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DIPLOMARBEIT

Titel der Diplomarbeit

Metabolite screening of *Medicago truncatula* applying salt and drought stress

verfasst von

Dipl.-Ing. Dr. Thomas Kolber

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Betreut von: Ass. Prof. Dr. Stefanie Wienkoop

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1 Introduction

There are a lot of reasons why we should understand the effects of salt and drought stress on plants. One is definitely the issue of feeding the world population and therefore having enough food. Due to the fact that currently most of the common growing areas on earth are already fit for agricultural use, it would be very helpful if we could extend the agricultural usable areas to areas that a currently not “friendly” enough for plant growth. These could be areas with a high salt content in soil and/or areas with less water availability. Other reasons are local or global changed climate conditions where global condition changes are caused by different kinds of pollution and lead therefore to an extension of areas that have less water than before. On the other hand, local climate changes are resulted by an increase of the concentration of substances like acids, salts and others incidentally exhausted by industry or willingly deployed by farming industries.

With the aim that plants can cope with that kind of stress for food production and thus can grow on such areas/soils a lot of investigations have been carried out in this context. Investigations about conserved and divergent metabolic responses to salt stress among different plant species have been carried out (Sanchez 1, 2008). Specific transcription factors in roots of *Medicago truncatula* have been identified which are salt-regulated (Gruber, 2008). A salt stress-responsive receptor homologue was isolated from *Medicago sativa* (Peña, 2008). The growth and nitrogen fixation in legume under salt stress was investigated (Lopez, 2008) (Salah, 2009). Cation concentrations in salt-tolerant *Medicago* species were examined (Sibole 2, 2003). Furthermore, plasma membrane ATPase expression in leaves of *Medicago* species (Sibole, 2005) and leave growth (Sibole 1, 2003) in relation to salt treatment was investigated. Beneficial plant growth with the aid of arbuscular mycorrhizal fungi under salt stress were detected (Evelin, 2009) (Dimkpa, 2009) (Ruiz-Lozano, 2003). Moreover, an expression database for roots of *Medicago*

truncatula under salt stress was created (Li, 2009). Interaction between salinity and iron deficiency in *Medicago ciliaris* was found (Rabhi, 2007). Antioxidant genes were expressed in *Medicago truncatula* in relation to salt stress (Mhadhbi, 2011). Dependency between ion concentration and bean growth to salinization was detected (Cabot, 2005). Expression of the TFIIIA regulatory pathway in the response to salt stress was investigated (Lorenzo, 2007). Research about *Medicago truncatula* improving salt tolerance when nodulated with rhizobium bacteria have been carried out (Bianco, 2009) (Verdoy, 2006). Regulatory pathways have been identified for root growth after salt stress in *Medicago truncatula* (Merchan, 2007). Metabolic responses to long-term salt stress (Sanchez_, 2010) or drought stress (Sanchez, 2012) in legume were investigated. Furthermore, the effect of salt stress to asparagine synthetase gene in wheat was detected (Wang, 2005).

1.1 Analysis of plant metabolites

According to the goal of the investigation there are three major plant metabolite analyses methods available: metabolite profiling, metabolite target analysis and metabolite fingerprinting (Nielsen, 2005).

Metabolite profiling is used to detect as many metabolites as possible within plant samples mostly with the aim to relatively compare them. Another approach is the metabolite target analysis, which is applied to determine often absolute concentrations of metabolites involved in a distinct pathway. The metabolite fingerprinting has not the intention to identify individual metabolites. It provides a fingerprint of all measured substances for sample comparison analysis. With other words to check if two samples are identical or not (Weckwerth & Kahl, 2013).

Once decided for a metabolite analysis method an appropriate analysis procession method has to be selected. Amongst others, there are three major options, which are chromatographic separation methods, electrophoretic separation methods or

spectroscopic analysis methods (Kleber & al, 1997). The basic idea behind spectroscopy is that organic substances absorb and/or emit photons which are accompanied by a change of molecular energy caused not only by electron transitions but also by oscillation or rotation changes. Common techniques in spectroscopy are for example UV spectroscopy, VIS spectroscopy, IR spectroscopy, ESR spectroscopy or NMR spectroscopy. They are for example used for the clarification of the structure of biological macro molecules.

The precondition that electrophoretic separation methods can be applied is that biomolecules have ionizable groups which enable the biomolecules being existent in solution as cations or anions. Additionally, molecules with the same charge but different molecular size show different charge density. Therewith, they can be separated by size and charge applying an electrical field. Gel electrophoresis or capillary electrophoresis (CE) are two typical techniques that belong to this separation method. CE in combination with mass spectrometry unites rapid analysis and efficient separation. The main advantage of CE-MS is the ability to separate most small and highly polar, charged metabolites (Weckwerth_, 2007).

Chromatography is the most important separation method in biochemistry. The general idea is to have two phases, a mobile phase that consists of the mixture of substances to be separated which is solved in a solvent (=elution) and the stationary phase. Herewith, the column represents the stationary phase consists mostly of a solid matrix (exception is liquid-liquid chromatography where a liquid thin layer represents the stationary phase). The principle of the separation is that different solved molecules in the mobile phase have a distinct interaction with the matrix of the stationary phase. This leads to different migration velocities and thus to a separation of the substance mixture.

The reason that chromatography is the most important separation method in biochemistry is that a huge amount of effort was put into improving and adapting

that method which lead to a lot of different analysis techniques. Each fits perfectly to the aim of the respective investigation. A few examples of widely-spread chromatographic techniques are ion-exchange chromatography (IEC), gas chromatography (GC), thin layer chromatography (TLC) or high performance liquid chromatography (HPLC).

IEC is often used for enrichment and/or quantitative analysis of certain proteins. The binding can be controlled by the pH value. The big advantage of TLC is the short separation time which can take only about 2 minutes and the simple setup. In opposite, the advantage of HPLC is the high separation effect (high resolution and high sensitivity) paired with an acceptable separation time of typically 1 hour achieved by smaller particles and applying high-pressure to the mobile phase. Disadvantages are higher costs and lower through-put.

1.1.1 Gas chromatography

GC uses an inert inorganic layer, e.g. silica gel covered with a very thin layer of inert liquid as stationary phase. This time, the mobile phase is an inert gas (=carrier gas) as hydrogen, argon, helium or nitrogen. Therewith, the stationary phase is attached to the interior part of a thin tube which is normally a few meters long and coiled many times to fit into an oven. Heating of the column is necessary because the sample has to been kept in the gas phase during passing the column. Generally, the liquid sample is quickly injected into an injection chamber, which is heated to several hundred degrees Celsius. Doing this, the sample is vaporized, mixed with the carrier gas and passes the heated column where the mixture is at the end separated into its different substances. Therewith, substances with a higher affinity to the stationary phase move more slowly through the column and reach the detector later at the end of the column.

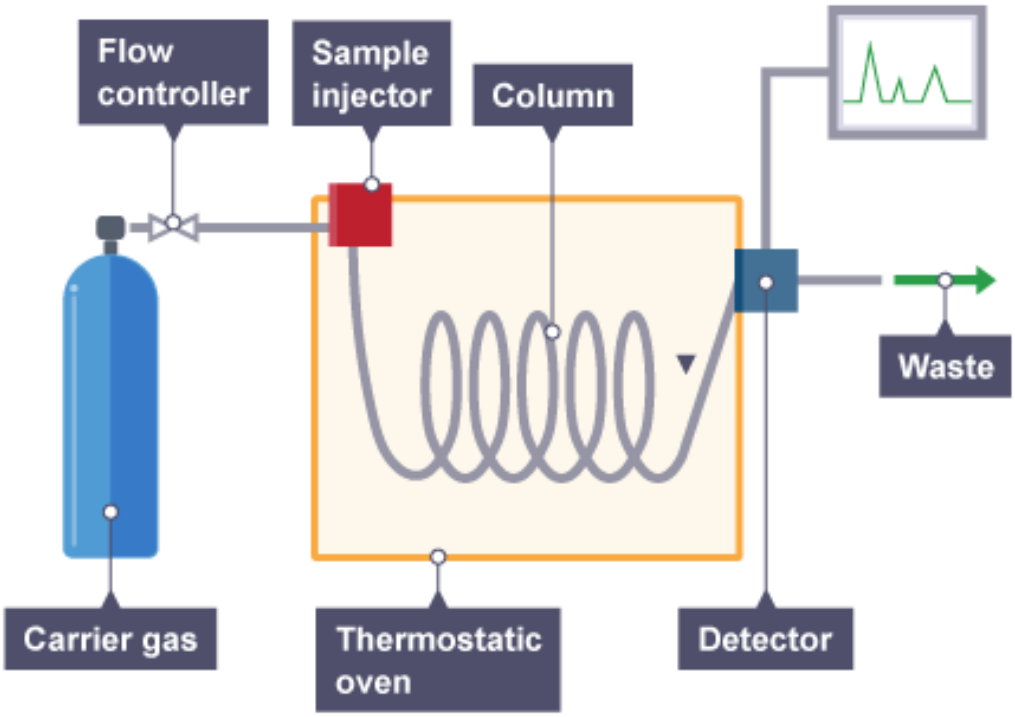


Figure 1 Schematic picture of a gas chromatograph

Gas chromatograph detectors

There are a series of different detectors that can be attached to a GC:

