trujillo, Teodor Parella, Roger Seco, Iolanda Filella, Josep Peñuelas. *Plant biology*, **2014,** 16 (2), 395-403.

DOI:10.1111/plb.12032

6.1 Abstract

Plants defense themselves against herbivory at several levels. One of these levels is the synthesis of inducible chemical defenses. By using NMR metabolomic technique described in chapter 1 we studied the metabolic changes of plant leaves after a wounding treatment simulating herbivore attack in the Mediterranean sclerophyllous tree *Quercus ilex*. First, an increase of glucose content was observed in wounded plants. There was also an increase of contents of C-rich secondary metabolites such as quinic acid and quercitol both related to the shikimic acid pathway and linked to the defense against biotic stress. There was also a shift in N-storing amino acids from leucine and isoleucine to asparagine and choline. The observed higher content of asparagine is related to the higher contents of choline through serine that has been proved to be the precursor of choline. Choline is a general antiherbivore and pathogens deterrent. The study shows the fast metabolic response of *Q. ilex* to defend its leaves based in a rapid increasing production of quinic acid, quercitol and choline. The results also confirm the suitability of ^{1}H NMR-based metabolomic profiling studies to detect the global metabolome shifts after a biotic stress in tree leaves, and therefore its suitability in ecometabolomic studies.

6.2 Introduction

Plants can respond to herbivore attack by several mechanisms. Some studies have observed changes in leaf molecular composition after herbivore attack (Ralph *et al.*, 2006) or after mechanical wounding (Ralph *et al.*, 2006; Grata *et al.*, 2007, 2008; Glauser *et al.*, 2008, 2010; Thiocone *et al.*, 2008). The resulting transcriptomic and metabolic changes provide resistance or tolerance to the herbivore or pathogen attack allowing plants to maintain high fitness in the presence of enemies. In wounded leaves metabolic process may not only protect them but also regenerate the injured tissue, e.g. suberization induction of the wound (Yang & Bernards, 2007).

The study of plant metabolic changes and processes related with herbivore attack has been mostly limited to the identification of single compounds or families of metabolites. Ecometabolomics aims to analyze the metabolome, the total number of metabolites and their shifts in response to environmental changes (Peñuelas & Sardans, 2009b; Sardans *et al.*, 2011; Rivas-ubach *et al.*, 2012). Metabolomics provides an analysis of the different response capacities conferred by the phenotypic plasticity of each species, thus allowing ascertaining what metabolic pathways are involved in a phenotypic response (Peñuelas & Sardans, 2009b; Sardans *et al.*, 2011; Rivas-ubach *et al.*, 2012). Some partial ecometabolomic studies of polar metabolites have found a great chemical variation in the metabolites used by plants to defend themselves against insect attacks (Widarto *et al.*, 2006; Jansen *et al.*, 2009; Kuzina *et al.*, 2009; Leiss *et al.*, 2009, 2011; Mirnezhad *et al.*, 2010) confirming the great variability of chemicals that are used by plant as chemical deterrents. The metabolomic techniques have been already used to investigate the effects of jasmonate on plant metabolism (Hendrawati *et al.*, 2006; Liang *et al.*, 2006a,b; Ozawa *et al.*, 2008) proving to be useful to reach an overview of global metabolism shifts in response to chemical signs. Among metabolomic approaches, NMR spectroscopy together with data mining allows the simultaneous detection of a wide range of metabolites and also to identify the metabolites underlying plant responses to herbivore attack (Verpoorte *et al.*, 2008; Leiss *et al.*, 2009, 2011).

The metabolome variations induced by wounding, which mimics the effects of herbivores, have been studied in herbaceous plants such as *Arabidopsis thaliana* (Grata *et al.*, 2007) and *Solanum lycopersicum*(Wasternack *et al.*, 2006). These studies have shown that an increase of oxygenated fatty acids (oxylipins) is a characteristic response to wounding and is often accompanied by the release of small lipid-fragmentation products, constituting the “oxylipins signature” (Mueller *et al.*, 2006; Wasternack *et al.*, 2006). However, not all the studies have

observed metabolomic shifts after wounding. Wounded leaves of *Solanum nigrum* did not change its metabolome structure 3 hours after simulated herbivore attack (Vandoorn *et al.*, 2011). Moreover, NMR based metabolomic studies in plants submitted to insect attack have been used to study the shifts at the level of constitutive defenses, by comparing resistant and non resistant genotypes of the same species (Leiss *et al.*, 2009, 2011; Mirnezhad *et al.*, 2010) and at the level of inducible defenses, by comparing attacked and non attacked plants of the same genotype (Widarto *et al.*, 2006; Plischke *et al.*, 2012). These few studies show that the chemical groups of metabolites underlying plant response to herbivore attack depend on plant taxonomic group. The main signal compounds observed in these studies range from secondary metabolites such as phenolics and pyrrolizidine alkaloids to primary metabolites such as sugars, amino acids and its derivatives.

6.3 Materials and methods

6.1.1 Plant material

We used 3-year-old potted *Quercus ilex* L. plants grown in a nursery (Tres Turons S.C.P., Castellar del Vallès, Catalonia, Spain), maintained under Mediterranean ambient conditions outdoors. They were grown in 2 L pots with a substrate composed of peat and sand (2:1), prior to being brought into the laboratory, where they were allowed to acclimate to laboratory conditions for several days before starting the experiment.

6.1.2 Plant and leaf chambers

We used two different chambers (Seco *et al.*, 2011). The whole aerial part of the monitored plant was enclosed in a 65 L cylindrical chamber made of transparent teflon film. This big plant chamber (PC) was illuminated from the top by fluorescent lights (Agrolite CFL, Barcelona) giving about 300 µmol $m^{-2} s^{-1}$ of photosynthetic active radiation (PAR) flux inside the chamber, with a 12:12h light:dark photoperiod. Ambient air from outside the building was introduced into the PC at a rate of about 15 L min^{-1} by means of an oil-free pump. A fan stirred the air inside the PC, and there was always an excess of air escaping from the PC through the tiny space between the trunk of the tree and the PC teflon film, thus preventing external air to enter the PC. CO_2 was added from a bottle (Abelló Linde S.A., Barcelona, Catalunya) through a mass flow controller (Bronkhorst High-Tech B.V., Ruurlo, Netherlands) during high photosynthetic activity periods, to keep atmospheric CO_2 concentrations ca. 385 µmol mol^{-1}.
A second, small leaf chamber (LC) was introduced inside the PC and clamped onto a leaf. This leaf cuvette was part of a LCpro+ Photosynthesis System (ADC BioScientific Ltd., Herts, England), which recorded photosynthesis (net CO_2 uptake), stomatal conductance, air humidity, and temperature data, while controlling the light radiation and the flow of air entering the leaf cuvette. A light diurnal cycle was programmed in the LC to simulate a typical sunny day, ranging from 0 to 1500 µmol $m^{-2} s^{-1}$ of PAR and with the same 12:12h photoperiod as the PC. The air entering the LC was taken from inside the PC: this way the air reaching all the leaves of the plant was the same. For more details see Seco *et al*. (2011).

6.1.3 Experimental design

In three plants we sampled well developed leaves randomly located in the outer canopy before cutting simulating herbivory and we sampled again randomly other well developed one-year old leaves of three plants after simulating herbivory by clipping.

This experimental setup resulted in two sampling and measuring times for plants: control (control before cutting) and wounding (after cutting leaves). Each one of these measures was taken on different consecutive days. On day 1, the plant was left untouched to acclimate to the chamber conditions. On day 2, at 15h, control leaves were sampled and afterwards some leaves were clipped with scissors leaving a small portion of petiole and leaf still attached to the ground to simulate herbivory. On day 3, the day after the clipping, we sampled again leaves in these plants clipped the day before. In all cases, the samples were immediately stored in liquid N_2.

6.1.4 Sample preparation

Frozen leaves were lyophilized. Dry leaves were kept into plastic cans and frozen at -20 ˚C. Samples were ground with a Mikrodismembrator-U (MARCA, CIUTAT) obtaining a fine leaf powder. All sample powders were then maintained at -20 ˚C until NMR extract preparation.

Leaf powder (200 mg) was introduced into a centrifuge tube. Thereafter, 6 mL of 50% water-methanol mixture and 6 mL of chloroform were added to each tube. Samples were mixed during 15 s by vortex and then 1 min sonicated. All tubes were centrifuged at 3.000 rpm during 30 min. Thereafter, 4 mL of each fraction (aqueous and organic), were collected independently into jars. This procedure was repeated twice obtaining 8 mL of aqueous and organic soluble fractions (ASF and OSF respectively) for each sample. Organic fractions were collected using crystal syringes.

In order to reduce the methanol percentage to 5% approximately and make the lyophilization process possible 100 mL of water were added to ASF samples. ASF samples were frozen at -80 ˚C. After that, samples were lyophilized to extract all water and methanol content. OSF samples were placed separately in a round-bottom evaporation flask and dried in a rotary vacuum evaporator.

For the NMR analyses, 1 ml of a 1:1 CD_3OD-D_2O phosphate buffer (90 mM, pH 6.0, containing 0.01% TSP, trimethylsilyl propionic acid-d4 sodium salt) was added to ASF dried samples obtaining a solution with final pH of 6.0. In parallel, 1 mL of chloroform-d containing 0.01% TMS (tetramethylsilyl) was added to OSF dried samples. TSP and TMS were used as

internal standards. All content was transferred into Eppendorfs and centrifuged 3 min at 6.000 rpm and 2 min at 10.000 rpm.

For each sample, 0.6 mL of supernatant was transferred into NMR sample tubes. The NMR tube caps with the OSF samples were covered with parafilm to avoid the chloroform evaporation. For more details on sample preparation see Rivas-Ubach *et al*. (2013).

6.1.5 ^{1}H NMR fingerprinting

High-resolution ^{1}H NMR spectroscopy measurements were conducted using a Bruker AVANCE 600 spectrometer equipped with an automatic sample changer and a multinuclear triple resonance TBI probe (Bruker Biospin, Rheinstetten, Germany) at a field strength of 14.1 T (600.13 MHz ^{1}H frequency). The probe temperature was set to 298.0 K. Following the introduction to the probe, samples were allowed to equilibrate (1 min) prior to the shimming process to ensure good magnetic field homogeneity. All liquid sample handling, automation and acquisition were controlled using TOPSPIN 2.1 software (Bruker Biospin, Rheinstetten, Germany).

For the water/methanol extract samples, one-dimensional (1D) ^{1}H NMR spectra were acquired with suppression of the residual water resonance. The water resonance signal was presaturated using a power level of 55 dB (Bruker nomenclature), corresponding to an effective field of 30 Hz during a relaxation delay of 2 s. Each spectrum was acquired into 32 k data points over a spectral width of 16 ppm as the sum of 128 transients and with an acquisition time of 1.7 s. The total acquisition time was ~8 min per sample during a relaxation delay 2s. For the chloroform extract samples, standard pulse-acquisition 1D ^{1}H-NMR spectra were acquired. The acquisition parameters were also set at 32 k data points, 16 ppm of spectral width and 128 transients, resulting in a total acquisition time of ~8 min per sample. All ^{1}H NMR spectra were phased, base line corrected and referenced to the internal standard (TSP for polar and TMS for nonpolar samples) resonance at δ 0.00 ppm using the same software TOPSPIN 2.1. For more details of sampling process and NMR determination see Rivas-Ubach *et al*. (2012).

6.1.6 Metabolite identification

The NMR spectrometer described for the fingerprinting was used for the acquisition of the 2D NMR experiments on selected representative samples of the water-methanol and of the chloroform extracts. The probe temperature was set to 298.0 K and the software utilized to acquire and process the experiments was TopSpin 2.1 (Bruker Biospin). 1D ^{1}H NMR experiment and 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY),

^1H-^{13}C heteronuclear single-quantum correlation (HSQC), and ^1H-^{13}C heteronuclear multiple-bond correlation (HMBC) were acquired using standard Bruker pulse sequences and routine conditions (Kim *et al*. 2010; Rivas-Ubach *et al*. 2012). For the water–methanol extract samples, 2D experiments were carried out with standard presaturation of the residual water peak during the relaxation delay. Spectra were referenced to TSP (^1H and ^{13}C at δ 0.00 ppm) in the case of polar samples and to the residual $CHCl_3$ (δ_H 7.260 ppm and δ_C 77.00 ppm for ^1H and ^{13}C, respectively) in the case of nonpolar samples.

The concerted analysis of 2D ^1H-^1H COSY, TOCSY, ^1H-^{13}C HSQC and HMBC experiments allowed the assignment of most of the peaks in the polar and nonpolar 1D ^1H-NMR spectra profiles. When possible, assignments were confirmed with described data bases (Breitmaier *et al.*, 1979; Bolinger *et al.*, 1984; Iles *et al.*, 1985; Ulrich & Zhulin, 2007; Llusià *et al.*, 2008; Fan & Lane, 2008, 2011).

6.1.7 NMR data treatment and statistical data analysis

1D ^1H-NMR spectra were used for statistical analyses. The statistical elaboration of the NMR data was performed using the AMIX software (Bruker Biospin, Rheinstetten, Germany). The intensity of the peaks of each variable of all samples was mean-centered and standardized. The integrate regions were normalized to the sum of all integral regions for each spectrum. NMR spectra were uniformly binned, being each region 0.002 ppm wide, and integrated automatically with *Bucketing* process ("*point size bucket table*"). In the case of polar spectra the region of the suppressed residual water signal, δ 4.8-5.1 ppm, and that of the residual methanol signal, δ 3.28-3.35 ppm, (used in the extraction process) were not considered. The polar spectra were reduced from 8193 to 6901 regions, 0.002 ppm wide each. In the case of nonpolar spectra, the region of suppressed residual cloroform signal, δ 7.1-7.3 ppm, was not considered. The nonpolar spectra were reduced to 7314 regions, 0.002 ppm wide each.

After that, the statistical analysis of the treated NMR data was performed separately for each set of spectra (polar and nonpolar). All ^1H NMR signals corresponding to the same molecular compound were summed to reduce the final number of variables. Signals in the phenolic region (6.6-7.2 ppm) were also summed and considered as one variable. We tested the normality of each variable by Kolmogorov–Smirnov tests. All variables followed normal distributions. Multivariate ordination analyses (PCAs based on correlations) were also performed to detect patterns of sample ordination in the metabolomic variables, which was performed with the software Unscrambler version 10.2 from CAMO (Computer Aided

Modelling, Trondheim, Norway). Differences in PCA scores between control and wounded plants were tested by t-test; wounding treatment was used as the independent factor (categorical variable) and the integral values of each metabolite were used as response dependent variables.

6.4 Results

6.1.8 Identification of metabolites. Metabolic profiles

The polar and nonpolar metabolic profiles of *Quercus Ilex* leaf are shown in Figure 6.1 and in Table S6.1 and S6.2 of the Appendix 3/CD. The ^{1}H NMR metabolic profile of the polar extract is shown in Figure 6.1A. The signals in the region between 3.2 and 5.9 ppm correspond mainly to sugars. Among them, α-glucose and β-glucose, with anomeric proton resonances at 5.29 ppm (d, J = 3.8 Hz) and at 4.70 ppm (d, J = 8.2 Hz) respectively, were identified. The disaccharide sucrose was also observed, with its characteristic anomeric proton doublet of the glucose unit at 5.45 ppm (d, J = 3.8 Hz). The singlet at 3.19 ppm which correlates to multiplets at 3.99 and 3.51 ppm via COSY (bonded respectively to carbon atoms at 53.8, 69.6 and 58.0 ppm correlates via HSQC) corresponds to choline, which is an important osmolyte. In the aliphatic region between 2.2 and 1.7 ppm, the secondary metabolites quinic acid and quercitol were identified, showing very intense signals. Among amino acids, asparagine was identified, by the doublet corresponding to its Hα carboxylic at 4.06 ppm (bonded to a carbon atom with 51.0 ppm chemical shift) and which correlates via COSY two doublet of doublets signals, corresponding to the protons of the methylene group at 2.93 and 2.99 ppm (bonded to a carbon atom at 34.5 ppm correlates via HSQC). Also glutamate, valine, alanine, isoleucine, and leucine were identified. Some organic acids such as formic acid (sharp singlet at 8.50 ppm), malic acid, citric acid, acetic acid and lactic acid were identified as well. The triplet at 1.33 ppm (t, J = 7.17 Hz), which correlates via COSY to a quartet at 4.36 ppm (t, J = 7.17 Hz), typically corresponds to N-acetyl group. Finally, signals at the 6.5 - 7.4 ppm region, area mainly of aromatic compounds, were also observed. All the identifications were based on the ^{1}H and ^{13}C NMR complete or partial assignment of the molecules based on 1D and 2D NMR experiments and on the comparison with reported data. For the complete description see Table S1 of the Appendix 3 in CD.

Figure 6.1B shows the ^{1}H NMR metabolic profile of the nonpolar extracts. Saturated and unsaturated fatty acid chains were identified, which come from free fatty acids, fatty alcohols, diacylglycerols and triacylglycerols. Also, *p*-coumaric acid derivatives were observed in the aromatic region. The complete description is shown in Table S6.2 of the Appendix 3 in the CD.