- Thermal conductivity detector (TCD)
  It measures changes in the thermal conductivity of the carrier gas, caused by the presence of the eluted substances (Sevcik, 1976).
- Electron capture detector (ECD)
  The carrier gas is ionized and forms electrons and positive gas ions while passing a radioactive nickel source. The electrons cause a 'standing' current. If a compound that can capture electrons is passing the detector the electron density is temporarily reduced which can be detected by a reduced current (Prichard, 2003).
- Flame ionization detector (FID)
  While organic compounds pass the flame, charged ions and electrons are formed. The migration between the electrodes of the detector causes an external current in relation to the concentration and nature of the compound (Prichard, 2003).
- Nitrogen-phosphor detector (NPD)
  A heated alkali bead (rubidium or cesium) emits electrons and creates a

background current. Compounds that contain nitrogen or phosphorus are combusted and parts of it are absorbed on the surface of the bead. The absorbed substances increase the emission of electrons which can be detected (Scott, 1996).

Furthermore, GC can be coupled with mass spectrometry (MS) or Fourier-transformation infra-red spectroscopy (FT-IR).

1.1.2 Gas chromatography coupled with mass spectrometry (GC-MS)

The main requirements in metabolite analysis are a wide dynamic detection range, high throughput, identification and quantification of metabolites as well as dealing with multiple metabolites at once (James, 2001). Mass spectrometry can cope with all these requirements and is therefore often used in combination with GC as analysis technique in metabolite analysis. Two very commonly used mass analyzers are quadrupole mass analyzer and time-of-flight mass analyzer.

1.1.3 Quadrupole mass spectrum analyser (Q-MS)

Quadrupoles are the most spread analysers due to the simple setup and lower costs.

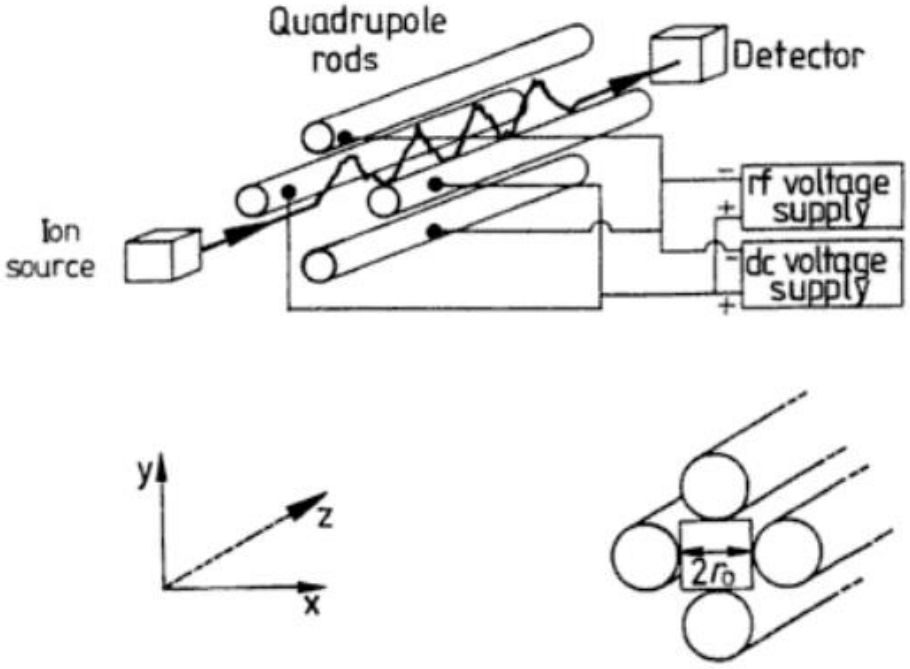


Figure 2 Scheme of a quadrupole analyser

A quadrupole consists of a set of 4 electrodes. Opposite rods are electrically connected. To be transmitted to the detector the oscillation of the ions must have finite amplitudes and may not collide with the rods or the walls of the chamber. Only ions with a narrow mass region are transmitted through the device *(Pasch & Schrepp, 2003)*.

1.1.4 Time-of-flight mass spectrum analyser (TOF-MS)

The time-of-flight (TOF) analyser is most frequently used for pulsed ion sources. Thereby, the principle is that the mass-to-charge ratio of an ion can be determined by measuring its velocity after accelerating it to a defined kinetic energy. At a fixed kinetic energy smaller ions travel at a higher speed than larger ones (Pasch & Schrepp, 2003).

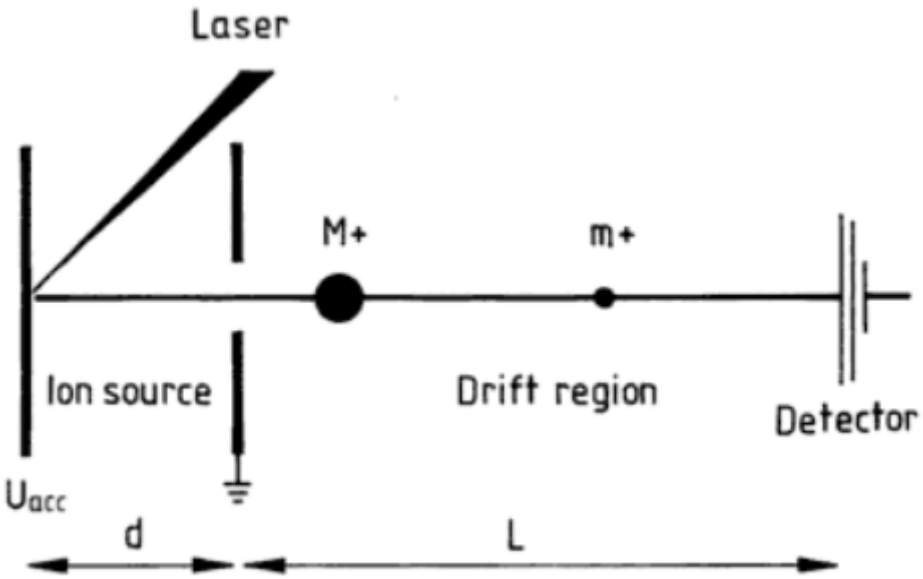


Figure 3 Scheme of a TOF analyzer

A special challenge in metabolite profiling is the identification of 100 to 1000 of unidentified metabolites that have been extracted from biological samples (Fernie & al, 2004).

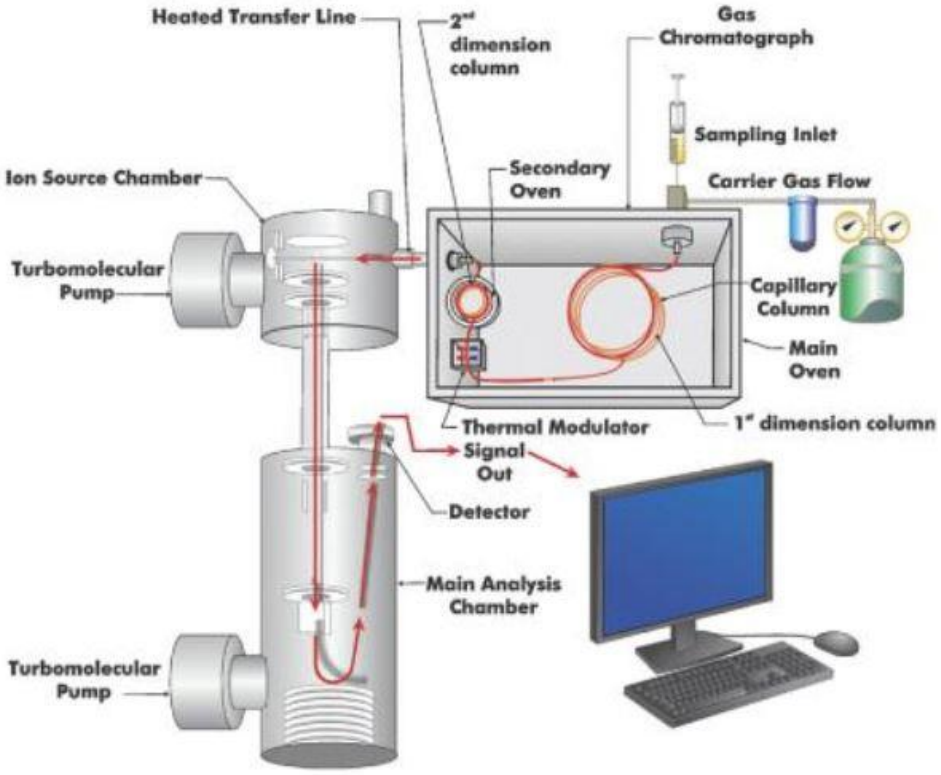


Figure 4 Leco GCxGC TOF MS system. It uses a quad-jet thermal modulator and a high speed time-of-flight mass spectrometer *(Pool & al, 2012)*.

GC-TOF-MS together with an approved metabolite library for identification is a very good combination to fulfil these requirements. For the measurements the following equipment and software was selected:

Equipment: Leco Pegasus ® 4D GCxGC TOFMS
Software: Leco® ChromaTOF-GC Software v4.50.8.0

1.2 Model plant *Medicago*

The species *Medicago truncatula* was selected because it belongs to the plant family legumes that are well known for their ability to form a symbiosis with bacteria to fixate nitrogen. This model organism has additionally the advantage to have a rapid generation time, has a small diploid genome which was already sequenced (Young & al, 2011). Therefore, this species was cultivated and used as model plant in different studies within our research department.

1.3 Objectives of this work

The model plant *Medicago truncatula* was grown under defined normal conditions for a certain amount of time. Then salt stress and drought stress was applied for 2 days' time. There was already a lot of research done with shoots of legume plants analysing their metabolites but less with roots. Therewith, the most investigations were done with plants without nitrogen fixing bacteria.

The objective of this work is to find out if statistical significant changes in metabolite concentration within the shoots and in roots of *Medicago truncatula* in relation to different plant treatments like salt and drought stress can be detected or not. Furthermore, it should be evaluated if there are differences in metabolite concentrations in relation to the presence or absence of nitrogen-fixing bacteria. Thus a metabolite profiling has been done.

2 Materials and Methods

2.1 Cultivation of plants

The cultivation of plants has been done according to the procedure described in (Staudinger, 2012).

First of all, the seeds of *Medicago truncatula* were sown in 24 pots which contained a mixture of perlite/vermiculite (2:5 - volume). Afterwards they were put into a growth camber and let them grown under controlled conditions (14h day, 10h night, 270µmol / (m^2.s) photosynthetic photon flux density, temp during day: 22°C, night: 16°C; 50-60 %rel humidity).

During the first 3 weeks, the plants were watered with nutrient solution (in first week 0.5 mM NH_4NO_3; in second and third week with a much higher concentration of 2.5 mM NH_4NO_3). After 3 weeks, 12 of the 24 plants were inoculated with *Sinorhizobizum spp*. All plants were watered for another 4 weeks; the ones without nitrogen-fixing bacteria (*Sinorhizobium*) were still watered with nutrient solution

(2.5mM NH_4NO_3) hence the water for plants with nitrogen-fixing bacteria was nutrient free.

After 7 weeks, the 12 plants without nitrogen-fixing bacteria were then split into 4 sub-groups (3 plants in each group) and subsequently treated differently (normal, lightly salted, heavily salted and water with-hold). The same split applied to the 12 plants with nitrogen-fixing bacteria. After 2 days of stress treatment, all plants were harvested, separated in roots and shoots, packed in aluminium folium and stored in the freezer at -80°C.

The cultivation and harvesting of the plant material was done by Staudinger and Mehmeti and kindly provided for this study.

2.2 Experimental setup - overview

As mentioned before in detail, plants of *Medicago truncatula* were grown under distinct environmental conditions and applied to stress for 2 days. Samples were divided in shoots / roots and additionally split up in samples that have nitrogen-fixing bacteria or not.

The applied environmental conditions were as follows:

- Normal (=control group)
- Lightly salted (Salt stress: 50mM NaCl added)
- Heavily salted (Salt stress: 200mM NaCl added)
- Water with-hold (Drought stress)

To gain a data set with statistical importance, 3 samples for each condition were taken for analysis.

The harvested and frozen samples were then homogenized to a fine-grained powder. Afterwards, the metabolites were extracted from the powder and further processed to be finally analysed by a GC-TOF-MS.

At the end, the metabolites of each sample were qualitatively as quantitatively analysed and compared to each other to figure out tendencies as a function of the treatment.

2.3 Plant material processing

2.3.1 Homogenization of the plant material

The procession of the plant samples start with homogenization of the harvested plant material by using a mortar cooled down with liquid nitrogen to about -20°C. The sample material is put into the mortar which bottom is covered with liquid nitrogen. Then the material was pestled. Important for this step is that the temperature of the plant material never raise above -20°C to avoid natural degradation processes within the plant material. Immediately after homogenization, the pulverized plant material is put into a falcon tube and put back into the freezer to be stored at -80°C.

2.3.2 Extraction of metabolites

First of all, the following solutions have to be prepared:

- Extraction buffer: CH_3OH : $CHCl_3$: H_2O (2,5: 1 : 0,5)
- Deionized water
- Internal standard: 0,1 g/l Sorbitol

Once available, the homogenized material is taken out of the freezer and an amount of approximately 40-60 mg of each sample is put into a save-lock Eppendorf tube. The exact fresh weight has to be determined and noted. Important for this step is that all tubes are stored in liquid nitrogen for the whole time and were only taken out just shortly before weighing to prevent degradation of plan material. Afterwards 1ml of extraction buffer is added to each tube, then shortly vortexed and put on ice for 8 minutes. 1ml extraction buffer is also added to a few empty Eppendorf tubes (=blanks). Blanks will be further identical processed as the samples and are included

in the term "sample" in the following paragraphs.

Next step is to centrifuge the samples applying 14g for 4 minutes at 4°C. In the meantime a new 2ml Eppendorf tube for each sample is filled with 0.5 ml deionized water. For each sample, the supernatants of the extraction buffer (now including metabolites) are added to the 2ml Eppendorf tubes for phase separation. The remaining pellets in the save-lock Eppendorf tubes have been discarded. Afterwards, the samples are vortexed and centrifuged for 2 minutes with 14g at room temperature. Once done, the supernatant of each sample is split into two aliquots of the same size using two 1,5ml Eppendorf tubes.

The last step is to add 20µl of internal standard to each sample, afterwards dry them on speed vac until they are completely dried and put them back into the freezer and kept at -20°C.

2.3.3 Derivatization of metabolites

For this step the following solutions have to be prepared:

- Methoximation mix (concentration: 40mg Methoxyaminhydroclorid in 1ml pyridine)
- Silylation solution: MSTFA (n-methyl-n-dimethylsilyltrifluoroacetamide)
- Alkane mixture: includes C10 - C40 alkanes with a concentration of 50mg/l each)

Important is that the methoximation mix has to be prepared freshly.