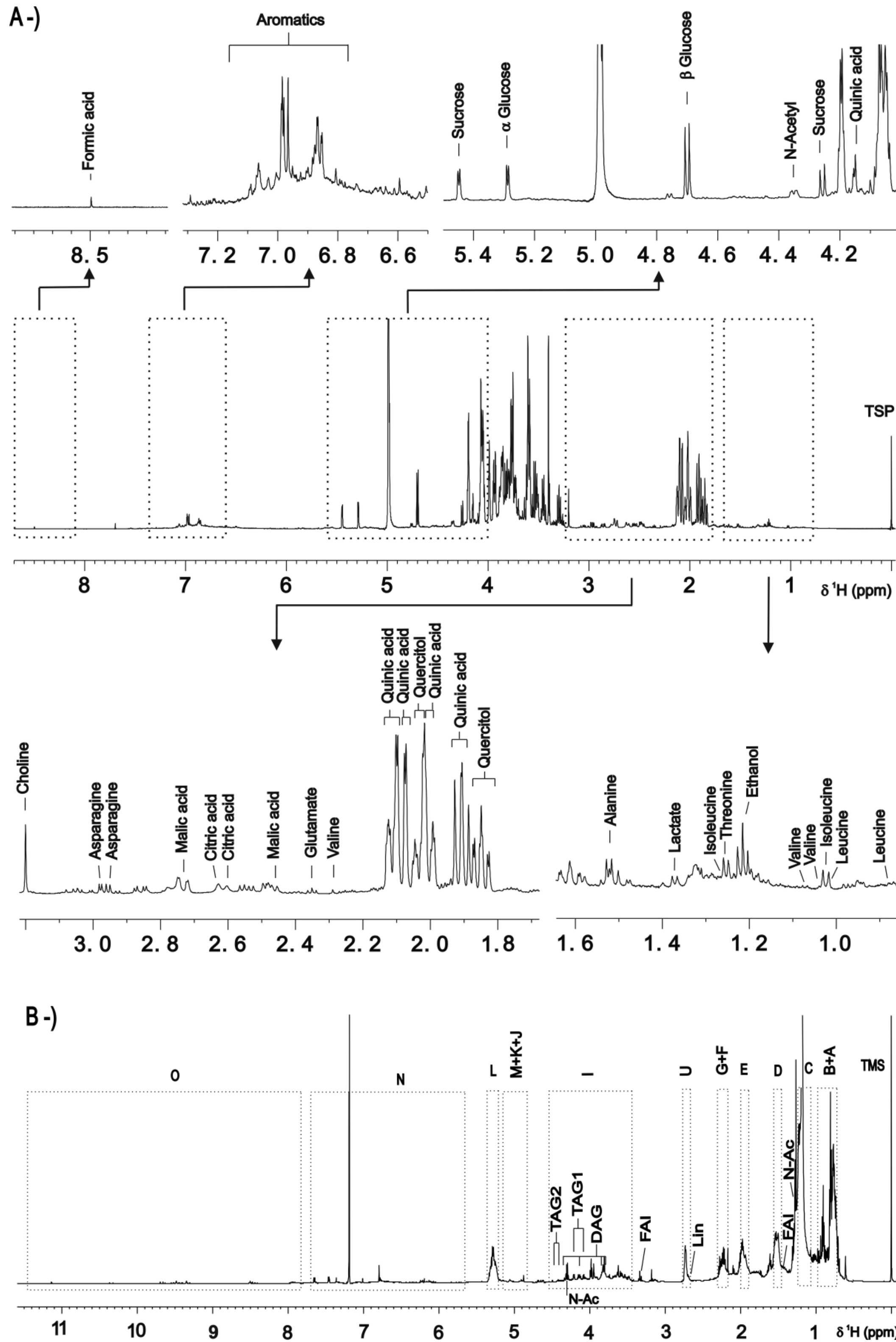


Figure 6.1. (A) Example of the ^{1}H NMR metabolic profile of the polar (water–methanol 1:1) extract sample of *Q. ilex* leaf. Assignments of the main peaks are indicated. The sample was dissolved in CD_3OD-D_2O 1:1 (pH 6.0) and referenced to TSP. (B) Example of the ^{1}H NMR metabolic profile of the nonpolar (chloroform) extract of *Q. ilex* leaf. Assignments of the main peaks are indicated. The sample was dissolved in $CDCl_3$ and referenced to TMS. Spectra were acquired at a magnetic field of 600 MHZ and at 298.0 K of temperature.

6.1.9 Control versus wounded plants

Glucose, quinic acid, quercitol and choline were the metabolites present in highest contents. Several individual compounds presented significant differences between control and wounded plants in polar extracts (Fig. 6.2, Tab. S6.3). Aromatics, valine and leucine presented lower contents in wounded plants than in control plants whereas glucose, quinic acid, choline, N-acetyl group, malic acid and quercitol presented higher contents in wounded than in control plants (Fig.6.2). Some detected and identified molecules such as sucrose, alanine and lactate presented no different contents between wounded and control plants. No significant differences between control and wounded plants were detected in non-polar extracts (Data not shown).

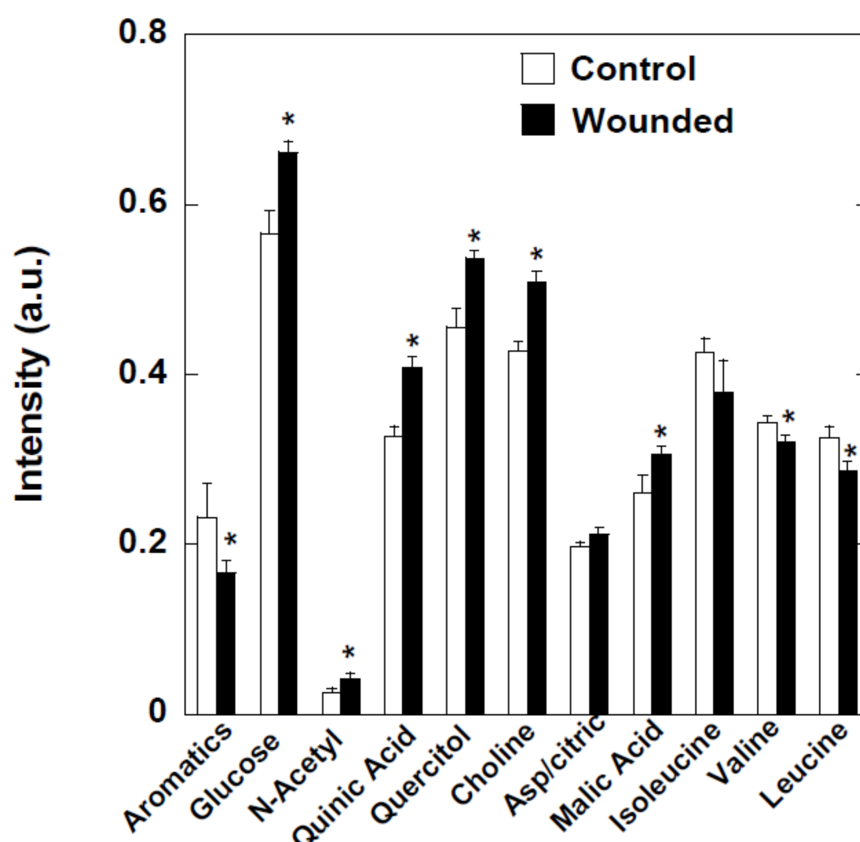


Figure 6.2. Comparisons of peak areas of different metabolites between control and wounded plants. * means statistical differences at the level $p < 0.05$. (t-test).

The PCA analysis of the different polar metabolites as variables and the different control and wounded plants as cases is shown in Figure 6.3. Wounded plants were clearly separated from control plants. Wounded plants presented lower contents of some amino acids such as isoleucine, leucine and valine and aromatics compounds than control leaves (Fig.6.3). On the contrary, wounded plants had significant higher contents of glucose, choline, N-acetyl derivative molecules, malic acid, quinic acid and quercitol than control plants (Fig. 6.3). Finally, no significant differences between control and wounded plants were detected in the PCA analysis of nonpolar extracts (Fig. 6.4).

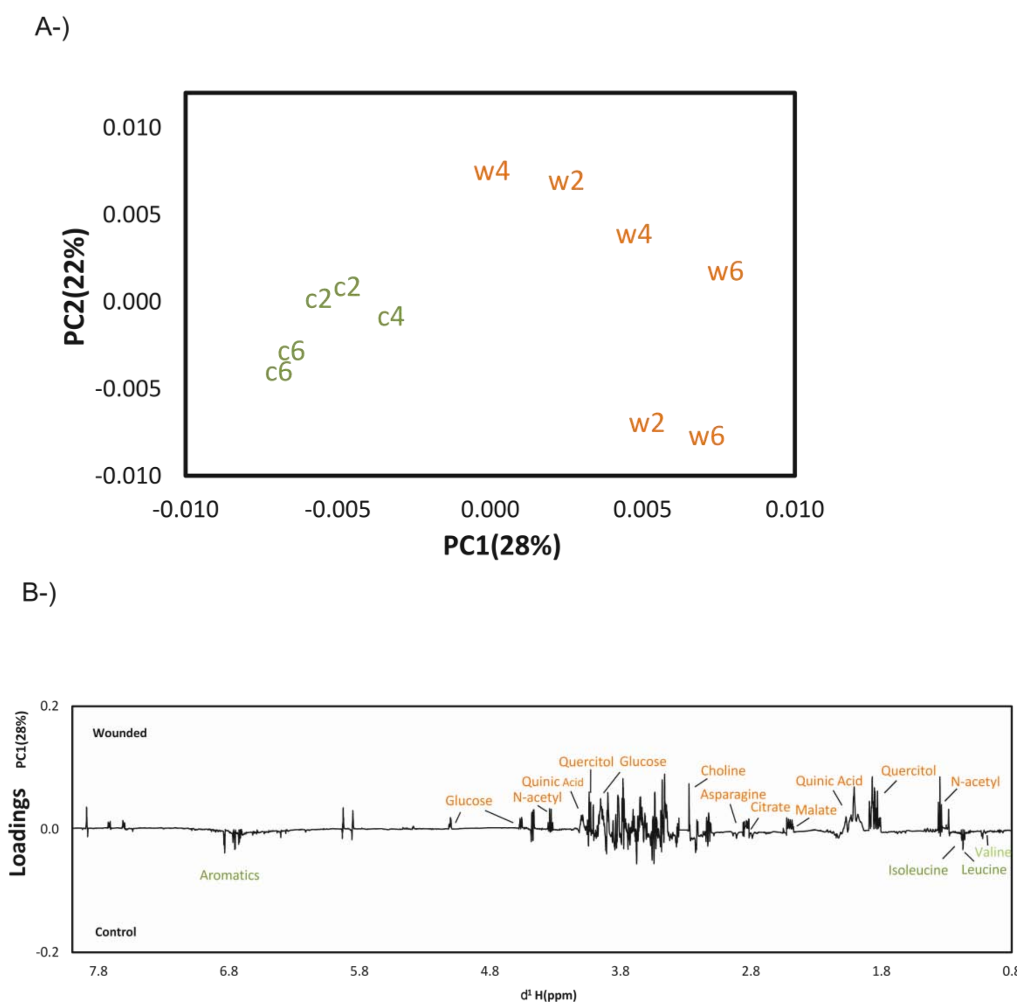
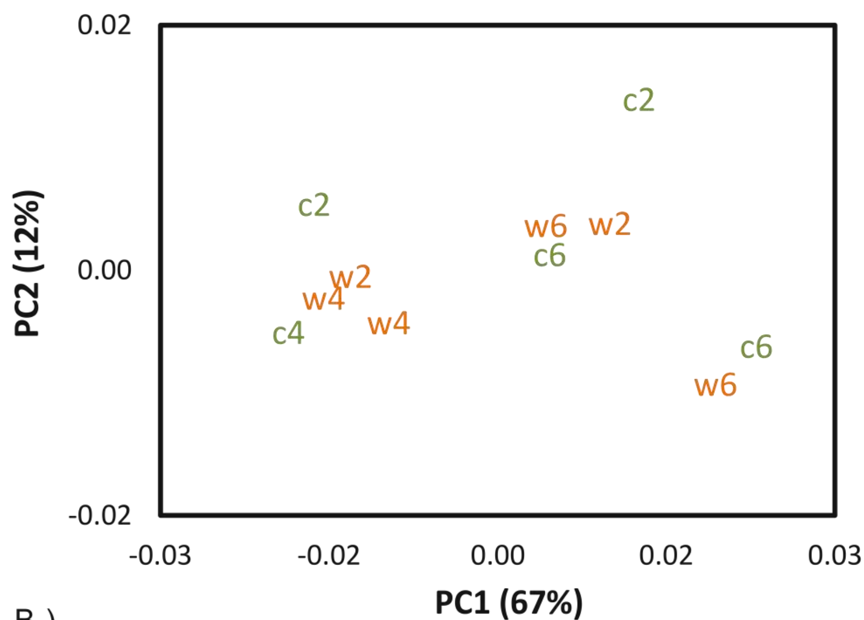


Figure 6.3. (A) Biplot of the second principal component (PC2) versus the first principal component (PC1) scores resulting from PCA conducted with ^{1}H NMR metabolomic variables from polar extracts of *Q. ilex* leaves. (B) Loading values of the first principal component (PC1) of the different peaks of the NMR spectrum, and their assignment, with a significantly different content in control than in wounded plants; the compounds with higher contents in not wounded plants (control) are green colored and those with higher contents in wounded plants are red colored.

A-)



B-)

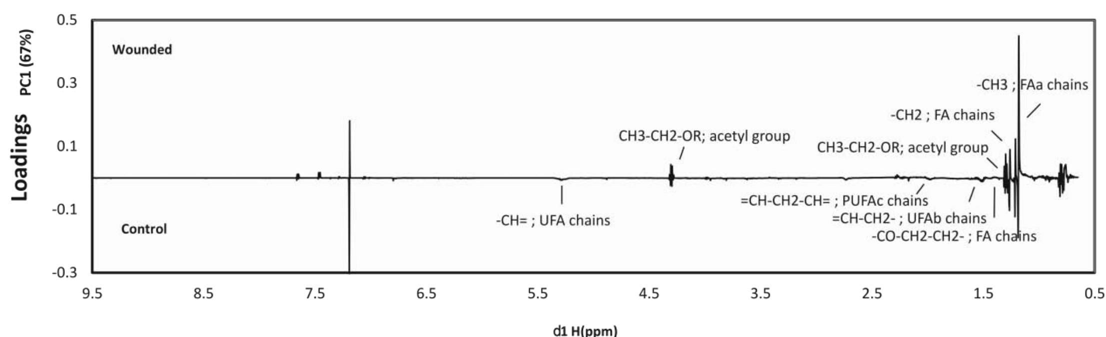


Figure 6.4. (A) Biplot of the second principal component (PC2) versus the first principal component (PC1) scores resulting from PCA conducted with ^{1}H NMR metabolomic variables from non-polar extracts of *Q. ilex* leaves. (B) Loading values of the first principal component (PC1) of the different peaks of the NMR spectrum.

6.5 Discussion

The increases in glucose content observed in wounded plants have been observed also in previous studies in herbaceous vegetation both in plants with wounded leaves (Widarto *et al.*, 2006) and roots (Lafta & Fugate, 2011). Increases in glucose content have been observed as a result of a cascade of defense responses against infection and wounding involving shifts in carbohydrate metabolism (Ehness *et al.*, 1997). The observed higher glucose contents in this study is related with the higher levels (19% more) of photosynthetic assimilation rates and in actual photosynthetic efficiency observed in these wounded plants (Seco *et al.*, 2011), all them suggesting an increase of carbon uptake after wounding. However, higher glucose concentrations could also be partly related to a decrease in C exportation from the leaves. It should be noted that although increased photosynthetic rates have been observed in wounded leaves of Q.ilex (Seco *et al.*, 2011) several studies have reported down regulation of genes involved in photosynthesis after herbivore attack (Royo *et al.*, 1999; Bilgin *et al.*, 2010; Kerchev *et al.*, 2012).

Amino acids and amino acid precursors and derivatives are considered defense related metabolites (Steinbrenner *et al.*, 2011) and their content has been observed to rise in response to wounding in different organs of *Solanum lycopersicum* (Steinbrenner *et al.*, 2011), in leaves of *Solanum tuberosum* (Plischke *et al.*, 2012) and also in leaves of *Brassica rapa* (Widarto *et al.*, 2006). Some studies have observed that Jasmonate-isoleucine molecule, a signal molecule, is produced after wounding (Yang & Bernards, 2007; Anssour & Baldwin, 2010). However, in this study lower contents of leucine and isoleucine have been observed in wounded plants, which instead presented higher asparagine content. Amino acids derived from aspartate have been observed to increase as response against pathogen attacks in *Arabidopsis thaliana* (Brauc *et al.*, 2011; Pétriacq *et al.*, 2012) and in carterpilar attack to *Solanum lycopersicum* plants (Steinbrenner *et al.*, 2011). Asparagine is a general nitrogen-transporter and has a key role in transamination process (Lam *et al.*, 1995). However, choline is synthesized from serine and serine synthesis in plants is related to transamination process from asparagine (Ta *et al.*, 1985; Joy & Prabha, 1986). Thus, the results suggest a shift of nitrogen (N) from isoleucine and leucine to asparagine and choline. The increase in choline content in wounded plants is related with the increase in the synthesis of membrane components after membrane injure (McNeil *et al.*, 2001). Some studies have observed that wounding induces choline synthesis by the jasmonic acid signal transduction pathway such as observed in *Arabidopsis thaliana* (Titarenko *et al.*, 1997). This effect has been observed not only at the site of wounding but also the

undamaged area of wounded plants (Ryu & Wang, 1996). The accumulation of N-rich soluble molecules has been suggested as a mechanism to store N and carbon (C) after wounding, helping to prevent N losses during the wound response (Zhou & Thornburg 1999). Thereafter, these wound-induced reserves could serve as a source for a new growth after wound-recovery phase (Zhou & Thornburg 1999). The increases of asparagine contents could be also related to an inhibition of protein synthesis that has been widely observed after herbivore attack (Royo *et al.*, 1999; Bilgin *et al.*, 2010; Kerchev *et al.*, 2012).

Quinic acid and quercitol have been observed to be the most abundant metabolites in leaves of some *Quercus* species such as *Q. suber* (Passarinho *et al.*, 2006). Thus, this study confirms that these two metabolites are very abundant in the leaves of the plants of the genus *Quercus*. Quinic acid is a metabolite related to metabolic response (inducible defense) in response to biotic stress (Koskimäki *et al.*, 2009; Murthy & Manonmani, 2009; Leiss *et al.*, 2009). Quercitol is a metabolite that has been proved to be related to osmotic stress avoidance under salt or drought stress in *Eucalyptus* (Adams *et al.*, 2005; Arndt *et al.*, 2008; Merchant *et al.*, 2009) and *Quercus* (Passarinho *et al.*, 2006; Spieß *et al.*, 2012) species, and also in response to warming in the genus *Quercus* (Chaves *et al.*, 2011). However, to our knowledge, no previous studies have shown a relationship between quercitol and response to herbivore.

6.6 Supporting information

The following tables are enclosed in the attached CD (named under Appendix 3).

Table S6.1 Assignment of the NMR signals of the polar metabolic profiles of *Q. Ilex* leaves

Table S6.2. Assignment of the NMR signals of the nonpolar metabolic profiles of *Q. Ilex* leaves

Table S6.3 shows the results of t-test statistics for the comparisons between control and wounded plants regarding the metabolite peak areas of the different metabolites.

Chapter 7

Study of the shifts in plant foliar and floral metabolome in response to the suppression of the associated microbiota

This study corresponds to the written unpublished work

Shifts in plant foliar and floral metabolome in response to the suppression of the associated microbiota.

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7.1 Abstract

The phyllospheric microbiota is assumed to play a key role in protecting host plants against diseases and thereby promoting growth. The metabolome of the microbial community and its relationship with the epispheric and internal plant metabolome remain to be investigated. We analyzed the LC-MS profiles of the epispheric and internal metabolomes of the leaves and flowers of *Sambucus nigra* with and without antibiotic treatment. We hypothesized that the microbiota would significantly influence the metabolomes of the plant epispheric communities but also those inside the plant organs. The metabolic profiles of the epispheric microbiota growing on different plant organs (flowers and leaves) were less variable than the metabolic profiles of these organs. The majority of the metabolites detected were present in the organs and in their epispheric microbiotas; the main differences were in the relationships among their metabolite concentrations. The suppression of microbial communities by topical applications of antibiotics had a greater impact on the epispheric metabolome than on the metabolomes of the plant organs, although the latter also changed significantly both in leaves and flowers. The plant organs had higher concentrations of metabolites (amino acids, some sugars, and secondary metabolites such as terpenes and phenols) than the epispheric habitat, especially in flowers. The application of antibiotics decreased the concentration of lactate in the metabolomes of the organs and epispheric habitats, suggesting a decrease in fermentation when the microbial populations were suppressed. These results strongly suggest that anaerobic and/or facultative anaerobic bacteria were present in high numbers in the phyllosphere and in the apoplasts of *S. nigra*. The *phyllospheric metabolism* showed a degree of complexity similar to that of the plant organs, but the suppression of several microbial taxa of the phyllosphere partially limited it. Antibiotic application affected the profile of the internal plant metabolome, indicating some level of interaction between the phyllospheric community and plant metabolic function and/or the presence of internal microbial communities that could be responsible for part of the plant metabolic profile. We cannot, however, exclude the possibility of antibiotic-induced damage to the plant tissues.

7.2 Introduction

Distinct microbial communities hosted in and on plant organs are especially important in roots (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012) but also in leaves (Peñuelas & Terradas, 2014; Rico *et al.*, 2014). Epispheric organisms, such as bacteria and fungi, colonize the surfaces of aerial plant organs. Microbes can arrive to or depart from surfaces of leaves through the action of rain, wind, or insects (Lindemann & Upper, 1985).

For phyllospheric microorganisms, the potential benefits of living on leaves are obvious and include supplies of nutrients (Giondo *et al.*, 2013; Scheublin *et al.*, 2014) and carbon (Madhaiyan *et al.*, 2005; Scheublin *et al.*, 2014). The bacteria themselves could also influence substrate availability by producing substances that increase substrate leaching from plant organs to the surface (Schreiber *et al.*, 2005). The advantages provided by phyllospheric inhabitants to their host plants, however, are not necessarily as apparent.

Some reports have shown that both internal and external foliar microbiotas exert several effects on plants, including indirect protection against pathogens (Arnold *et al.*, 2003; Vorholt, 2012; Bulgarelli *et al.*, 2013), protecting plants from diseases and promoting plant growth by various mechanisms (Fry, 1989; Giondo *et al.*, 2013), and plant communication by affecting emissions of volatile organic compounds (Bulgarelli et al., 2013; Peñuelas, et al., 2014; Vorholt, 2012). The relationships between microorganisms and their hosts include parasitic, commensal, and mutualistic interactions. The classification of these relationships can be difficult, principally the discrimination between commensals and mutualistic symbionts, which represent a continuum (Thrall *et al.*, 2007). Many members of the human gut bacterial community were previously considered commensals but are now regarded as beneficial symbionts because of their contributions to host metabolism and immunity (Hooper, 2009). Similar questions of host benefit and microorganism-microorganism interactions should be asked about the microbial communities associated with plants (Wilson *et al.*, 1999; Hirano & Upper, 2000).

Foliar surfaces are habitually poor in nutrient availability, but significant amounts of organic carbon have been detected, including carbohydrates, amino acids, organic acids, and sugar alcohols (Tukey, 1970; Fiala *et al.*, 1990; Weibull *et al.*, 1990). The heterogeneous nature of nutrient availability has been clearly observed on foliar surfaces (Leveau & Lindow, 2000; Miller *et al.*, 2001). The correlations of foliar mass per area and nitrogen and phosphorus concentrations with foliar bacterial community structure have been well documented (Yadav *et al.*, 2005; Kembel *et al.*, 2014). In addition to the carbon sources, volatile plant-derived

metabolic substrates, including isoprenes and C1 compounds (Fall & Benson, 1996), have been identified. Methanol, primarily a by-product of cell-wall metabolism by pectin methyl esterases, is a prominent C1 source for phyllospheric microorganisms and is released in diurnal cycles (Fall & Benson, 1996). Methanol can serve as a substrate for a methylotrophic epispheric bacterium (*Methylobacterium extorquens*) that confers a growth advantage to these organisms *in situ* (Abanda-Nkpwatt *et al.*, 2006; Kawaguchi *et al.*, 2011). Bacterial communities on well-fertilized plants may be limited primarily by carbon availability and only secondarily by nitrogen availability (Wilson & Lindow, 1994). Bacteria can use several nitrogen sources, including organic nitrogenous compounds such as amino acids, which could be valuable sources of nitrogen for phyllospheric bacteria. Ammonia may also be used as a nitrogen source in the phyllosphere (Papen *et al.*, 2002), and nitrogen fixation by phyllospheric bacteria has been reported (Fürnkranz *et al.*, 2008; Rico *et al.*, 2014). Phyllospheric bacteria also need to take up other macro- and microelements for growth.

Plants produce a wide range of secondary metabolites with antimicrobial activity (Wink, 2008), and microorganisms can also produce antimicrobial metabolites (Giddens *et al.*, 2003). Competition for space and nutrient resources, the production of antibiotics, and interference with cell-signaling systems in microbial communities are the principal mechanisms by which indigenous bacteria and fungi antagonize each other (Wilson & Lindow, 1994; Lindow & Brandl, 2003; Hibbing *et al.*, 2010). The complete set of metabolites of the epispheric habitat, however, has not yet been analyzed. Ecometabolomics (Peñuelas & Sardans, 2009a,b; Sardans *et al.*, 2011) could provide such information. A metabolome is the entirety of the small molecules in an organism as the final expression of its genotype (Fiehn, 2002) and can be considered as the organism’s chemical phenotype (Peñuelas & Sardans, 2009a,b). Metabolomic techniques could be combined with the application of antibiotics against bacteria and fungi to discern the role in plant function of microbial communities living on and in plant organs. We have analyzed the metabolomes of the epispheric habitats of leaves and flowers and of the organs themselves of the species *Sambucus Nigra* L.

7.3 Materials and methods

We used 20 four-year-old potted *Sambucus nigra* L. plants, grown in a nursery (Tres Turons S.C.P., Castellar del Vallès, Catalonia, Spain) in 15-L pots with a 2:1 peat:sand substrate and maintained with regular irrigation under outdoor Mediterranean ambient conditions to ensure that the substrate was held at field capacity throughout the experimental period. Ten of the plants were fumigated with with 1600 ppm streptomycin, 400 ppm oxytetracycline, and 200 ppm chloramphenicol in 50 mL of H_2O with 1% glycerol to eliminate the floral and foliar epispheric microbiota. The other 10 plants were kept as control plants and were sprayed with 50 mL of H_2O with 1% glycerol but without antibiotics.

7.1.1 Collection and preparation of tissue samples

Samples of leaves and flowers were collected in spring before treatment and after 1, 7, 15, and 30 days of fumigation. The samples were washed in glasses with water for 2 min. The water was immediately frozen at -80 °C, and the flowers and leaves were lyophilized to avoid leaching. The experimental design contained a total of 200 samples: five sample-collection days, two organs per plant (leaves and flowers), two fractions (epispheric and plant), two treatments (fumigated and unfumigated), and five replicates. The sample preparation is described in detail by Rivas-Ubach et al. (2013). Briefly, the flowers and leaves were frozen immediately in liquid nitrogen and then lyophilized and stored in plastic cans at -80 °C. The samples were then ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for 4 min, producing a fine powder that was stored at -80 °C. The metabolomes of the solid contents of the lyophilized solutions from the washed floral and foliar surfaces were extracted by the same methodology as for the organs. See Appendix 1 or the supplementary material of Gargallo-Garriga *et al.* (2014) for details.

7.1.2 Analysis by liquid chromatography-mass spectrometry (LC-MS)

The LC-MS platform (all from ThermoFisher Scientific, San Jose, CA, unless otherwise noted) consisted of an Accela U-HPLC system with quaternary pumps, an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switchland), a Keystone hot pocket column heater, and an Exactive Orbitrap mass spectrometer controlled by Xcalibur 2.1. Reversed-phase LC separation used a Synergy Hydro-RP column (100 × 2 mm, 2.5 µm particle size, Phenomenex, Torrance, CA) with the ion-pairing agent tributylamine in the aqueous mobile phase to enhance retention and separation. The LC used a column with a small particle size (2.5 µm instead of 4 µm) to reduce

peak widths and expedite analysis. The total run time was 25 min, and the flow rate was 200 µL/min. Solvent A was 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B was methanol. The gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B, and the column was then washed and stabilized for 5 min before the next sample was injected. Other LC parameters were: autosampler temperature, 4 °C; injection volume, 10 µL; and column temperature, 25 °C. HESI (heated electrospray ionization) was used for MS detection. All samples were injected twice, once with the ESI operating in negative ionisation mode ($^{-}$H) and once in positive ionisation mode ($^{+}$H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60 000). The resolution and sensitivity of the spectrometer were monitored by injecting a caffeine standard after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analyzed during the sequence. The assignment of the metabolites was based on standards, with the retention time and mass of the assigned metabolites in both positive and negative ionisation modes.

7.1.3 Statistical analyses

The LC-MS data were analyzed by univariate and multivariate statistical analyses. Permutational multivariate analyses of variance (PERMANOVAs) (Anderson et al., 2008) were conducted using the Euclidean distance, with organ (flowers and leaves), treatment (control and antibiotic treated), date (pre-treatment, 1, 7, 15, and 30 days), and fraction (epispheric and plant) as fixed factors and individuals as random factors. Multivariate ordination principal component analyses (PCAs) (based on a matrix of correlations) and partial least squares discriminant analyses (PLS-DAs) were also performed to detect patterns of sample ordination in the metabolomes. The PCAs and PLS-DAs initially analyzed the HPLC-MS data with the various detected and quantified metabolites as variables and different samples as cases. The detailed analysis of the data from the above set of experiments allowed the analysis of the differences between the metabolic profiles and enabled the identification of clusters, groups, outliers, and, in general, the differences between the metabolic profiles of the plant tissues and between those of the tissues and the phyllosphere with and without antibiotic treatment (Sandasi *et al.*, 2011) (Fig. 7.1). A Kolmogorov-Smirnov (KS) test was performed on each variable for normality. All identified and unidentified metabolites were normally distributed. The PERMANOVA, PLS-DAs, ANOVAs, post-hoc tests, and KS tests used R software (R

Development Core Team 2008) and were performed to detect shifts in both the metabolomes and individual metabolites and in the variables controlling them.

7.4 Results

7.1.4 Univariate analyses

Chloramphenicol and streptomycin were present in all organ and epispheric samples of the antibiotic-treated plants from day 1. The concentration of the streptomycin decreased with time and was no longer detected at day 15 in the organs and epispheric extracts. Chloramphenicol, however, was detected throughout the monitored period (30 days), though at day 30 it was detected only in leaves. Oxytetracycline was found only in the epispheric extracts and only until day 15.

7.1.4.1 Organ versus epispheric extracts

The concentrations of 80% of the detected metabolites differed significantly between the leaves and their epispheric extracts (1020 of the 1277) and between the flowers and their epispheric extracts (1014 of the 1271). More metabolites were detected in the plant organs than in the epispheric biofilms. A total of 1626 metabolic variables were detected, 1594 in the plant organs and 1220 in the epispheric extracts (Tab. S7.1 and 7.2). A total of 1140 metabolites were detected in both the organs and the epispheric extracts; 80 were detected in the epispheric extracts but not in the organs, and 406 were detected in the organs but not in the epispheric extracts. A total of 1277 metabolites were detected in leaves and in foliar epispheric extracts; 196 (including aspartic acid, fisetin, nicotine, rhamnetin, and vitexin) were detected in leaves but not in foliar epispheric extracts, and 28 were detected only in foliar epispheric extracts (Tab. S7.1). A total of 1271 metabolites were detected in flowers and floral epispheric extracts; 194 (including aconitic acid and L-ornithine) were detected in flowers but not in their epispheric extracts, and 28 (including adenosine and glycerol 3-phosphate) were detected only in floral epispheric extracts (Tab. S7.2).

7.1.4.2 Effects of antibiotic treatment

All metabolites detected in leaves were found in control and in treated samples. The antibiotic treatment caused a shift in the concentrations of 118 of the 1277 (9.2%) metabolites detected in leaves (Tab. S7.3). The concentrations of 55 metabolites (including secondary metabolites such as caffeic acid) increased after treatment, and the concentrations of the other 63 metabolites decreased.

All except two of the detected metabolites in the foliar epispheric extracts were detected in both control and treated samples. The concentrations of 133 of the 1132 (11.8%) detected metabolites changed after the antibiotic treatment (Table S7.4). The concentrations of 33 metabolites increased after the treatment, including d-tocopherol, glucose, a non-determined disaccharide, a non-determined hexose, raffinose pentahydrate-maltotriose, and glutamine (Tab. S7.4).

The antibiotic treatment affected 97 of the 1271 (7.6%) metabolic variables detected in the flowers (Table S5). All of these metabolites were found in control and treated samples, except for two that were in the control but not the treated samples. The concentrations of 25 compounds (including a non-determined pentose, pyridoxine, loganin, catechin, threonine, phenylalanine, saponarin, and citrate) were higher in the antibiotic-treated than in the control plants (Tab. S7.5).

The antibiotic treatment affected 74 of the 1271 (7.6%) metabolites detected in the floral epispheric extracts (Tab. S7.6). All of these metabolites were found in the control and treated samples, except three metabolites that were in control but not the treated samples. Pyrogallol was present in treated but not control samples. The concentrations of six unidentified metabolites (X254, X92, X1338, X1576, X1329, and X1068) were higher in antibiotic-treated than control plants (Tab. S7.6).

The concentration of only one identified metabolite (caffeic acid) was higher in leaves after the antibiotic treatment, whereas the concentrations of five identified metabolites were lower (Fig. 7.1). The antibiotic treatment caused the decrease of the concentrations of acetyl-CoA and some of the related amino acids such as alanine. The concentrations of all amino acids involved in the ketoglutarate pathway also tended to decrease, as did the concentration of lactate. In contrast, the concentrations of the amino acids glutamic acid and glutamine involved the ketoglutarate pathway tended to increase in the foliar epispheric extracts after antibiotic application. Concentrations of vitamin B5 and some hexoses increased, while concentrations of vitamin B1 and pentoses decreased in the foliar epispheric extracts under antibiotic treatment.