Before starting with the first step, the standards for quantification (QC mixes) have to be prepared by pipetting 5µl, 10µl, 20µl 30µl and 40µl of the stock solution into a separate Eppendorf tube and dry them using the speed vac. Therewith, the labelling of the standards consists of a prefix “QC” that is followed by the amount of the standard volume pipetted, e.g. QC20 = 20µl pipetted. The stock solution of the QC mix includes about 40 distinct metabolites with known concentration. Thereby, the

concentrations of theses metabolites are the same within a metabolite group and are shown in the following table.

Amount of metabolites [pmol]	QC5	QC 10	QC 20	QC 30	QC 40
Sugars	50	100	200	300	400
Amino acids	12,5	25	50	75	100
org. acids	20	40	80	120	160
Polyamines	50	100	200	300	400

Table 1 Concentration of metabolite groups in the stock solution of the QC mix

Once everything is prepared, just one of the aliquots for all samples have been taken out the freezer and let them warm until room temperature before opening the lids of the Eppendorf tubes to prevent water condensation at the tube walls. The other aliquots are kept in the freezer if something goes wrong to have a backup. Then 20µl of methoximation mix is added to the dried samples and to QC mixes. To achieve a complete dissolution of the pellets, all samples are put into the thermo mixer at 30°C for 90 minutes. The silylation mixture is provided in a flask of 1ml. The flask will be opened just shortly before adding it to the samples - but before that 30µl of alkane mixture is added. Then 80µl of the silylation solution is added and again shaken in the thermo mixer this time at 37°C for half an hour. Then the samples are centrifuged for 2 minutes and the supernatant is carefully transferred into a glass vial with micro inserts. These glass vials were then put into the GC sampler for analyzation.

2.4 Measurement with GC-MS

Each batch contains the samples to be measured, one blank, one alkane mixture as well as the QC mixes (QC5-QC40). When the sampler of the GC-MS is loaded with the respective glass vials, each sample has to be measured twice (one time in split less mode and another time in split 25 mode - this is necessary due to the higher sugar concentration in the samples which otherwise would reach the upper detector limit of the GC-MS applying split less mode). After selecting the appropriate

temperature programme and determining the desired measurement sequence, the measurement can be started. The separation is done by gas chromatography and further analysed by TOF-MS detector. Therefore, the output file includes information about substance peaks (metabolites) with their respective retention time and their mass spectra.

2.5 Data mining

2.5.1 Reference file

The obtained measurement files are analysed with Leco® ChromaTOF-GC Software v4.50.8.0. Before starting with the automatic evaluation of the measurement files using the software, a reference file has to be created. This was done by detecting all significant peaks of a randomly selected sample (selected was one of the 3 control samples of leaves without nitrogen-fixing bacteria). These peaks were then identified by matching them with the built-in mass spectrum library. At the end, the reference file includes 127 detected metabolites with their respective retention times. 52 of them are unknowns (no match found with mass spectrum library).

#	Substance name	Retention time [s]
1	#Unknown 01	331,05
2	#Unknown 02	337,45
3	#Pyruvic acid sodium salt [MPI-MDN35]	347,65
4	#Lactic acid, DL- (2TMS) [gmd-mean]	356,8
5	#Unknown 03	358,05
6	#Unknown 04	359,65
7	#Unknown 05	364,7
8	#Glycolic acid (2TMS) [gmd-mean]	367,15
9	#Unknown 06	368,15
10	#Valine (1TMS) [gmd-mean]	376,65
11	#D-Alanine [MPI-MDN35]	390,8
12	#Hydroxylamine (3TMS) [gmd-mean]	398,8
13	#Unknown 07	413,1
14	#Unknown 08	415,65
15	#Leucine (1TMS) [gmd-reduced]	433,7
16	#Unknown 09	442,1
17	#Unknown 10	444,1
18	#Proline (1TMS) [gmd-reduced]	450,15
19	#Unknown 11	452,1

20	#Phosphoric acid monomethyl ester (2TMS) [gmd-mean]	456,3
21	#Malonic acid (2TMS) [gmd-mean]	479,35
22	#3-Butyn-1-ol [MPI-MDN35]	512,7
23	#Unknown 12	513,25
24	#Unknown 13	513,55
25	#Serine (2TMS) [gmd-mean]	524,55
26	#Unknown 14	524,8
27	#2-Piperidinecarboxylic acid (1TMS) [gmd-mean]	528,95
28	#Ethanolamine (3TMS) [gmd-mean]	533,5
29	#Unknown 15	534,15
30	#Phosphocreatine sodium salt [MPI-MDN35]	542,45
31	#Glycerol [MPI-MDN35]	542,6
32	#Unknown 16	550,5
33	#Threonine, allo- (2TMS) [gmd-mean]	557,9
34	#Unknown 17	561,85
35	#Maleic acid [MPI-MDN35]	565,65
36	#Unknown 18	566,2
37	#Unknown 19	569,35
38	#Glycine [MPI-MDN35]	569,65
39	#Succinic acid [MPI-MDN35]	572,15
40	#Unknown 20	576,75
41	#Unknown 21	577,25
42	#Unknown 22	579,55
43	#L-(-)-Glyceric acid Hemicalcium salt [MPI-MDN35]	592,7
44	#Lumichrome (2MEOX) [gmd-mean]	593,25
45	#Fumaric acid [MPI-MDN35]	599,65
46	#Unknown 23	614,7
47	#D-Serine [MPI-MDN35]	617,9
48	#Argininosuccinic acid [MPI-MDN35]	630,4
49	#Unknown 24	634,45
50	#Unknown 25	638,2
51	#Unknown 26	641,75
52	#Methionine (1TMS) [gmd-mean]	654
53	#Unknown 27	655,7
54	#Octylamine (2TMS) [gmd-mean]	658,5
55	#Butanoic acid, 2,4-dihydroxy- (3TMS) [gmd-mean]	662,2
56	#Aspartic acid (2TMS) [gmd-reduced]	666,85
57	#Unknown 28	667,1
58	#Alanine, beta- (3TMS) [gmd-mean]	671,9
59	#Cysteamine (3TMS) [gmd-mean]	676,1
60	#Unknown 29	692,75
61	#Oxalic acid dihydrate [MPI-MDN35]	703,7
62	#Malic Acid RI 441990 [MPI-MDN35]	727,25
63	#Asparagine [-H2O] (2TMS)	733,45
64	#Unknown 30	741,15
65	#DL-Methionine methylsulfonium chloride [MPI-MDN35]	748,85

66	#DL-Pyroglutamic acid [MPI-MDN35]	751,35
67	#Glutamic acid (2TMS) [gmd-reduced]	755,65
68	#Butanoic acid, 4-amino- (3TMS) [gmd-mean]	757,25
69	#Unknown 31	762,25
70	#Unknown 32	766,45
71	#Unknown 33	767
72	#Phenylalanine (1TMS) [gmd-mean]	767,45
73	#Unknown 34	769,9
74	#Unknown 35	774,65
75	#Unknown 36	780,7
76	#Butane, 1,2,4-trihydroxy- (3TMS) [gmd-reduced]	783,75
77	#L-Threonic acid calcium salt [MPI-MDN35]:2	789,9
78	#alpha-ketoglutaric acid monosodium salt [MPI-MDN35]	794,3
79	#Unknown 37	795,65
80	#D-Glucose-3-sulfate sodium salt [MPI-MDN35]	822,1
81	#D-Glutamic acid [MPI-MDN35]	829,05
82	#NM- Butanoic acid, 2,4-dihydroxy- (3TMS) [gmd-mean]	860,7
83	#DL-Arabinose [MPI-MDN35]	867,8
84	#L-Asparagine (3TMS) - [MSRI]	868,8
85	#Unknown 38	869,9
86	#Unknown 39	874,1
87	#Unknown 40	882,9
88	#Unknown 41	885,05
89	#Unknown 42	887,75
90	#D-(+)-Arabitol [MPI-MDN35]	897,1
91	#Unknown 43	902,25
92	#Unknown 44	913,1
93	#Putrescine (4TMS) [gmd-mean]	916,2
94	#Adonitol [MPI-MDN35]	918,2
95	#Unknown 45	928,55
96	#D-Galactonic acid Hemicalcium salt [MPI-MDN35]:2	930,65
97	#D-Gluconic acid sodium salt [MPI-MDN35]	938,85
98	#Unknown 46	943,05
99	#Unknown 47	944,45
100	#D-Tagatose [MPI-MDN35]	962,5
101	#Shikimic acid [MPI-MDN35]	969,8
102	#DL-Isocitric acid [MPI-MDN35]	980
103	#L-Ascorbic acid [MPI-MDN35]	1000,15
104	#D-(-)-Fructose [MPI-MDN35]	1023,35
105	#L-(-)-Sorbose [MPI-MDN35]:5	1030,3
106	#Methyla-D-mannopyranoside [MPI-MDN35]	1037,5
107	#beta-D-(+)-Glucose [MPI-MDN35]	1041,95
108	#Erythrose (1MEOX) (3TMS) BP [gmd-reduced]	1048,15
109	#Unknown 48	1048,45
110	#alpha-D-Talose [MPI-MDN35]:2	1053,3
111	#Unknown 49	1060,65

112	#Sedoheptulose [MPI-MDN35]	1062,9
113	#Sorbitol [gmd-mean]	1067,25
114	#Galactinol Dihydrate [MPI-MDN35]	1091,6
115	#Palmitic acid [MPI-MDN35]	1112,75
116	#Citric acid [MPI-MDN35]	1123,7
117	#myo-Inositol [MPI-MDN35]	1162,65
118	#1-O-Methyl-beta-D-Galactoside [MPI-MDN35]	1218,6
119	#Octadecanoic acid (1TMS) [gmd-reduced]	1230,55
120	#Unknown 50	1360,35
121	#Palatinitol [MPI-MDN35]	1426,75
122	#D-(+)-Digitoxose [MPI-MDN35]	1440,75
123	#Sucrose [MPI-MDN35]	1473,05
124	#Laminaribiose (mixed anomers) [MPI-MDN35]	1523,95
125	#D-Gentiobiose [MPI-MDN35]	1536,05
126	#Unknown 51	1609,25
127	#Unknown 52	1861,95

Table 2 *Detected substances in one of the 3 control samples of leaves without nitrogen-fixing bacteria*

After the reference file was created, in a first run the software automatically compares the reference file with the detected peaks of other samples to identify them. Additionally, the software automatically defines the limits of the peak area below the curve to calculate the respective mass detector signal in arbitrary units. In a second run, the identification as well as the pre-defined areas of the detected peaks has to be manually verified.

For peaks beginning from #1 (#Unknown 01) until #101 (#Shikimic acid) the split-less data were analysed. For the remaining peaks (mostly sugars) the split 25 data had to be analysed because the split-less data were overloaded by sugar. Unfortunately, the QC mix standards for split 25 were partly not accessible therefore all substances beginning from #102 until #127 could not be evaluated. This applies mostly to sugars but also to acids of the TCA cycle (citric acid and iso-citric acid).

2.5.2 Normalization

2.5.2.1 Normalization with QC mix

As a first step, normalization to our external standard (=QC mixes) is required. Our external standard (QC mix) consists of about 40 metabolites (i.e. sugars, amino acids,

organic acids and polyamines) with known concentration. For each metabolite M which was in the QC mix as well as in our reference sample (reference file), the concentration of the 5 dilutions is plotted against the respective mass detector signal in arbitrary units. Before doing that, the Blank value of each obtained metabolite M has to be subtracted of the respective mass detector signal. Therewith, for each metabolite M a regression line was computed. The results of the computation are the slope of the regression line k_M [pmol/100µl] and the intersection of the regression line $YAxis_M$ with y-axis. 16 metabolites, which are in both the reference as well as in the QC mix, can be quantitatively analysed. All other metabolites, which are only in the reference but not in the QC mix can only be analysed qualitatively.

Metabolites in sample and in QC mix

For the 16 metabolites that are in the samples as well as in the QC mix an absolute quantification was computed as follows:

$$Conc_M = k_M \times (S_M - Blank_M - YAxis_M) \times dil \times V_{inj} / 1000$$

$Conc_M$ ..... Concentration of metabolite M in sample [nmol / 100µl]
k_M ..... Slope of metabolite M [pmol / 100µl]
$YAxis_M$ ..... Intersection of regression line with y-axis
S_M ..... Mass detector units for metabolite M in sample
$Blank_M$ ..... Mass detector units for metabolite M in Blank
dil ..... Dilution: Number of aliquots (=2)
V_{inj} ..... Injection volume (=100)

Metabolites only in sample but not in QC mix

The normalization for all other metabolites is done with the mean of the slopes for all metabolites within a substance category (i.e. one mean slope for all sugars, one for all amino acids, one for all organic acids and one for all polyamines).

$$k_{SC} = \sum_{M=1}^{n} k_M \times \frac{1}{n}$$

SC .....Substance category, i.e. sugars
M.....all metabolites that belongs to the substance category
n .....Number of metabolites that belong to a substance category
k_M .....Slope of metabolite M [pmol / 100µl]
k_{SC}.....Mean of slopes for all metabolites M [pmol / 100µl]

If a metabolite cannot be allocated to a certain substance category, the normalization is done with the mean for all existing slopes independent of any substance category. The reference value is then computed as follows:

$$RefV_M = k_{SC} \times (S_M - Blank_M)$$

$RefV_M$ .....Concentration of metabolite M in sample
k_{SC}.....Mean of slopes for all metabolites M [pmol / 100µl]
S_M .....Mass detector units for metabolite M in sample
$Blank_M$ .....Mass detector units for metabolite M in blank

2.5.2.2 Normalization with weight

Due to the fact that the initial weight of each sample was different, it is necessary to normalize to the weight. Only after that step it is then possible to compare concentrations or reference values to each other. In our case, the weight normalized to 1g fresh weight (FW). For absolute concentrations the following equation was applied:

$$Conc_{M,FW} = Conc_M \ / FW_M$$

$Conc_M$ .....Concentration of metabolite M [nmol / 100µl]
FW_M.....Fresh weight of metabolite M [g]
$Conc_{M,FW}$ ...Concentration of metabolite M [nmol / 100µl/1g FW]

Parallel to that, for relative comparison the reference values are normalized as follows:

$$RefV_{M,FW} = RefV_M \ / \ FW_M$$

$RefV_M$ .....Reference value of metabolite M
FW_M.....Fresh weight of metabolite M [g]
$RefV_{M,FW}$ ...Reference value of metabolite M [1/1g FW]

QC_M .....Mass detector units for metabolite M in QC
$Blank_M$ .....Mass detector units for metabolite M in blank

2.5.3 Analysis

After the normalization, for each metabolite of a sample exists either an absolute concentration or a reference value that allows comparison to other samples. Before starting with the comparison, the mean and the standard deviation of the 3 samples with identical treatment were calculated – one for each metabolite.

2.5.3.1 Comparison to respective control

Firstly, we compare the treated samples (salt stress or drought stress) with the respective control sample and check if there is a significant difference applying t-test. A significant difference is given, if the value of t-test is equal or below 0.05.

2.5.3.2 Comparison between samples with and without nitrogen-fixing bacteria

Furthermore, we compare plants with nitrogen-fixing bacteria with the respective plants that were not in symbiosis with rhizobacteria. Again, significant changes are detected by applying t-test. A difference is then significant, if the value of t-test is equal or below 0.05.

3 Results

The results below just depict these metabolites that show significant changes according to t-test (<= 0.05).

3.1 Comparison to respective control

With this series we compare the treated plants (salt stress or drought stress) with the respective unstressed plant (control group).