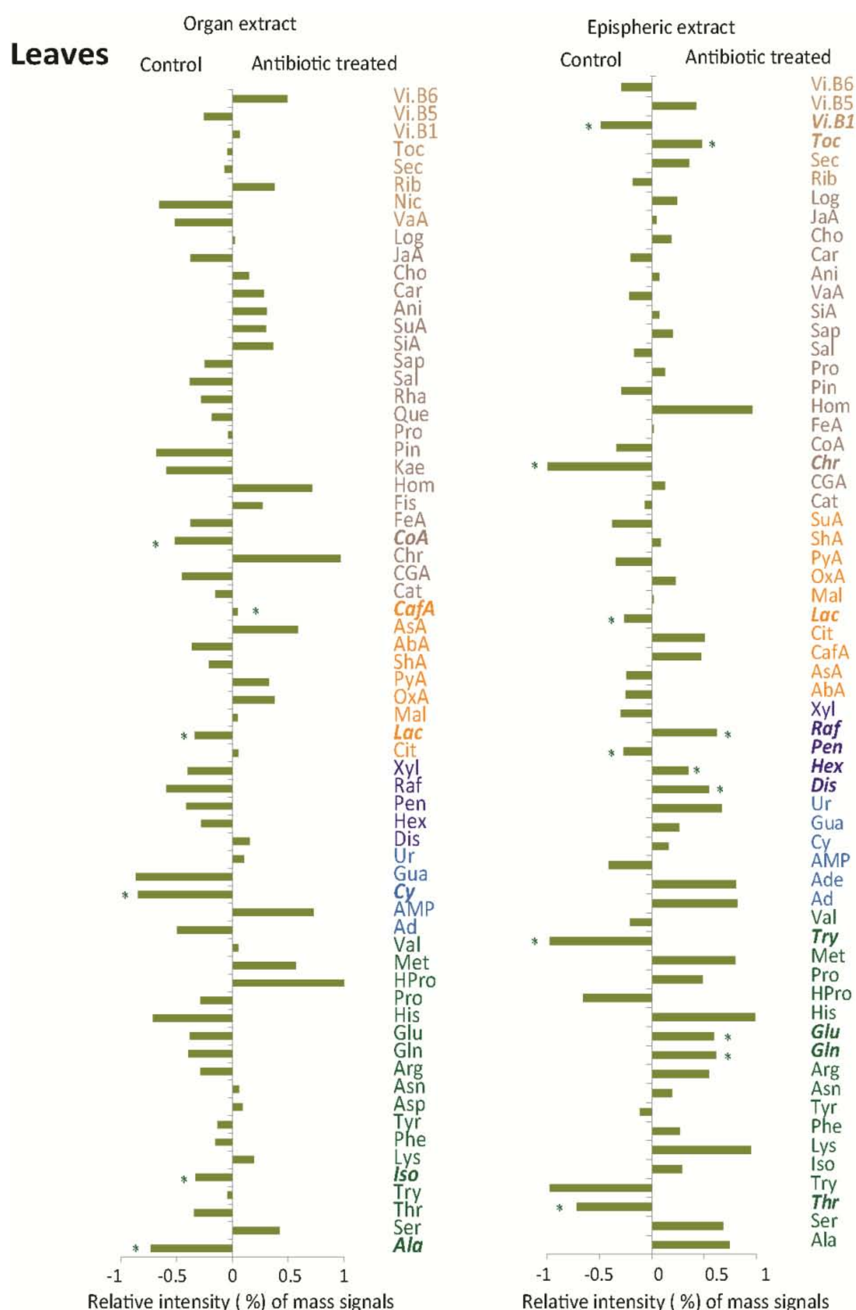

Figure 7.1. Differences between the standardized signal intensities of the identified metabolites in the LC-MS profiles of the antibiotic-treated and control leaves. The various metabolomic families are represented by different colors: green, amino acids; yellow, compounds associated with the metabolism of amino acids and sugars; cyan, nucleotides; brown, terpenes and phenolics; dark blue, sugars; dark brown, others. Metabolites: amino acids: Glu, glutamic acid; Asp, aspartic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Gln, glutamine; His, histidine; HPro, hydroxyproline; Iso, isoleucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Try, tryptophan; Tyr, tyrosine. Nucleobases: Ad, adenine; Ur, uracil. Nucleosides: Ade, adenosine; Cy, cytidine; Gua, guanosine; Ur, uridine. Nucleotide: AMP, adenosine monophosphate. Compounds associated with the metabolism of amino acids and sugars: Cit, citric acid; Lac, lactic acid; Mal, malic acid; OxA, oxaloacetic acid; PyA, pyruvic acid; ShA, shikimic acid; SuA, succinic acid; AbA, abscisic acid (ABA); AsA, ascorbic acid (vitamin C); Cat, catechin. Others: Ani, adonitol (ribitol); Toc, d-tocopherol; JaA, jasmonic acid; Vi.B6, pyridoxine (vitamin B6); Rib, riboflavin (vitamin B2, formerly vitamin G); Vit, vitexin; Car, carvone; Sec, secologanin; Log, loganin; Cho, choline; Nic, nicotine; Vi.B5, pantothenic acid (vitamin B5); Vit.B6p, pyridoxine (vitamin B6); Vi.B1, thiamine (vitamin B1). Terpenes and phenolics: CafA, caffeic acid; CGA, chlorogenic acid; Chr, chrysin; CoA, coumaric acid; Pin, d-pinitol; FeA, ferulic acid; Hom, homoorientin; Kae, kaempferol; Pro, protocatechuic acid; Que, quercetin; Rha, rhamnetin; Sap, saponarin; SiA, sinapinic acid; Sal, sodium salicylate; VaA, vanillic acid; Fis, fisetin; Rhap, rhamnetin. Sugars: Dis, disaccharides; Hex, hexoses; Pen, pentoses; Raf, raffinose pentahydrate - maltotriose; Xyl, xylitol - arabitol. Asterisks and bold italic text indicate statistical significance ($P < 0.05$) in one-way ANOVAs.

The effects of the antibiotic treatment on the identified metabolites were even stronger in flowers and in floral epispheric extracts, with a general trend towards lower concentrations (Fig. 7.2). The concentration of only one identified metabolite, the iridoid loganin, increased after antibiotic treatment, whereas the concentrations of most of the other identified secondary compounds and sugars clearly tended to decrease. Concentrations decreased significantly for phenylalanine but tended to increase for the amino acids associated to the pyruvate pathway (serine, alanine, glycine, and threonine, the latter significantly) in the floral metabolomic profile after antibiotic application. The concentrations of the identified metabolites in the floral extracts did not increase significantly after the antibiotic application, but the concentrations of the identified sugars and amino acids tended to decrease.

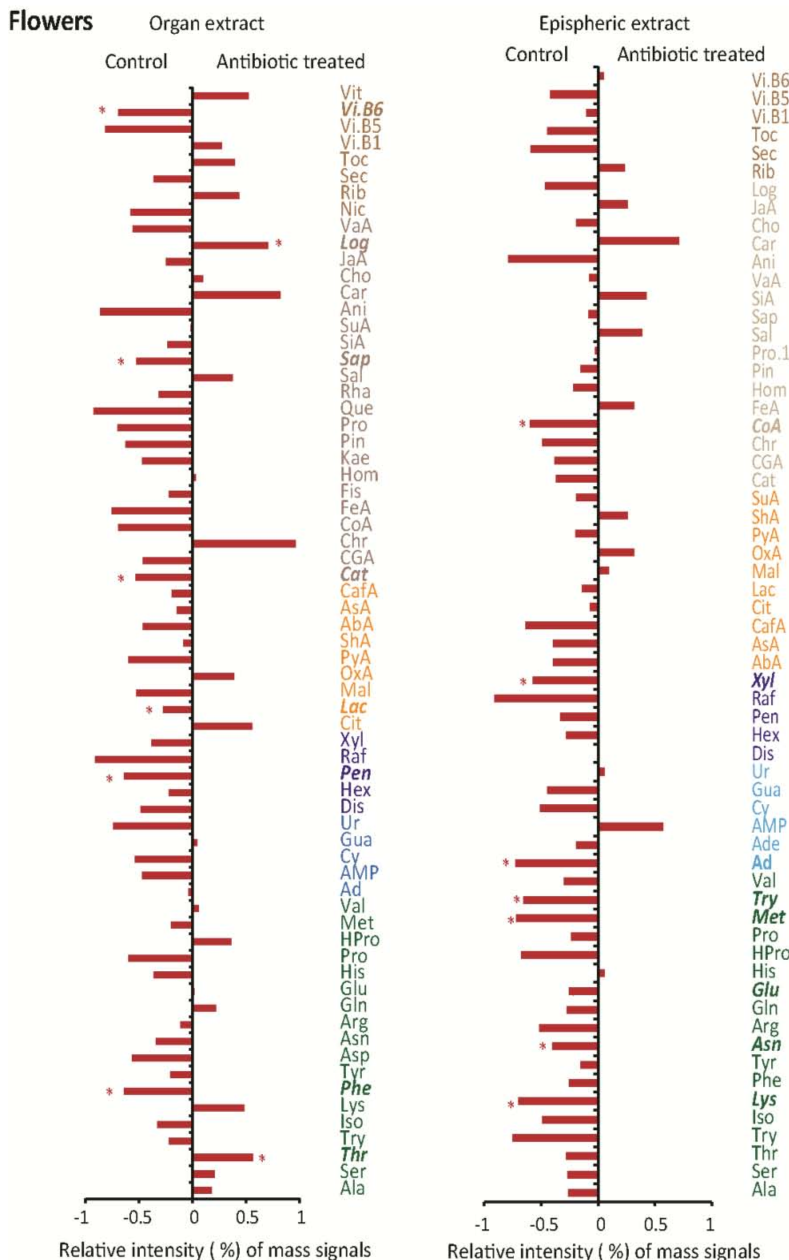

Figure 7.2. Differences between standardized signal intensities of the identified metabolites in the LC-MS profiles of the antibiotic-treated and control flowers. Variables are colored and labelled as described for Fig. 1. Asterisks and bold italic text indicate statistical significance (P < 0.05) in one-way ANOVAs.

7.1.5 Multivariate analyses

The metabolic profiles clearly differed between the plant organs and their microbial epispheric communities (Fig. 7.3). The PCAs of all the metabolomic data separated the epispheric microbial and organ metabolomic profiles along PC1 for both organs. The changes epispheric in metabolomic structure were more significant between the leaves and their epispheric extracts. The metabolomes of the flowers and leaves were separated along PC2 for both the organs and their epispheric microbial communities (Figs. 7.3-7.5). The PERMANOVA analysis confirmed these results, indicating very different metabolomes between the organs and the epispheric extracts (pseudo-F = 361; $P < 0.001$). The overall metabolomes also differed significantly depending on the organ (pseudo-F = 159; $P < 0.001$) date of sampling (pseudo-F = 22.7; $P < 0.001$), individual plant (pseudo-F = 6.61; $P < 0.001$), and epispherictreatment (pseudo-F = 5.00; $P < 0.01$) (Tab. 7.1). Some two-level interactions between factors were also significant: individual plant with plant organ and epispheric environment (pseudo-F = 2.23; $P < 0.05$), date of sampling with plant organ (pseudo-F = 2.47; $P < 0.05$), date of sampling with organ and epispheric environment (pseudo-F = 6.44; $P < 0.01$), and plant organ with organ and epispheric environment (pseudo-F = 108; $P < 0.001$). The interaction between treatment with organ and epispheric environment, however, was only marginally significant (pseudo-F = 1.74; $P < 0.1$).

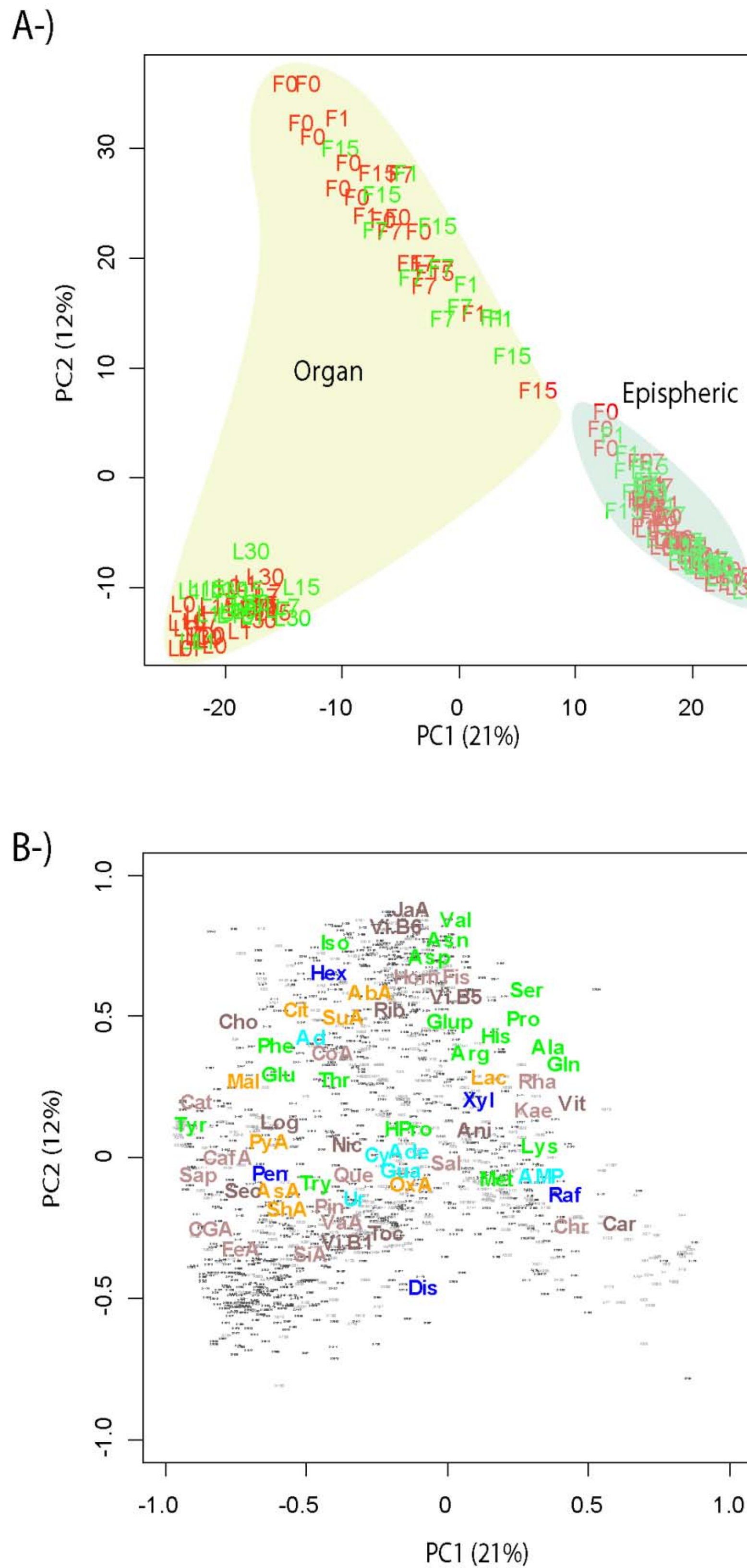

Figure 7.3. Case scores (A) and metabolite loading (B) of the PCA conducted with the variables of the metabolomes. Letters indicate different organs: F, flowers; L, leaves) and colors indicate different treatments (green, control; red, antibiotic treated). Numbers indicate the day the samples were collected (0 without treatment and 1, 7, 15, and 30 days after treatment). Variables are colored and labeled as described for Fig. 7.1.

The metabolic profile of the flowers showed a higher proportion of most amino acids, some sugars such as hexoses and xylitol-arabitol, and some secondary metabolites such as terpenes and phenols (Fig. 7.3). The metabolic profile of the leaves showed higher concentrations of some metabolites associated to the Krebs cycle such as malic acid, pyruvate, chlorogenic acid, quercetin, and oxaloacetate; nitrogenous bases such as adenosine, guanosine, and uridine; and most secondary metabolites.

The metabolic profiles of the plant organs showed higher proportions of most amino acids, some sugars such as hexoses and pentoses, and some secondary metabolites such as terpenes and phenols than epispheric communities. The epispheric communities showed higher proportions of some amino acids such as lysine and methionine, some sugars such as raffinose, some secondary compounds such as chrysin and carvone, and of AMP than plant organs. The epispheric communities showed notably higher concentrations of lactate.

The epispheric metabolomes were less variable than the organ metabolomes (Fig. 7.3). Epispheric metabolomic variability was much less significant and lower between leaves and their epispheric extracts than between flowers and their epispheric extracts. The coefficients of variation of the PC2 scores were 16% for leaves and 58% for flowers.

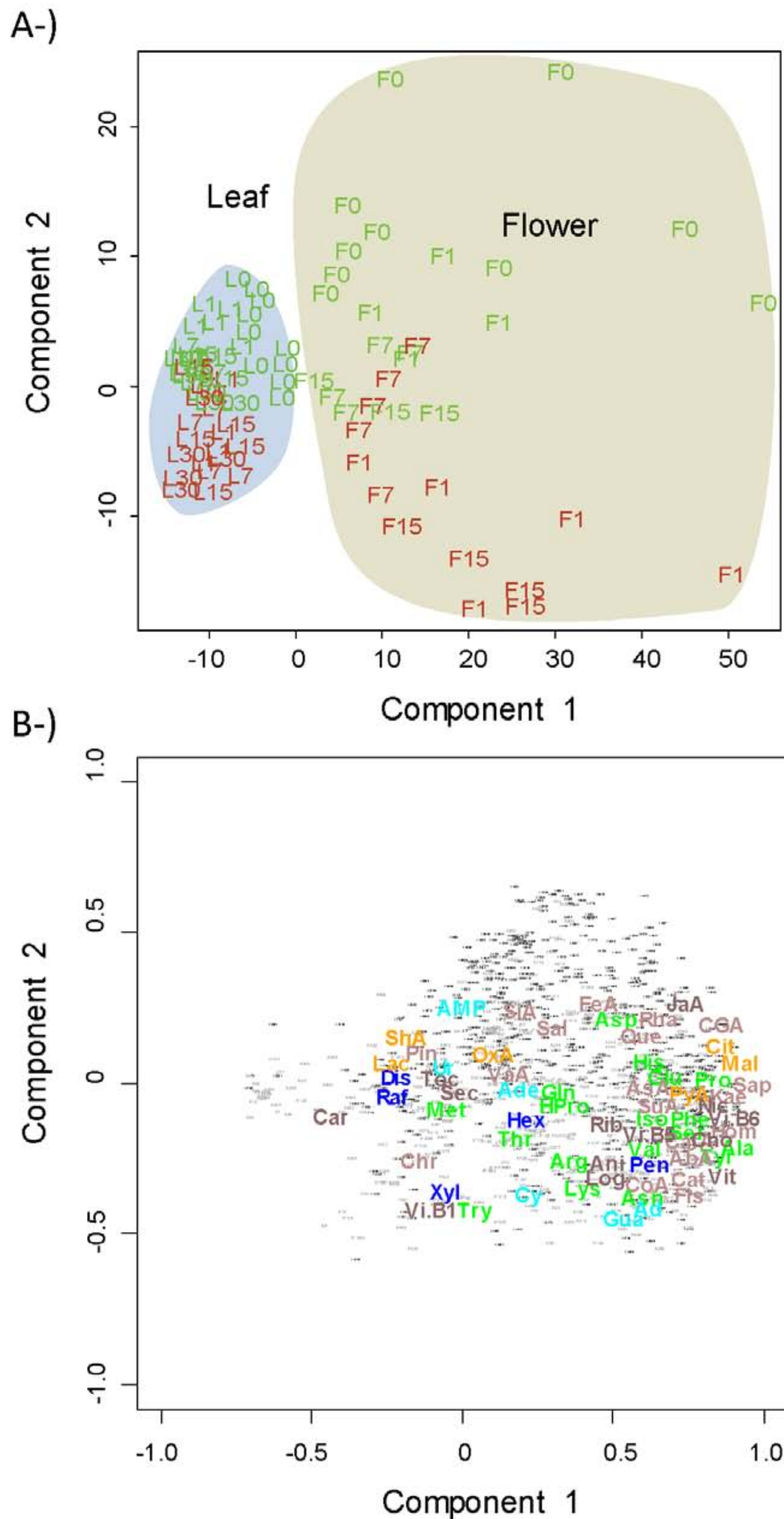

Figure 7.4. Component 1 *vs* component 2 of the partial least squares discriminant analysis (PLS-DA) of the changes of the metabolomes of the epispheric extracts in response to the antibiotic treatment. Case scores are represented in A-) and metabolite loading in B-). Letters indicate different organs (F, flowers; L, leaves), and colors indicate different treatments (green, control; red, antibiotic treated). Numbers indicate the day the samples were collected (0 without treatment and 1, 7, 15, and 30 days after treatment). Variables are colored and labeled as described for Fig. 7.1.

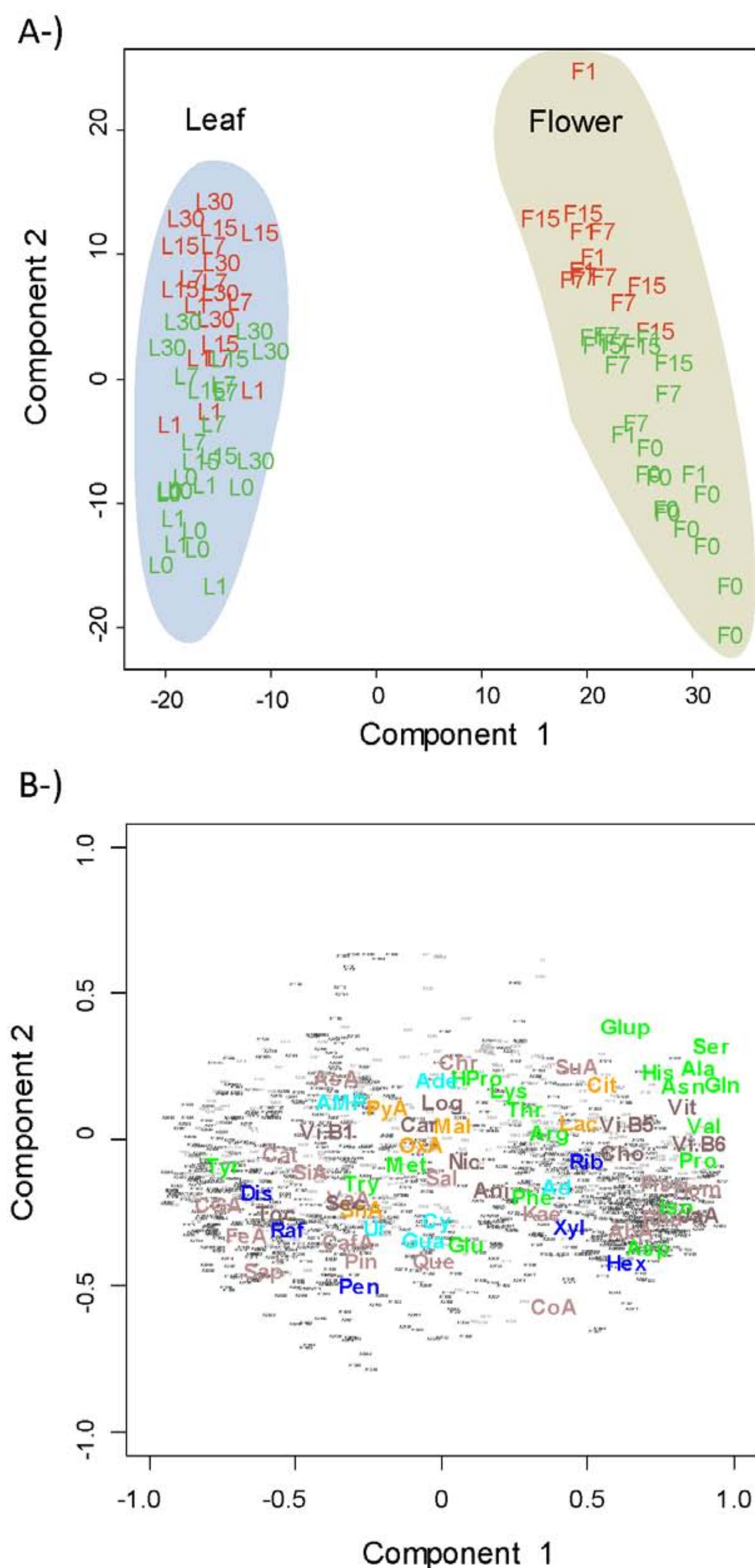

Figure 7.5. Component 1 *vs* component 2 of the partial least squares discriminant analysis (PLS-DA) of the changes of the metabolomes of the plant organs extracts in response to the antibiotic treatment. Case scores are represented in A-) and metabolite loading in B-). Letters indicate different organs (F, flowers; L, leaf), and colors indicate different treatments (green, control; red, antibiotic treated). Numbers indicate the day that samples were collected (0 without treatment, 1, 7, 15, and 30 days after the treatment). Variables are colored and labeled as described for Fig. 7.1.

The effect of the antibiotic treatment was greater in the epispheric environment than in the organs, despite it was also significant in them. The PERMANOVA indicated overall shifts in the metabolomic profiles of leaves and flowers due to the treatment, being flowers more sensitive to the treatment than leaves. The decrease in lactate concentrations due to the antibiotic treatment was general in all samples, of organs and epispheric extracts. Also, the antibiotic treatment caused the decrease of the concentrations of citraconic acid in the foliar and floral epispheric communities and the presence of pyrogallol in the floral epispheric community.

Table 7.1. PERMANOVA results. Bold type indicates significant effects ($P < 0.05$). Italics type indicates marginally significant effects ($P < 0.1$).

	F.Model	*P*-value
P (plant)	6.11	**<0.0001**
D (date)	22.71	**<0.0001**
FL (flowers and leaves)	159.3	**<0.0001**
O (organ and epispheric extract)	361.23	**<0.0001**
TRT (antibiotic treatment)	5	**0.0070**
P:D	2.23	*0.080*
P:FL	1.96	*0.056*
P:O	2.23	**0.028**
P:TRT	1.83	0.13
D:FL	2.47	**0.026**
D:O	6.4	**0.002**
D:TRT	1.35	0.25
FL:O	108.99	**<0.0001**
FL:TRT	1.5	0.15
O:TRT	1.74	*0.098*

7.5 Discussion

7.1.6 Effects of suppression of the epispheric community on metabolic profiles

The effect of the antibiotic treatment on the metabolic profiles was evident in both the epispheric communities and the plant organs. The shifts in the metabolic profiles emphasized the importance of the microbiota in the metabolic function of plants. This study is, to the best of our knowledge, the first work that demonstrates a shift in the global metabolomic expression of a plant due to the suppression of its microbial community. The antibiotics were applied to all plant surfaces with an expected direct impact on the surface of the plant, but this antibiotic application also affected the internal plant organ metabolome, suggesting some levels of impact on endophyte microbial communities. These results were consistent with previous studies, which reported that some target metabolites or specific functions of a plant are related to microbial communities (Lindow & Brandl, 2003; Schreiber *et al.*, 2005; Vorholt, 2012). Moreover, some studies have shown that in internal plant organs it exists a wide microbial endophyte community, which is apparently not related to parasitism or symbiosis functions (Rosenblueth & Martínez-Romero, 2006; Baldan *et al.*, 2014).

The concentration of citraconic acid in the epispheric extracts decreased after the antibiotic treatment. Citraconic acid is produced by microorganisms (Strobel, 2001), particularly by the degradation of isoprenoid compounds (Seubert, 1959). This result was also consistent with the change in the molecular composition of terpene emissions observed after antibiotic treatment (Peñuelas et al., 2014). The decrease in terpene emissions reported by Peñuelas et al (2014) was likely due to the impact of the antibiotics on the floral epispheric microbiota, reducing *de novo* biosynthesis (Schulz & Dickschat, 2007; Davis *et al.*, 2013; Lemfack *et al.*, 2014) or biotransformation (de Carvalho & da Fonseca, 2006; Mirata *et al.*, 2008; Ponzoni *et al.*, 2008; Peñuelas & Terradas, 2014; Peñuelas *et al.*, 2014a). Terpene biosynthesis is common in microbial metabolism, but only a few bacterial and fungal *TPS* genes have yet been reported (Sandhu & Waraich, 1985; Ponzoni *et al.*, 2008), likely due to the low identities of the amino acid sequences of these enzymes compared with those in eukaryotes (Peñuelas et al., 2014). For example, the presence of some epispheric microbes can induce an immune response by the plant and the subsequent emission of defensive terpenes from flowers to control their microbial communities (Arnold *et al.*, 2003).

Another interesting result was the decrease in the proportion of lactate in both organs and epispheric extracts after the antibiotic treatment, suggesting an inhibition of fermentation. In

fermentation, electrons are transferred from reduced substrates to oxidized intermediates to generate reduced fermentation products such as lactate (Drake *et al.*, 1997; Zumft, 1997). Our results, thus, strongly suggest that the anaerobic and/or facultative anaerobic bacteria are present in significant proportions in the phyllosphere of *S. nigra*. A quantitative and qualitative study of the phyllospheric microflora of *Aloe vera* showed that bacteria and yeast densities were relatively high, and bacteria were represented mainly by facultative anaerobic genera, dominated by Enterobacteriaceae (Amir *et al.*, 2007).

The metabolite pyrogallol appeared in the floral epispheric community after the antibiotic treatment. Pyrogallol is a phenolic compound synthesized in plants by the shikimate pathway and is believed to function as a defensive agent against invading microbes and as a signal molecule in plant interactions with pathogens (Kocaçalişkan *et al.*, 2006). The presence of microorganisms may thus have either inhibited the synthesis of pyrogallol or biotransformed it, or, alternatively, the plant may have produced more when the potentially defensive role of the microbes was suppressed.

The antibiotic treatment caused the decrease of the concentrations of acetyl-CoA and its derived amino acid isoleucine in the leaves. Isoleucine is a precursor in the synthesis of several secondary compounds, many of which decreased in concentration after antibiotic application. The concentrations of the amino acid precursors of the ketoglutarate-synthesis pathway tended to decrease in the leaves and to increase in the foliar epispheric extracts in response to antibiotic application. The concentrations of most of the detected metabolites generally decreased in flowers after antibiotic application, though the concentration of the others increase, such is the case of the amino acids associated to the pyruvate-synthesis pathway. These results indicated that the impacts of the antibiotic treatment on the metabolic profile of the internal plant were also significant, even though the impacts were stronger on the metabolic profile of the episphere. As previously mentioned, we observed that the concentration of lactate increased in the internal plant organs after being treated by the antibiotic. The environment of apoplasts (the intercellular space surrounding plant cells) would be competitive for oxygen. Fermentation is favored in these anaerobic situations, and high concentrations of lactate would be produced by the microorganisms. Our results further suggest that 1) the antibiotics penetrated the interiors of the leaves and 2) the antibiotic treatment may have had a direct effect on plant metabolism (Wilson *et al.*, 1999; Hirano & Upper, 2000). Unfortunately, this study did not allow to determine the cause of these effects; for instance, if they are the results of the internal microbial communities that respond to the antibiotic present in the plant cells. We can not exclude a potential antibiotic-induced damage

to the plant tissues. The results of this study showed a certain role of the microbial communities in the metabolomes of the plant organs.

7.1.7 Metabolic profile differences between leaves and flowers

The two plant organs studied had different metabolic profiles, with flowers having the more variable profile. The differences between these two organs explained the 58% of the total variance of the metabolomes. These results were consistent with those of previous studies showing different metabolic profiles among organs of the same plant, for example between shoot and root biomass in grasses (Gargallo-Garriga et al., 2014, 2015). Functional specialization among plant organs is thus high and should be taken into account in ecometabolomic studies, because the metabolic functional response and the relationships between environmental variables and shifts in plant metabolomes can be very different, and even opposite, depending on the organ (Gargallo-Garriga et al., 2014, 2015).

S. nigra is pollinated by insects (mainly hoverflies), so the higher concentrations of some sugars and amino acids in the flowers than the leaves may be related to nectar synthesis and to spore and gamete formation. Floral secondary metabolites such as terpenes and phenols are produced to attract insects (Strack & Fester, 2006; Jobic *et al.*, 2007; Abdel-Farid *et al.*, 2009). Leaves are the main photosynthetic tissue in trees and thus the site of primary production of the main biomolecules. This is consistent with the higher concentrations in leaves than in flowersof some metabolites, associated with the Krebs cycle (malic acid, pyruvate, chlorogenic acid, quercetin, and oxaloacetate, and the nitrogenous bases adenosine, guanosine, and uridine) in leaves than in flowers. Foliar secondary metabolites such as terpenes and phenols are produced in defensive reactions (Lipka *et al.*, 2005; Allwood *et al.*, 2006).

7.1.8 Organ versus epispheric metabolism

The high percentage of the detected metabolomic variables that differed significantly between the organs and epispheric extracts, and the low percentage of compounds in the organs but not in the epispheric extracts, or in the epispheric extracts but not in the organs, indicated similar metabolomes, (with many common metabolites but with different relative proportions of these common metabolites), as indicated by the PCA.

The complex metabolic profiles were more similar between the two epispheric communities than between the corresponding metabolic profiles of the organs. The metabolomes of the two epispheric microbial communities, however, differed significantly in some aspects. The metabolism of the epispheric extracts may thus be much more conservative and homeostatic than that of the organs, and the metabolism of the flowers may be more variable than that of the leaves.

Plant organs have complex functions, and the leaves and flowers in our study had higher proportions of most amino acids, some sugars such as hexoses and pentoses, and some secondary metabolites such as terpenes and phenols than the corresponding epispheric communities. The large variety of these compounds are thus provided by the plants and not by the microorganisms (Arriaga-giner, 1986; Weibull *et al.*, 1990; Bachmann & Keller, 1995). The epispheric community, however, had a higher proportion of some amino acids such as lysine and methionine, some sugars such as raffinose. In especial, the increase of the concentration of some secondary compounds such as chrysin and carvone indicates that the microorganisms play an important role in the plant and can produce some metabolites for the immune response of the plant (Arnold *et al.*, 2003).

7.6 Supporting Information

Supporting information of this study is gathered in the enclosed CD (named under Appendix 4)

Table S7.1 One-way ANOVAs of identified metabolites in leaf organ and epispheric.

Table S7.2 One-way ANOVAs of identified metabolites in flowers organ and epispheric

Table S7.3 One-way ANOVAs of identified metabolites in in leaf organ in different antibiotic treatment within the plants receiving control levels of water in leaf**.**

Table S7.4 One-way ANOVAs of identified metabolites in identified in leaf epispheric in different antibiotic treatment within the plants receiving control levels of water in leaf.

Table S7.5 One-way ANOVAs of identified metabolites in in flowers organ in different antibiotic treatment within the plants receiving control levels of water in leaf.

Table S7.6 One-way ANOVAs of identified metabolites in in flowers epispheric in different antibiotic treatment within the plants receiving control levels of water in leaf**.**

Table S4.7 Abbreviation, family and the real name of the metabolites detected.