3.1.1 Fertilized plants

3.1.1.1 Plants - 2 days with 50mM NaCl

M. truncatula - 2 days with 50mM NaCl	effect
Shoots	
Roots	
Pyruvic acid sodium salt	⇩

Pyruvic acid sodium salt / roots **T-Test = 0.01**

	Mean		Standard Deviation	
	Control	50mM	Control	50mM
Reference value	382	166	113	107
Change	100%	43%	30%	28%

Table 3 Overview and details of significant metabolite changes of *M. truncatula* after 2 days treatment with 50 mM NaCl

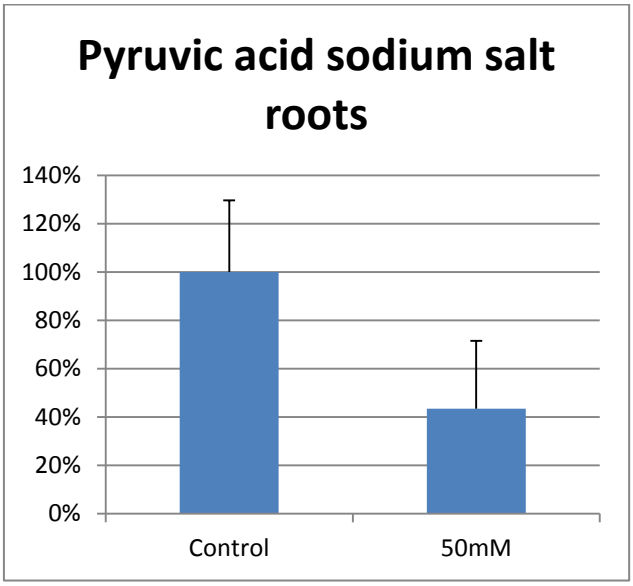


Figure 5 Significant metabolite changes of M. truncatula after 2 days treatment with 50 mM NaCl in roots

3.1.1.2 Plants - 2 days with 200mM NaCl

M. truncatula - 2 days with 200mM NaCl	effect
Shoots	
Lactic acid	⇧
Roots	
Butanoic acid, 2,4-dihydroxy-	⇧

Lactic acid / shoots **T-Test = 0.03**

	Mean		Standard Deviation	
	Control	200mM	Control	200mM
Reference value	414	1479	464	553
Change	100%	357%	112%	133%

Butanoic acid, 2,4-dihydroxy- / roots **T-Test = 0.01**

	Mean		Standard Deviation	
	Control	200mM	Control	200mM
Reference value	83	168	15	26
Change	100%	202%	18%	32%

Table 4 Overview and details of significant metabolite changes of *M. truncatula* after 2 days treatment with 200 mM NaCl

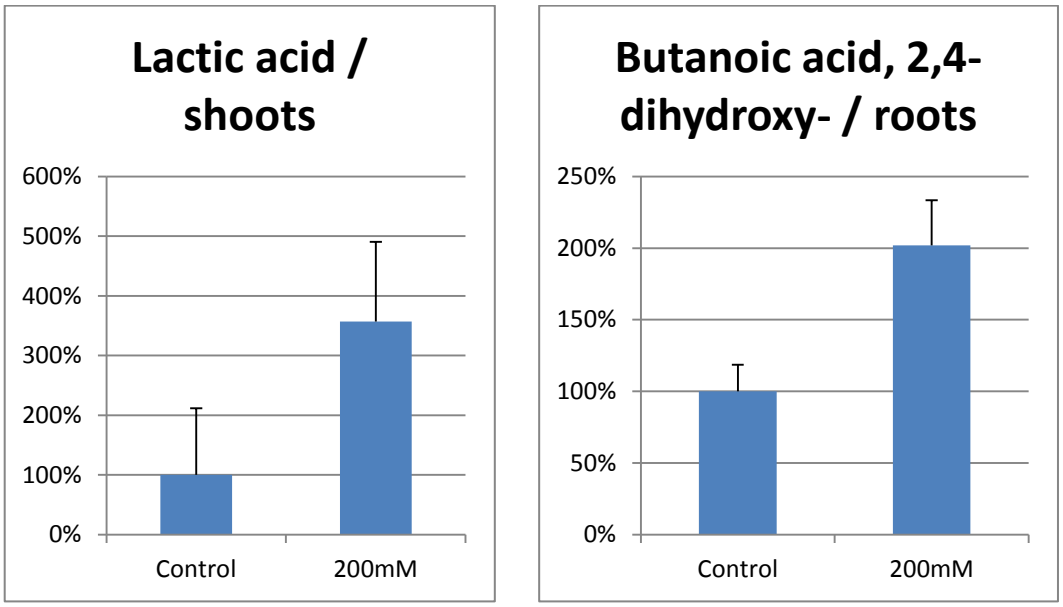


Figure 6 Significant metabolite changes of *M. truncatula* after 2 days treatment with 200 mM NaCl in roots and shoots

3.1.1.3 Plants - 2 days dry

M. truncatula - 2 days dry	effect
Shoots	
Lumichrome	⇧
Argininosuccinic acid	⇧
D-(+)-Arabitol	⇧
Roots	
Phosphoric acid monomethyl ester	⇧
Aspartic acid	⇧
Glutamic acid	⇧
Succinic acid	⇧

Table 5 Overview of significant metabolite changes of *M. truncatula* after 2 days drought treatment

Shoots

Lumichrome / shoots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	57	105	40	42
Change	100%	184%	70%	73%

Argininosuccinic acid / shoots **T-Test = 0.03**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	62	172	24	45
Change	100%	279%	39%	73%

Arabitol / shoots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	181	409	49	86
Change	100%	226%	27%	47%

Table 6 Significant metabolite changes of *M. truncatula* after 2 days drought treatment in shoots

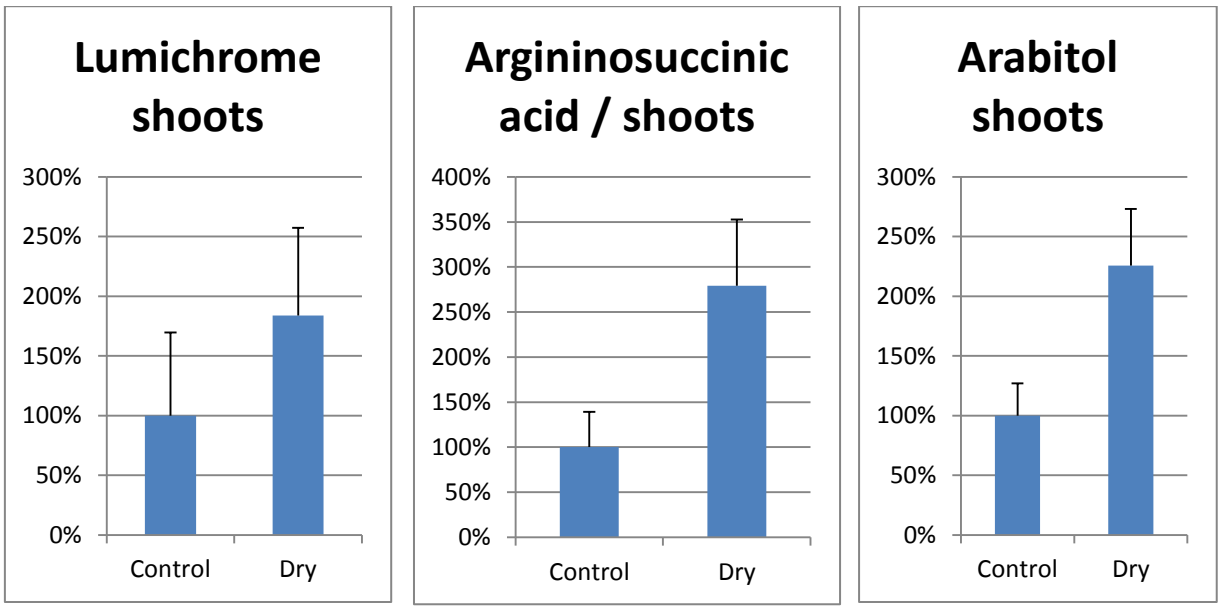


Figure 7 Significant metabolite changes of *M. truncatula* after 2 days drought treatment in shoots

Roots

Phosphoric acid monomethyl ester / roots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	47	79	4	9
Change	100%	169%	8%	20%

Aspartic acid / roots **T-Test = 0.02**

	Mean		Standard deviation	
	Control	Dry	Control	Dry
Conc [nmol/l]	152	393	102	153
Change	100%	258%	67%	100%

Glutamic acid / roots **T-Test = 0.03**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Conc [nmol/l]	367	947	298	429
Change	100%	258%	81%	117%

Succinic acid / roots			T-Test = 0.04	
	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Conc [nmol/l]	167	365	68	85
Change	100%	219%	41%	51%

Table 7 Significant metabolite changes of *M. truncatula* after 2 days drought treatment in roots

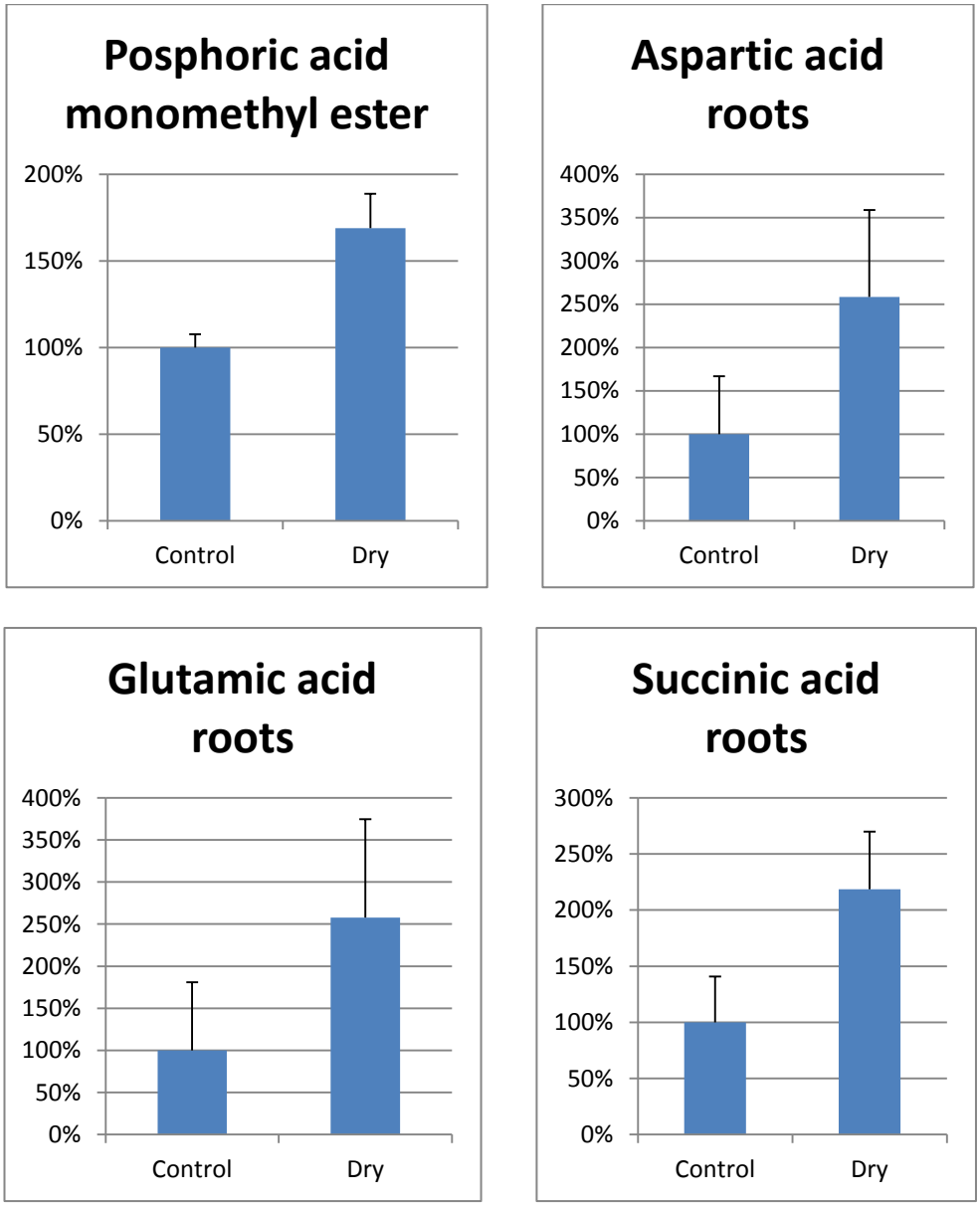


Figure 8 Significant metabolite changes of *M. truncatula* after 2 days drought treatment in roots

3.1.2 Nitrogen-fixing plants

3.1.2.1 Plants - 2 days with 50mM NaCl

M. truncatula / Rhizobium - 2 days with 50mM NaCl	effect
Shoots	
2-Piperidinecarboxylic acid	⇧
Alanine, beta-	⇧
Pyroglutamic acid	⇧
Asparagine	⇩
Roots	
Butanoic acid, 2,4-dihydroxy-	⇧
Butanoic acid, 4-amino-	⇧
Oxalic acid dihydrate	⇩
Fumaric acid	⇩
Succinic acid	⇧

Table 8 Overview of significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 50 mM NaCl

Shoots

2-Piperidinecarboxylic acid / shoots **T-Test = 0.01**

	Mean		Standard Deviation	
	Control	50mM	Control	50mM
Reference value	1007	3575	328	646
Change	100%	355%	33%	64%

Alanine, beta / shoots **T-Test = 0.05**

	Mean		Standard Deviation	
	Control	50mM	Control	50mM
Reference value	61	103	19	10
Change	100%	168%	31%	16%

Pyroglutamic acid / shoots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	50mM	Control	50mM
Reference value	3815	8135	438	500
Change	100%	213%	11%	13%

Asparagine / shoots **T-Test = 0.02**

	Mean		Standard deviation	
	Control	50 mM	Control	50 mM
Conc [nmol/l]	15517	9156	4899	3687
Change	100%	59%	32%	24%

Table 9 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 50 mM NaCl in shoots