III. CONCLUSIONS

We have presented a thesis centered in the application of metabolomics to the field of ecology. The procedures followed have been specially developed to reduce the experimental errors and to be applied to a large number of samples, as it is often required in ecology. It has been demonstrated that it is sensitive enough to detect changes in the metabolic profile of different plants and plant organs when subjected to different biotic and abiotic stimuli. The methodology has been designed for studying the metabolome and stoichiometry of wild plants, being possible to be used for targeted and untargeted studies. Though compromised to some extent by its sensitivity, ^{1}H NMR spectroscopy is an effective technique for fingerprinting and metabolic profiling applied to samples of plant origin. This method has the advantage of combining also high-throughput analyses of LC-MS. The parallel LC-MS and NMR analysis on the same sample allows to have a wider view of the metabolome. The combination of these analyses with stoichiometry makes a step forward in the understanding of the processes driving the ecosystem structure and function (Chapter 3).

The four metabolomic studies performed yield the following conclusions.

The simultaneous ecometabolomic analysis of roots and shoots provides a complete view of the entire plant, including the response of different organs to environmental changes, the global phenotypic response, and the metabolic mechanisms underlying these responses. Such a simultaneous analysis has shown that shoots and roots have different metabolomes and nutrient concentrations, the shoot metabolome is much more variable than the root metabolome, and roots and shoots respond to drought with opposite metabolic changes. When metabolism is activated in roots, metabolism is deactivated in shoots. These opposite metabolic responses may account for the frequent lack of large reductions in productivity in drought experiments, at least for short term (Peñuelas, et al. 2007)(Chapter 4).

The study of the influence of warming on the effects of drought on stoichiometry and metabolomics in shoots and roots in connected to the previous work. In this study we conclude that the stoichiometric and metabolomic responses of plants to warming strongly depend on water availability, and the response differs in shoots and roots. Warming under drought conditions stimulates root primary metabolic activity more than drought alone. Compared to drought alone, shoots under simultaneous warming and drought shifts their osmoprotective and antis-tress strategies by down- and up-regulating the synthesis of various secondary metabolites and by activating some primary metabolic pathways. Our results thus demonstrate different metabolomic expressions in different parts of the plant and a large plasticity in the responses to environmental changes (Chapter 5).

In the study of the responses of *Quercus Ilex* seedling to simulated wounding, we confirm the suitability of the 1H NMR metabolic profiling approach to monitor and characterize the

metabolome shift in response to wounding. The NMR profiles of Quercus Ilex leaves shifts after wounding. A marked increase in the C-rich secondary metabolites, quinic acid and quercitol, which are related to the shikimic acid metabolic pathway (Wilson *et al.*, 1998; Chaves *et al.*, 2011), is observed. Glucose content also increases, while among N-containing metabolites there is a shift from leucine and isoleucine to asparagine and choline, molecules that are implicated in the defence against biotic stress. The comparison of these results with those of other similar studies conducted in other plants, suggests a strong variability in the responses of plant metabolome to wounding depending on taxonomic groups. The metabolites induced by herbivore or pathogen attackes have been found to be different depending on the herbivore or pathogen species and also on the attacked plant species (Widarto *et al.*, 2006; Jansen *et al.*, 2009; Kuzina *et al.*, 2009; Leiss *et al.*, 2009, 2011; Mirnezhad *et al.*, 2010; Steinbrenner *et al.*, 2011). These results also confirm the presence in great content of quinic acid and quercitol in the genus *Quercus* and their role in mechanisms against biotic stress (Chapter 6).

Finally, the suppression of the epispheric microorganisms in *Sambucus Nigra* leaves and flowers turns out into shifts in the concentrations of some metabolites of these organs, being some metabolites no longer detected. These results suggest the significant functional metabolic activity of the microorganisms. Antibiotic treatments kills or slows the reproduction of the microbes, changing the phyllospheric production of metabolites. We observed that the antibiotic penetrates the plant organs, and their effects also suggested the important role of the microbiota in the metabolome of the organs. The differences in the metabolomic compositions between flowers and leaves are greater than their difference between their corresponding epispheric environments. Thus, the epiphytic metabolism is more conservative and homeostatic than that of internal plant organ.

As general remarks of the described work, we conclude the following.

The quality of the defined procedure for the performance of NMR- and LC-MS-based ecometabolomics combined with stoichiometrical analysis has been demonstrated and consolidated with the works described in Chapter 4 and 5 and the subsequent publications (Gargallo-Garriga et al., 2014 and 20015).

The studies in chapter 4 and 5 contribute to better understand the relationship between the information obtained from metabolome analysis and from stoichiometric analysis.

The described studies contribute to better understand how different stimuli (environmental changes related to climate change, like drought or warming; the attack of a herbivore or the

own microbiota) affect the metabolism (and stoichiometry) of terrestrial organisms and of their organs.

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V. APPENDIX

8.1 Appendix

All the experiments and the raw data are enclosed in the link:
http://sermn.uab.cat/2015/06/phd-thesis-albert/

8.2 Appendix 1

8.3 Supplementary information of the chapter 4

8.1.1 Material & Methods

8.1.1.1 Study site

Sampling was part of the EVENT II experiment(Walter *et al.*, 2013), where precipitation patterns were experimentally modified in a semi-natural, extensively managed grassland in the Ecological-Botanical Garden of the University of Bayreuth, Germany (49°55'19'' N, 11°34'55'' E, 365 m a.s.l.). The climate is characterised as temperate and moderately continental with a mean annual air temperature of 8.2 °C and 724 mm of mean annual precipitation (1971-2000, data from the German Weather Service). The soil in the experiment is classified as a Gleysol. The homogeneous, loamy Ap horizon (42% sand, 43% silt, and 15% clay) has a depth of 30 cm, followed by a clayey Bg horizon. The water table drops to -1.5 to -2 m in summer and can reach -30 cm in winter and after longer periods of rain. The main rooting zone is within the upper 15 cm, and few roots penetrate the Bg horizon. The mean pH of the topsoil is 4.1 (1 M KCl). The volumetric soil moisture content for the permanent wilting point is near 15% and that for field capacity is near 40%. The experimental site is a semi-natural grassland that has neither been ploughed nor fertilised for at least 20 years prior to the installation of the EVENT II experiment in 2008. The meadow was mown twice a year for hay production prior to the start of the experiment. The semi-natural grassland community is dominated by tall grasses such as *Alopecurus pratensis* L. (meadow foxtail) and *Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl (tall oat-grass) and harbours on average 16 species per m^2. All species are C3 plants.

8.1.1.2 Experimental design

The field experiment had a two-factorial design manipulating (1) variability of intra-annual precipitation (low, medium, and high, the latter including an extreme drought) and (2) warming (ambient, winter warming, and summer warming). The design consisted of 45 plots, each 1.5 × 1.5 m in size, with five replications of all factorial combinations. The warmed and unwarmed plots were blocked and randomly assigned within each manipulation of the precipitation.

The annual precipitation was kept constant since 2009, while the temporal pattern of precipitation in time was altered during the growing season (April-September). Three precipitation treatments, irrigated control, ambient control, and drought were established. The plots in the **irrigated control** treatment received at least the long-term (1971-2000) average weekly precipitation per week. The plots were exposed to ambient rainfall, but if the weekly rainfall was less than the long-term average, the deficit was added by irrigation. If the weekly ambient rainfall exceeded the long-term average, the excess was not subtracted for the next irrigation. The amount of precipitation in this treatment served as the reference amount for all other treatments. The plots in the **ambient control** treatment received ambient levels of precipitation plus four irrigations (before and after drought, six weeks after drought, and in late September near the end of the growing season) to compensate for the differences with the irrigated control treatment at those four times. The drought treatment also received these additions of water, so all three treatments received the same annual amount of precipitation. Rain was excluded from the plots of the **drought** treatment to simulate the local 1000-year recurrence of drought calculated by Gumbel statistics based on the 1961-2000 time series recorded at a local weather station. Drought was defined by the number of consecutive days with <1 mm daily precipitation and was simulated by the exclusion of natural rainfall for 42 days using rain-out shelters. The shelters had steel frames (Hochtunnel, E & R Stolte GmbH, Germany) and were covered with transparent plastic sheets (0.2 mm polyethylene, SPR 5, Hermann Meyer KG, Germany) during the period of simulated drought that permitted nearly 90% penetration of photosynthetically active radiation, based on tests prior to set-up. An 80-cm gap was left between the ground and the plastic sheets to allow air exchange near the surface, which reduced microclimatic artefacts such as increased temperatures or reduced wind speed. The amount of excluded rain was applied together with the adjustment to the irrigated control treatment at the end of the period of artificial drought as one heavy episode of rain within two days.

The warming manipulations were performed either during the winter (October-March) or the summer (April-September) starting in October 2009. Temperatures were increased using infra-red overhead heating lamps equipped with reflector domes (IOT/90 250W Elstein, Northeim, Germany) at a height of 0.8 m, theoretically providing 60 W/plot. The lamps were raised to 1 m when tall grasses reached 80 cm. Unwarmed plots were equipped with dummy heaters. The air temperature at 5 cm above the ground was raised on average by 0.9 °C in winter and by 1.3 °C in summer. Note that the focus of the study was the effect of drought, not of warming.

8.1.1.3 Target species

Two C3 grasses were selected as target species for this study: *A. pratensis* L. and *Holcus lanatus* L. Both species were selected based on their high frequency in the experimental plots and their importance in semi-natural grasslands across central Europe. *A. pratensis* is the dominant species at the experimental site, producing about 18% of the annual aboveground biomass. It is a tall (up to 110 cm) and productive species of agricultural importance in moist and nutrient-rich meadows. *H. lanatus* is also frequent at the site, but less productive (3% share of the annual aboveground biomass). It occurs in semi-natural grasslands throughout Europe, Asia, and North Africa and is invasive in North America and Australia. It tolerates a wide range of conditions but prefers moist meadows.

8.1.1.4 Collection and preparation of the tissue samples

Samples were collected at the end of the drought manipulation before irrigation in July and again at the end of the growing season in September. Above- and belowground tissues were sampled from each of the 45 plots (3 precipitation treatments × 3 warming treatments × 5 replicates). We collected a total of 347 samples. In July we collected 44 samples of leaf blades and 43 samples of fine roots of *H. lanatus* and 45 samples of leaf blades and 44 samples of fine roots of *A. pratensis*. In September we collected 42 samples of leaf blades and 41 samples of fine roots *of H. lanatus* and 45 samples of leaf blades and 44 samples of fine roots of *A. pratensis*. The samples were roughly cleaned and immediately frozen in liquid nitrogen. The procedure for sample preparation is described in detail by Rivas-Ubach et al. (2013)(Rivas-Ubach *et al.*, 2013). Briefly, the frozen samples were lyophilised and stored in plastic cans at -80 ºC. Soil contamination was removed from the root samples. Finally, the samples were ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for 4 min, producing a fine powder that was stored at -80 ºC until the extraction of the metabolites.

8.1.1.5 Elemental analysis

For the analysis of C and N, 1.5 mg of each powdered sample was analysed, and their concentrations were determined by combustion coupled to gas chromatography using a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).
P, K, Fe, Mn, Mg, Ca, and S were analysed by extraction by acid digestion in a microwave reaction system under high pressure and temperature. Briefly, 250 mg of leaf powder were placed in a Teflon tube with 5 mL of nitric acid and 2 mL of H_2O_2. A **MARSX press** microwave reaction system (CEM, Mattheus, USA) was used for these acid digestions. The digested material was transferred to 50-ml flasks and resuspended in Milli-Q water to a final volume of 50 mL. The element concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (Perkin-Elmer Corporation, Norwalk, USA).

8.1.1.6 Extraction of metabolites

Two sets of 50-mL centrifuge tubes were labelled as set A for liquid chromatography-mass spectrometry (LC-MS) analysis and set B for nuclear magnetic resonance (NMR) analysis. Each tube of set A received 150 mg of a powdered sample then 6 mL of water/methanol (1/1), and the samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All tubes were centrifuged at 1100 × g for 15 min. Next, 4 mL of each tube of set A were transferred to its corresponding tube of set B. This procedure was repeated twice for two extractions of the same sample. The resulting extracts were used for metabolomic analysis.

8.1.1.7 Preparation of extracts for NMR analysis

Eight millilitres of the extracts were resuspended in water to reduce the proportion of methanol (<15% methanol). The solutions were lyophilised, and 4 mL of water were added to each tube, which was vortexed and centrifuged at 23 000 × g for 3 min. The samples were frozen at -80 ºC and lyophilised again. Finally, 1 mL of KD_2PO_4-buffered D_2O solution containing 0.01% TSP (trimethylsilyl propionic acid sodium salt) (pH 6.0) was added to each dried fraction. TSP was used as the internal standard for the NMR experiments. The solutions were transferred to 2-mL centrifuge tubes with a micropipette and centrifuged at 23 000 × *g* for 3 min, and 0.6 mL of the supernatants were transferred to NMR sample tubes.
The procedure for the extraction of metabolites is described in detail by Rivas-Ubach et al.(Rivas-Ubach *et al.*, 2013).

8.1.1.8 Preparation of extracts for LC-MS analysis

Two millilitres of the supernatants of each tube of set A were collected using crystal syringes, filtered through 0.22 µm microfilters, and transferred to a labelled set of LC vials. The vials were stored at -80 ºC until the LC-MS analysis.

8.1.1.9 LC-MS analysis

LC-MS chromatograms were obtained with a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex, Waltham, Massachusetts, USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with an HESI II (heated electrospray ionisation) source. Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3-µ particle size; Thermo Scientific, Waltham, Massachusetts, USA) at 30 ºC. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B). Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use. The elution gradient, at a flow rate of 0.3 mL per minute, began at 10% A (90% B) and was maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10% A and 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilised for 5 min before the next sample was injected. The injection volume of the samples was 5 µL. HESI was used for MS detection. All samples were injected twice, once with the ESI operating in negative ionisation mode (-H) and once in positive ionisation mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60 000). The resolution and sensitivity of the spectrometer were monitored by injecting a standard of caffeine after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence. The assignment of the metabolites was based on the standards, with the retention time and mass of the assigned metabolites in both positive and negative ionisation modes.

8.1.1.10 NMR analysis

See the main text for the description of the NMR analysis. 1H NMR-based fingerprints were obtained for all samples. One-dimensional (1D) ^{1}H NMR spectra were acquired with suppression of the residual water resonance. The water-resonance signal was presaturated using a power level of 55 dB during a relaxation delay of 2 s. Each spectrum acquired 32 k data points over a spectral width of 16 ppm as the sum of 128 transients and with an acquisition

time of 1.7 s. The total acquisition time was ~8 min per sample with a relaxation delay of 2s. All ^{1}H NMR spectra were phased and baselines were corrected and referenced to the resonance of the internal standard (TSP) at δ 0.00 ppm using TOPSPIN 3.1. See Rivas-Ubach et al. (2013) (Rivas-Ubach *et al.*, 2013)for more details of the sampling and NMR determination. The data were subsequently used for the statistical analysis. A variable-size bucketing was applied to all ^{1}H NMR spectra with AMIX software (Bruker Biospin, Rheinstetten, Germany), scaling the buckets relative to the internal standard (TSP). The output was a data set containing the integral values for each assigned ^{1}H NMR spectral peak in the described pattern. The buckets corresponding to the same molecular compound were summed.

The NMR spectrometer described for the fingerprinting was used for the acquisition of the 2D NMR on selected representative samples. The probe temperature was set to 298.0 K, and TopSpin 2.1 (Bruker BioSpin) acquired and processed the experiments. The data for 1D ^{1}H NMR, 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC), and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) were acquired using standard Bruker pulse sequences and routine conditions(Rivas-Ubach *et al.*, 2013).

8.1.1.11 Statistical analyses

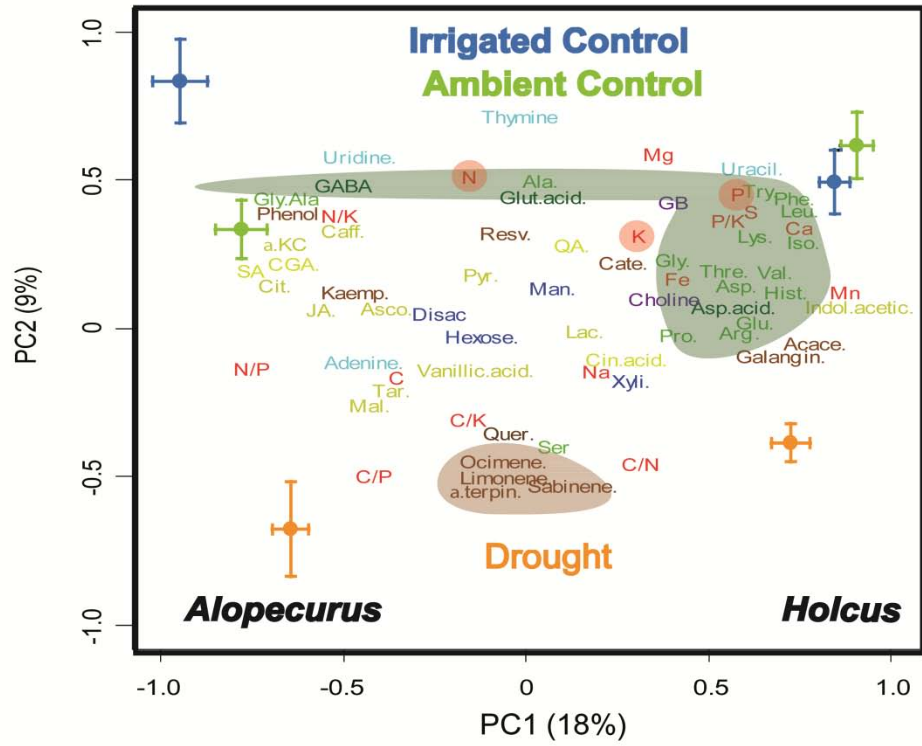
To test for differences in plant elemental stoichiometries and metabolomes between seasons and drought treatments, we conducted a PERMANOVA analysis of the LC-MS and NMR metabolomic fingerprints from *H. lanatus* and *A. pratensis* using Euclidean distances, with season (September and July), climatic treatment (ambient control, irrigated control, and drought), and organ as fixed factors. The number of permutations was set at 999. The PERMANOVA analysis was conducted with R (R Development Core Team 2008).

The plant stoichiometric and metabolomic fingerprints were also subjected to principal component analysis (PCA) to understand how the stoichiometries and metabolomes of *H. lanatus* and *A. pratensis* shifted with the factors studied (organ, season, and climatic treatment). Fingerprints from leaves and roots for July and September were additionally submitted to separate PCAs (Fig. 2). The PCAs were performed by the *pca* function of the *mixOmics* package of R (R Development Core Team 2008). The score coordinates of the variables were subjected to one-way ANOVAs to find statistical differences among groups (see Supporting Information in Rivas-Ubach et al. (2013)). A Kolmogorov-Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally

distributed was removed from the data set. Statistica v8.0 (StatSoft) was used to perform ANOVAs, post-hoc tests, and KS tests.

8.2 Figure caption

Figure S4.1. Plot of cases and variables in the PCAs conducted with the elemental, stoichiometric, and metabolomic variables in plants sampled in September. (A) Plot of cases and variables of shoots. (B) Plot of cases and variables of roots. C/N/P/K ratios are shown in red. The various metabolomic families are represented by colours: blue, sugars; green, amino acids; dark-green, amino-acid derivatives; yellow, related compounds to amino acid and sugar metabolism; cyan, nucleotides; violet, osmolytes; and brown, terpenes and phenols. Metabolites: acacetin (Acace), adenine (Adenine), alanine (Ala), arginine (Arg), ascorbic acid (Asco), asparagine (Asn), aspartic acid (Asp), caffeic acid (Caff), catechin (Cate), chinic acid (Cin.acid), chlorogenic acid (CGA), choline (Choline), citric acid (Cit), disaccharide (Disac), galangin (Galangin), gamma-aminobutyric acid (GABA), glutamic acid (Glu), glutamine (Gln), glycine (Gly), glycine betaine (GB), glycine-alanine (Gly-Ala), hexose (Hexose), histidine (Hys), indol acetic acid (Indol.acetic), isoleucine (Ile), jasmonic acid (JA), kaempferol (Kamp), lactic acid (Lac), leucine (Leu), limonene (Limonene), lysine (Lys), malic acid (Mal) mannose (Man), ocimene (Ocimene), phenolic group (Phenol), phenylalanine (Phe), proline (Pro), pyruvate (Pyr), quercetin (Quer), quinic acid (QA), resveratrol (Resv), sabinene (Sabinene), serine (Ser), shikimic acid (SA), tartaric acid (Tar), threonine (Thr), thymine (Thymine), tryptophan (Try). The means of cases are indicated by: blue, irrigated control; green, ambient control; and orange, drought. *Holcus lanatus* is indicated as *Holcus* and *Alopecurus pratensis* as *Alopecurus*.


A
SHOOTS
Irrigated Control
Ambient Control
Drought
Alopecurus
Holcus
PC1 (18%)
PC2 (9%)
Thymine
Uridine.
Mg
Uracil.
GABA
Ala.
Glut.acid.
GB
Gly.Ala
Phenol
N/K
Caff.
Resv.
QA.
a.KC
CGA.
SA
Cit.
Kaemp.
Pyr.
Man.
Cate.
JA.
Asco.
Disac
Hexose.
Lac.
Adenine.
Vanillic.acid.
N/P
Cin.acid.
Na
Xyli.
Galangin.
Acace.
Tar.
Mal.
C/K
Quer.
Ser
Ocimene.
Limonene.
a.terpin.
Sabinene.
C/N
C/P

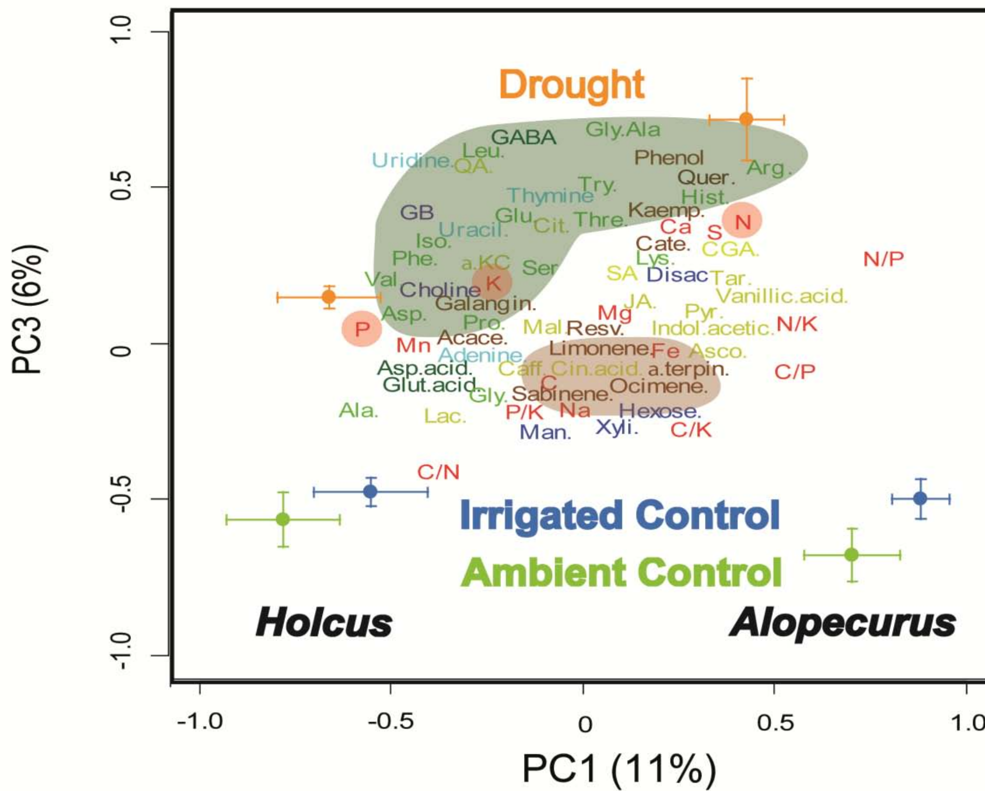

B
ROOTS
Drought
Irrigated Control
Ambient Control
Holcus
Alopecurus
PC1 (11%)
PC3 (6%)
GABA
Gly.Ala
Uridine.
Leu.
QA.
Phenol
Arg.
Quer.
Thymine
Try.
Hist.
GB
Glu.
Cit.
Thre.
Kaemp.
Uracil.
Iso.
Cate.
CGA.
N/P
Phe.
a.KC
Ser
SA
Disac
Tar.
Val
Choline
Galangin.
Vanillic.acid.
Asp.
Pro.
Mal
Resv.
Indol.acetic.
N/K
Mn
Acace.
Adenine
Limonene.
Asco.
Asp.acid.
Glut.acid.
Caff.
Cin.acid.
a.terpin.
C/P
Gly.
Sabinene.
Ocimene.
Ala.
Lac.
P/K
Na
Hexose.
Man.
Xyli.
C/K
C/N

Figure S4.2. Loading of elemental stoichiometric and metabolomic variables in the PC axes separating drought treatments (Fig. S1). Variables are coloured and labelled as described in the caption for Fig. 1.

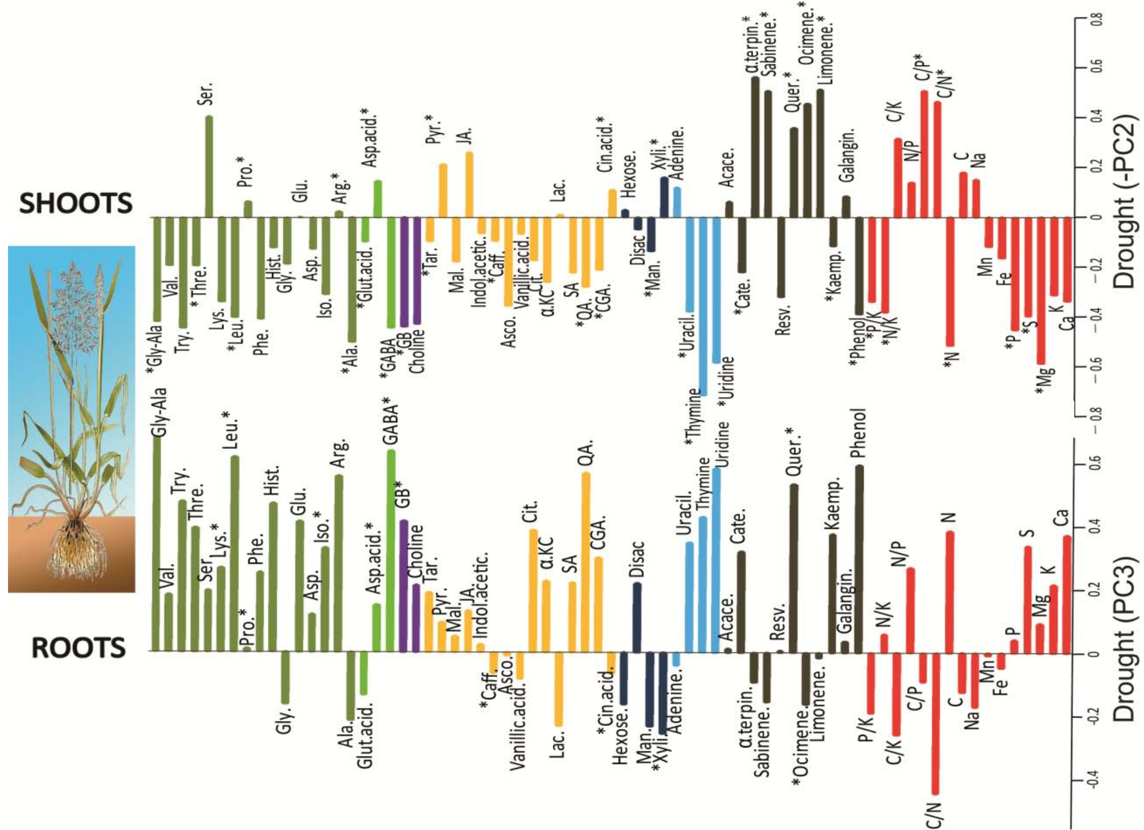


Figure S4.3. Clustered image maps of the metabolites in stems treatments based on the simulated data with the PLS can method. The red and blue colours indicate positive and negative correlations respectively.

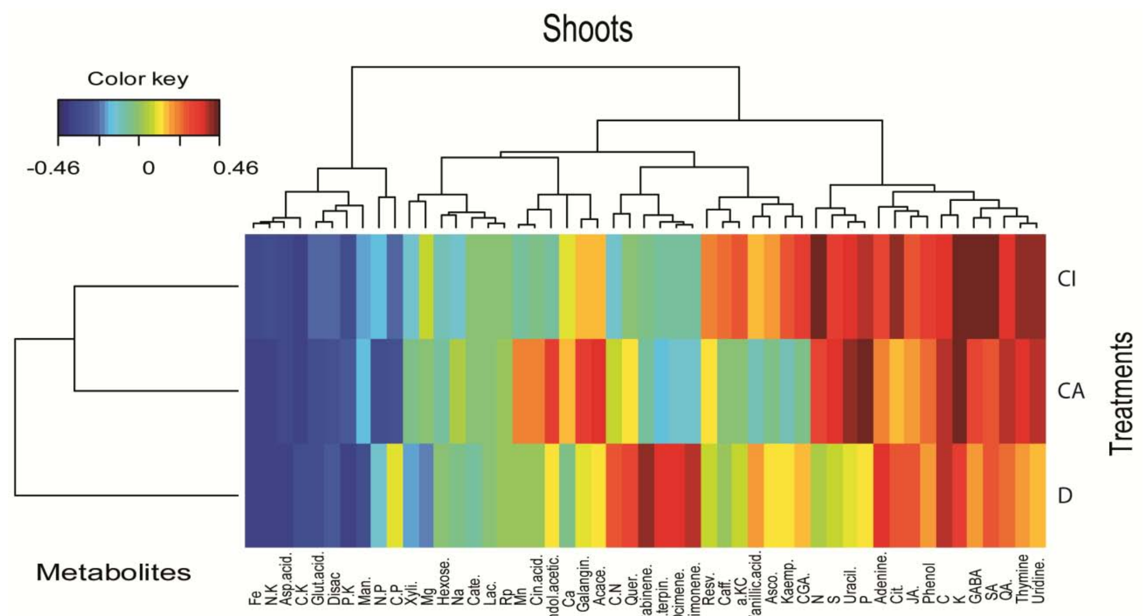
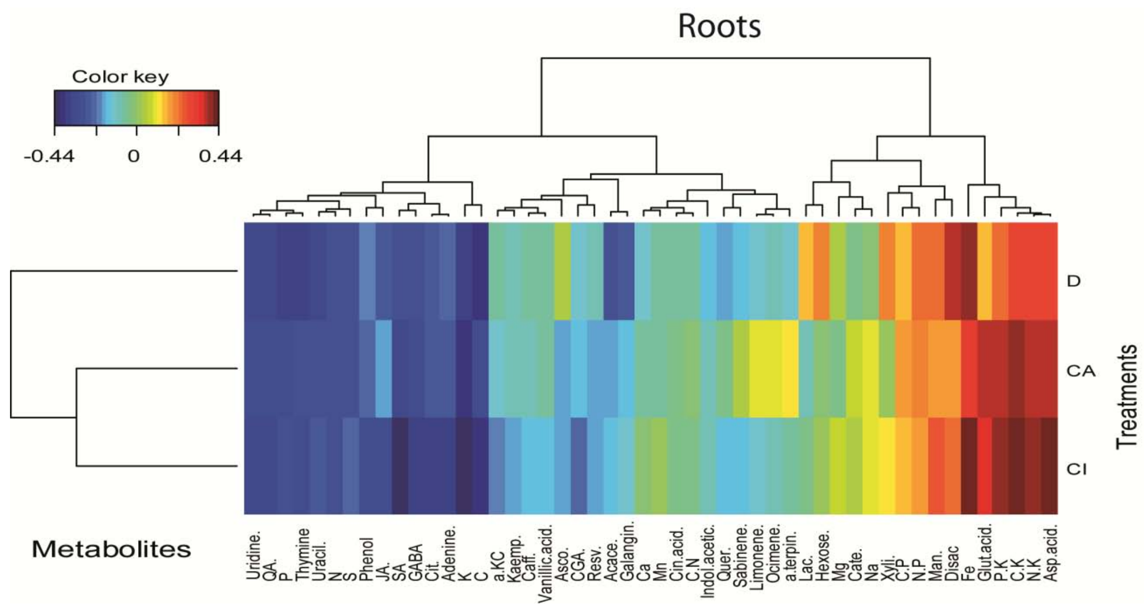


Figure S4.4. Clustered image maps of the metabolites in roots treatments based on the simulated data with the PLS can method. The red and blue colours indicate positive and negative correlations respectively.



9.1 Appendix 2

9.2 Supplementary information of the chapter 5

Methods S5.1 NMR elucidation

The signals in the region between 3.2 and 5.9 ppm correspond mainly to sugars. Among them, α-glucose and β-glucose, with anomeric proton resonances at 5.29 ppm (d, J = 3.8 Hz) and at 4.70 ppm (d, J = 8.2 Hz) respectively, were identified. The disaccharide sucrose was also observed, with its characteristic anomeric proton doublet of the glucose unit at 5.45 ppm (d, J = 3.8 Hz). The proton singlet at 3.19 ppm is correlated via HSQC to the carbon signal at 53.8 ppm, corresponding to choline, proton singlet at 3.88 ppm is correlated via HSQC to the carbon signal at 54.6 ppm corresponding to glycine betaine and the proton triplet at 2.31 ppm is correlated via HSQC to the carbon at 34.3 ppm corresponding to γ-aminobutyric acid, which is an important osmolyte. In the aliphatic region between 2.2 and 1.7 ppm, the secondary metabolite chinic acid was identified, showing very intense signals. Among amino acids, valine, alanine, isoleucine, serine, threonine and lysine were also detected and identified. The proton doublet of glycine-alanine at 1.57 ppm is correlated via HSQC to the carbon at 16.9 ppm. Some organic acids such as indol acetic acid and lactic acid were identified as well. Finally, signals at the 6.5 - 7.4 ppm region, correspond mainly to phenol compounds. All the identifications were based on the ^{1}H and ^{13}C NMR complete or partial assignment of the molecules based on 1D and 2D NMR experiments and on the comparison with previously reported data.

Figure S5.1. A. Even II experiment carried out at Bayreuth (Germany). Colors indicate the applied treatment (blue, irrigated control; red, drought; yellow, control ambient), except for the gray color that is out of the limits of our project but it is shown to demonstrate the random distribution of the plots. Letters indicate the drought treatment (CA, control ambient; D, drought; IC, irrigated control). Warming is indicated by letters (W_s, summer warming; W_w, winter warming). B. Scheme of the experimental design.

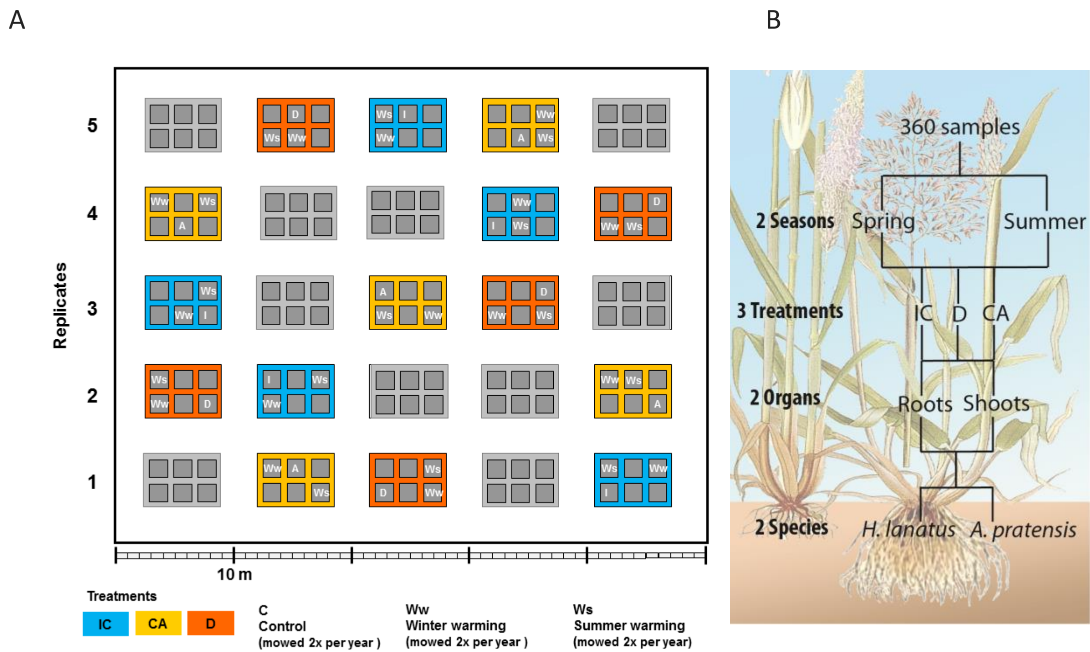
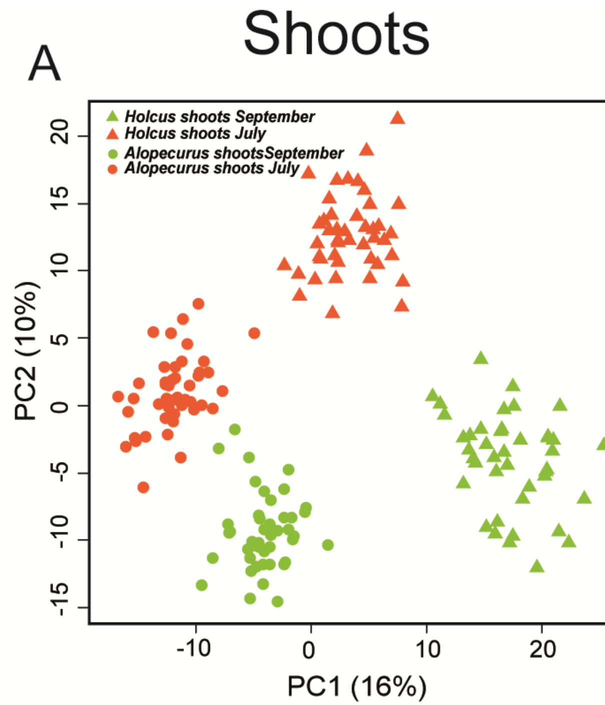


Figure S5.2. Plots of cases and variables in the PCA conducted with the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* using PC1 versus PC2. (A) The cases are categorised by shoot seasons. Seasons are indicated by different colours (green, September; red, July). The two species are represented by geometric symbols (circles, *A. pratensis*; triangles, *H. lanatus*). (B) Loadings of the various elemental stoichiometric and metabolomic variables in PC1 and PC2.Metabolites as in Figure 1.



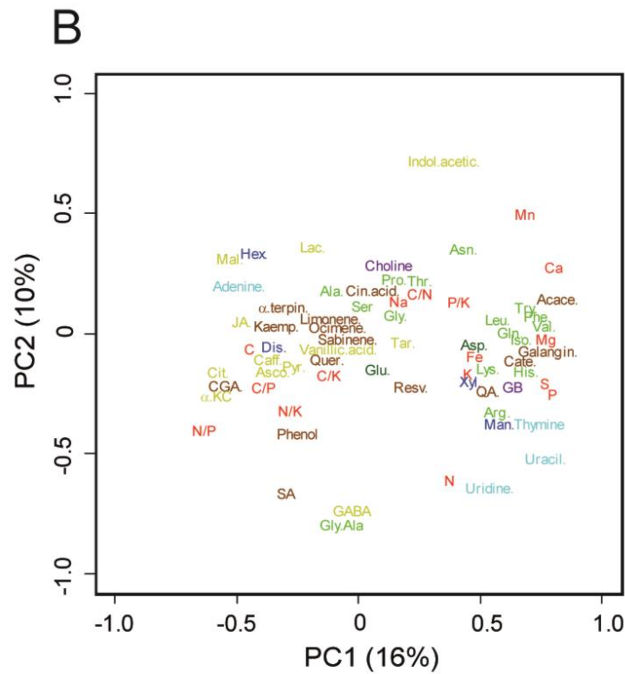
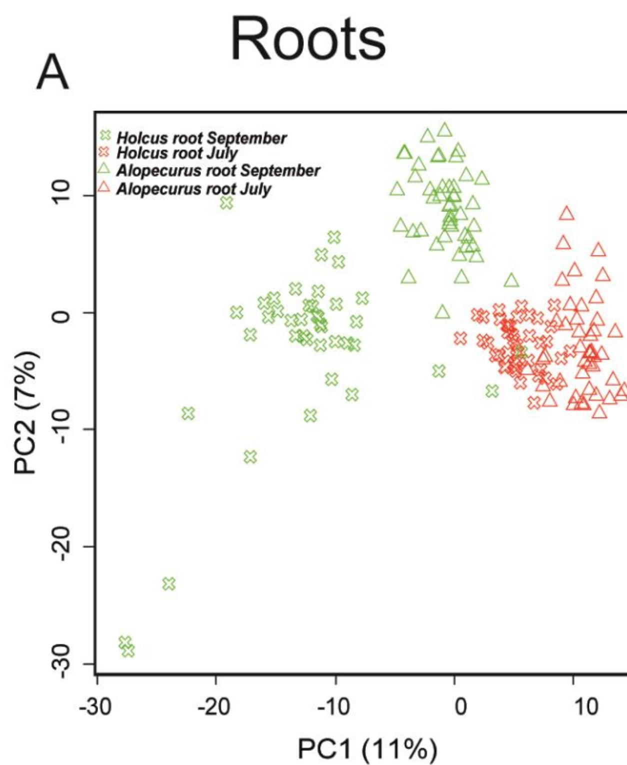


Figure S5.3. Plots of cases and variables in the PCA conducted with the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* using PC1 versus PC2. (A) The cases are categorised by root seasons. Seasons are indicated by different colours (green, September; red, July). The two species are represented by geometric symbols (circles, *A. pratensis*; triangles, *H. lanatus*). (B) Loadings of the various elemental stoichiometric and metabolomic variables in PC1 and PC2. Metabolites as in Figure 1.



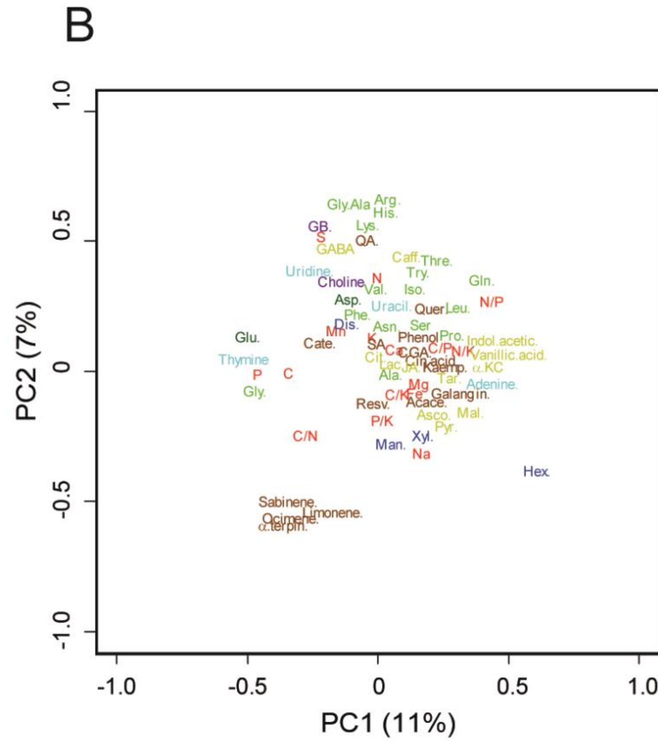
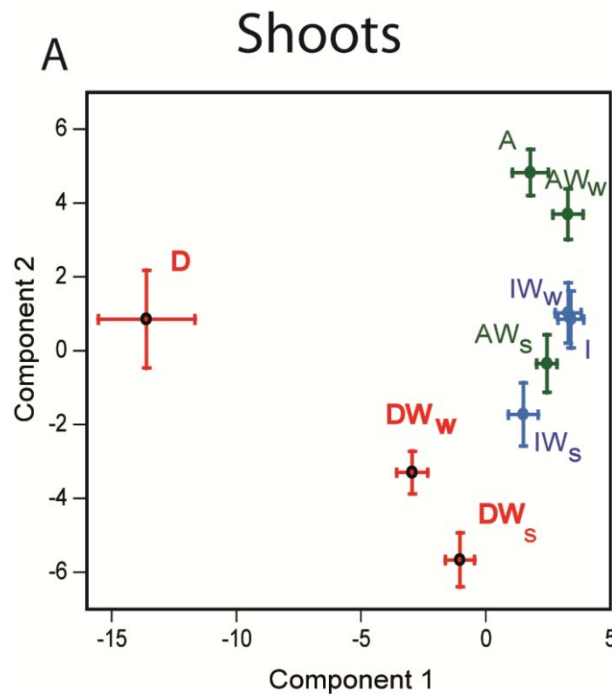


Figure S5.4. Component 1 vs component 2 of the partial least squares discriminant analysis with data of both seasons and shoots of the elemental, stoichiometric, and metabolomic variables in *Holcuslanatus* and *Alopecuruspratensis*. (A) Samples categorised scores (mean ± S.E.) by shoots in the warming plus drought (factorial) treatment. Drought is indicated by different colours (green, ambient control; red, drought; blue, irrigated) and letters (A, control ambient; D, drought; I, irrigated). Warming is indicated by letters (W_s, summer warming; W_w, winter warming). (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Figure 1.



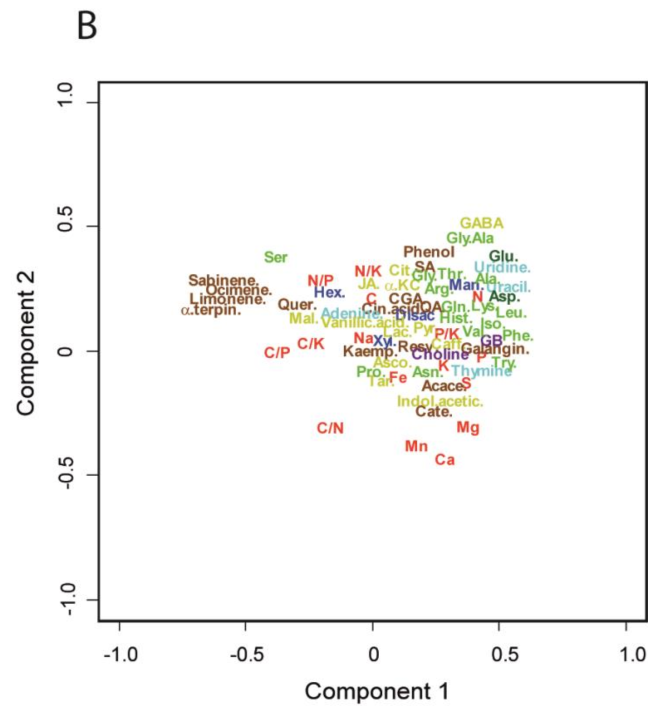


Figure S5.5. Component 1 vs component 2 of the partial least squares discriminant analysis of the elemental, stoichiometric, and metabolomic variables in *Holcuslanatus* and *Alopecuruspratensis* for data of both seasons and roots. (A) Samples categorised scores (mean ± S.E.) by roots in the warming plus drought (factorial) treatment. Drought is indicated by different colours (green, ambient control; red, drought; blue, irrigated) and letters (A, control ambient; D, drought; I, irrigated). Warming is indicated by letters (W_s, summer warming; W_w, winter warming). (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Figure 1.

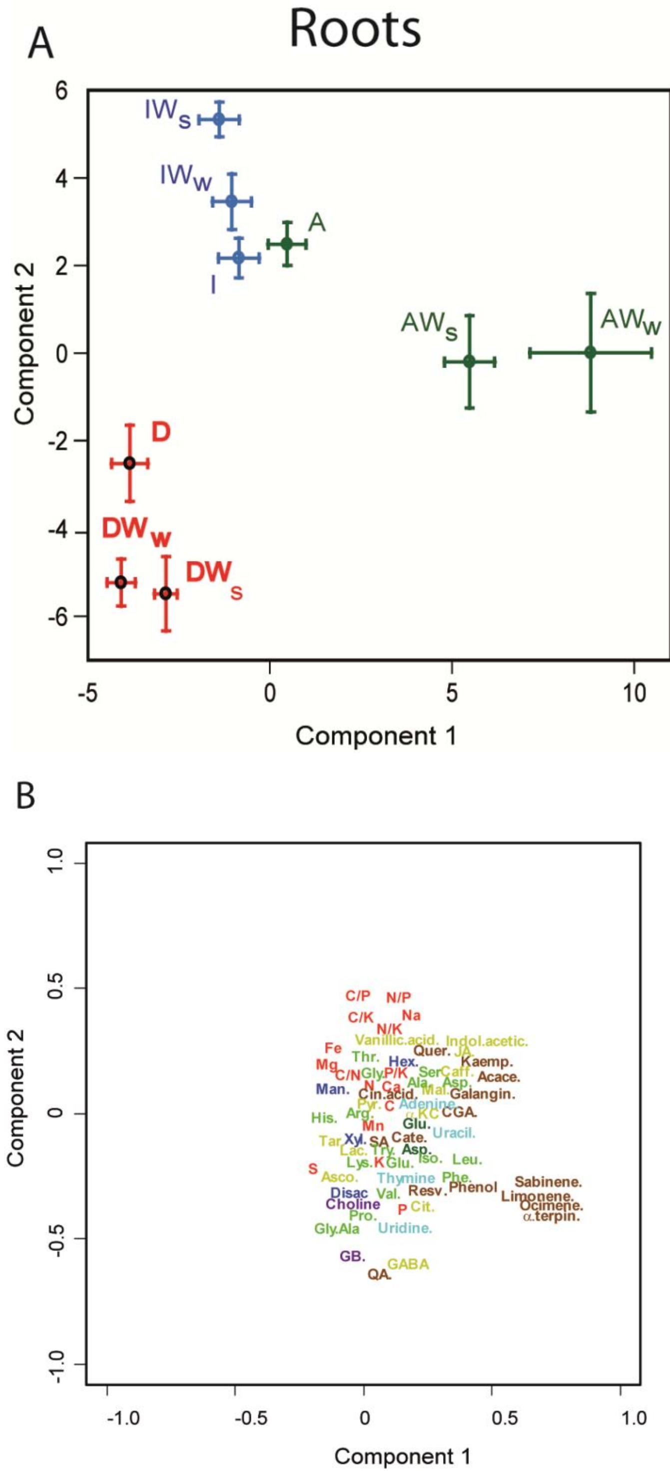
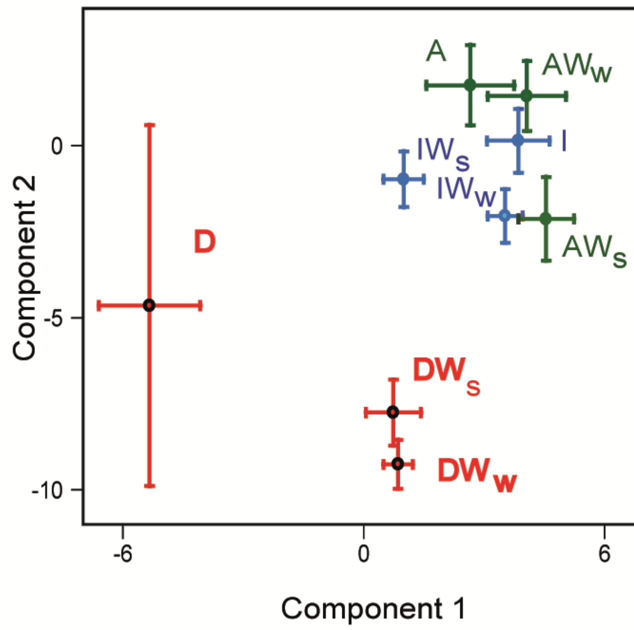


Figure S5.6. Component 1 vs component 2 of the partial least squares discriminant analysis of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised scores (mean ± S.E.) by shoots and roots in the warming plus drought (factorial) treatment. Drought is indicated by different colours (green, ambient control; red, drought; blue, irrigated). Warming is indicated by letters (W_s, summer warming; W_w, winter warming). (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Figure 1.

Warming & Drought

A



B