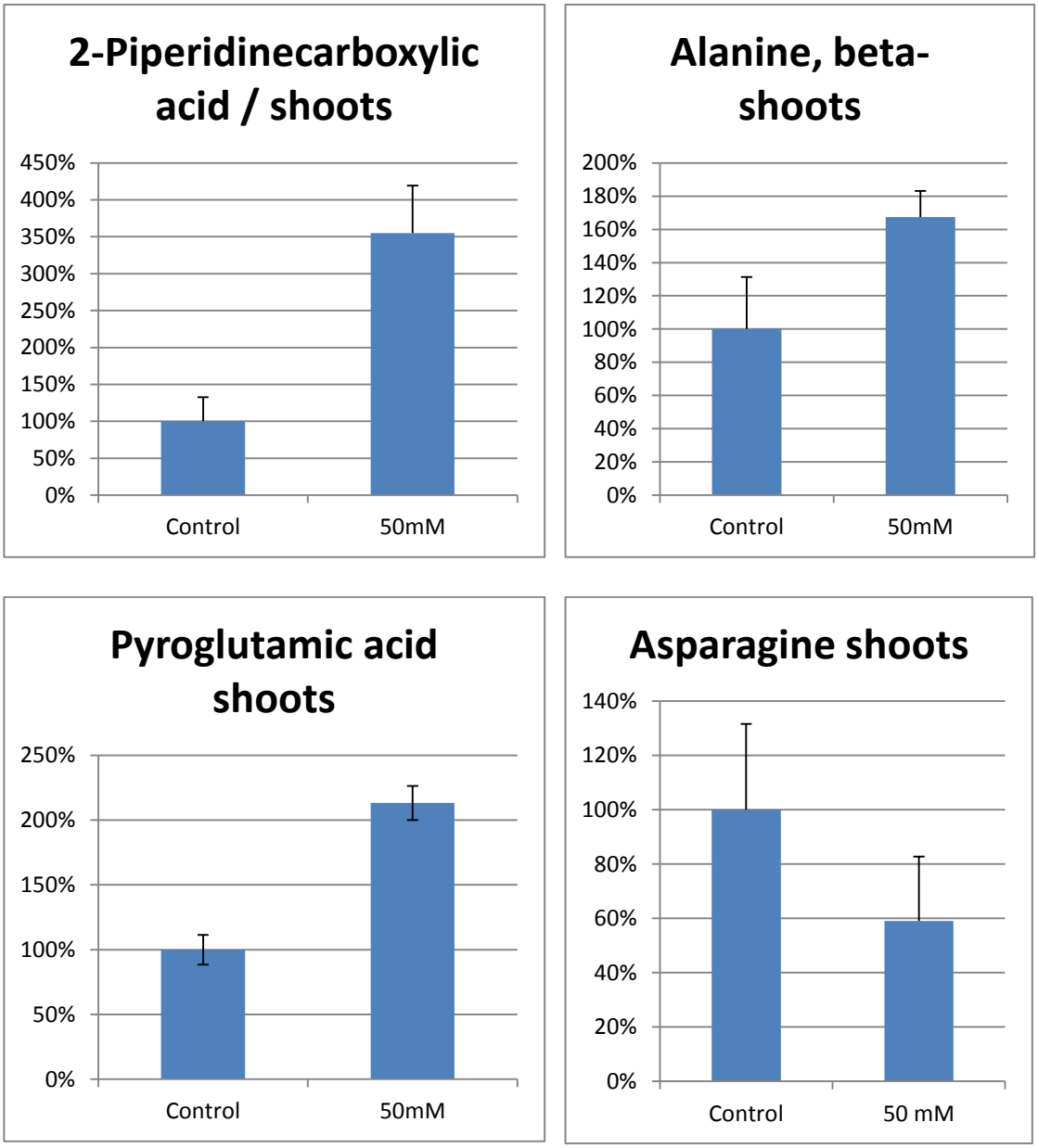


Figure 9 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 50 mM NaCl in shoots

Roots

Butanoic acid, 2,4-dihydroxy- / roots **T-Test = 0.04**

	Mean		Standard Deviation	
	Control	50 mM	Control	50 mM
Reference value	87	206	16	39
Change	100%	238%	19%	45%

Butanoic acid, 4-amino- / roots **T-Test = 0.04**

	Mean		Standard Deviation	
	Control	50 mM	Control	50 mM
Reference value	249	543	66	29
Change	100%	218%	26%	12%

Oxalic acid dihydrate / roots **T-Test = 0.04**

	Mean		Standard Deviation	
	Control	50 mM	Control	50 mM
Reference value	57	35	10	8
Change	100%	61%	18%	15%

Fumaric acid / roots **T-Test = 0.02**

	Mean		Standard deviation	
	Control	50 mM	Control	50 mM
Conc [nmol/l]	330	275	8	19
Change	100%	83%	3%	6%

Succinic acid / roots **T-Test = 0.04**

	Mean		Standard Deviation	
	Control	50 mM	Control	50 mM
Conc [nmol/l]	140	395	23	30
Change	100%	281%	17%	21%

Table 10 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 50 mM NaCl in roots

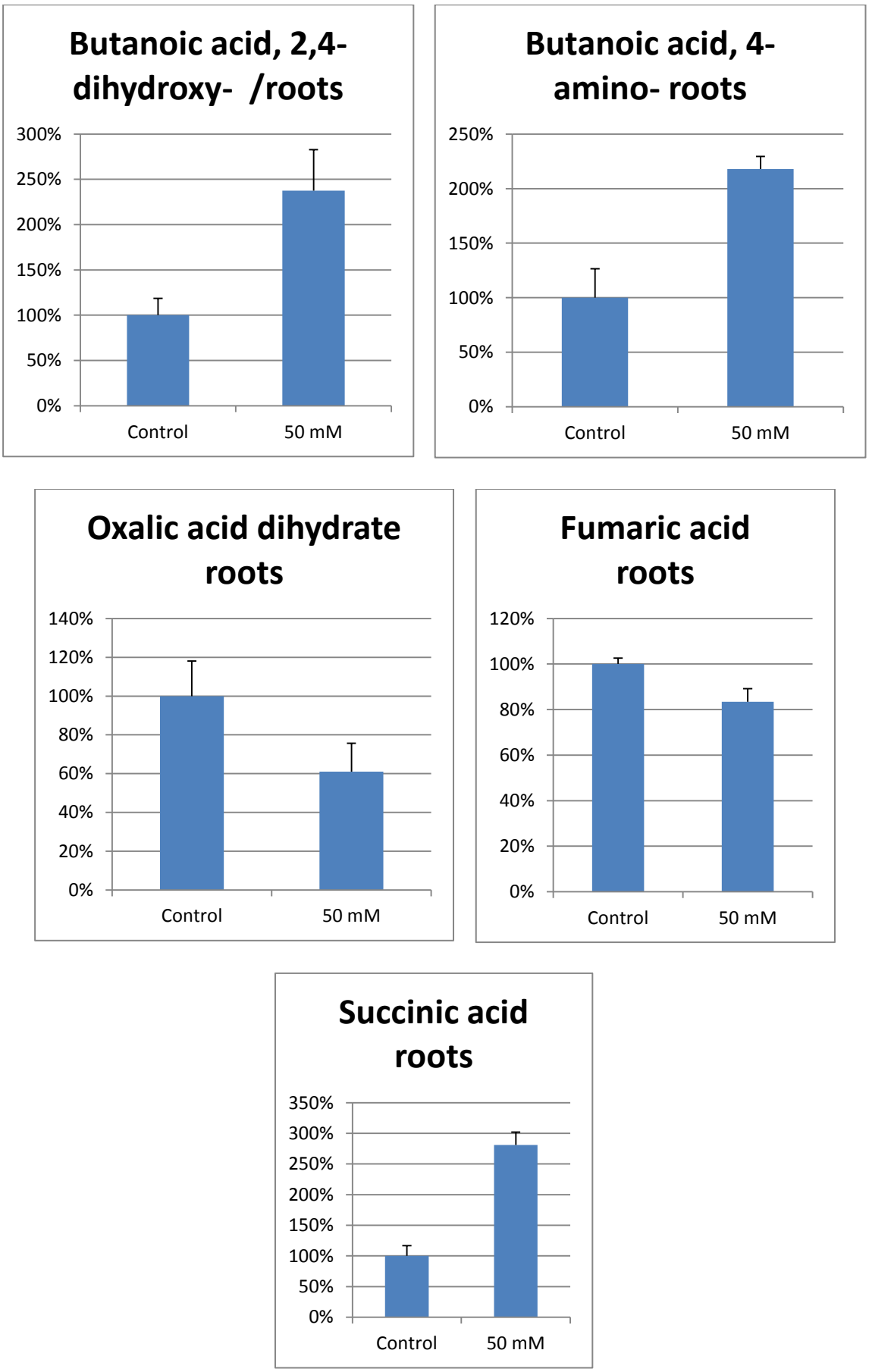


Figure 10 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 50 mM NaCl in roots

Metabolites that are part or support the citric acid cycle are also affected by salt treatment. In this context, the treatment of 50 mM NaCl shows a slight decrease of about 15% of fumaric acid but an obvious increase of about 3 times the concentration of succinic acid (**Table 10**). A decrease of fumaric acid as a result of salt stress was also detected in *T. halophila (Gong & al, 2005)*, in the shoot tips from grapevine, *V. vinifera cv. Cabernet (Cramer & al, 2007)*, in the shoots of *A. thaliana* and *L. japonicus*, but no significant change in shoots and roots of *O. sativa (Sanchez 1, 2008)*. Interestingly, a more than 2 times increase of fumaric acid was detected in roots of *M. truncatula* with nitrogen-fixing rhizobium symbionts but treated with 200 mM NaCl for 6 days *(Staudinger, 2012)*. The increase of succinic acid was also detected in *T. halophila* after a short-time salt stress of 150 mM NaCl *(Gong & al, 2005)* but an decrease of it was reported in the roots of *O.sativa* after 3 weeks salt stress *(Sanchez 1, 2008)*.

3.1.2.2 Plants - 2 days with 200mM NaCl

M. truncatula / Rhizobium - 2 days with 200mM NaCl	effect
Shoots	
Phosphocreatine sodium salt	⇧
Roots	
Oxalic acid dihydrate	⇩
2-Oxoglutaric acid	⇧
Alanine	⇧
Glutamic acid	⇧
Succinic acid	⇧
Threonine	⇧

Table 11 Overview of significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 200 mM NaCl

Shoots

Phosphocreatine sodium salt /shoots **T-Test = 0.04**

	Mean		Standard Deviation	
	Control	200 mM	Control	200 mM
Reference value	1587	4379	1211	1834
Change	100%	276%	76%	116%

Table 12 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 200 mM NaCl in shoots

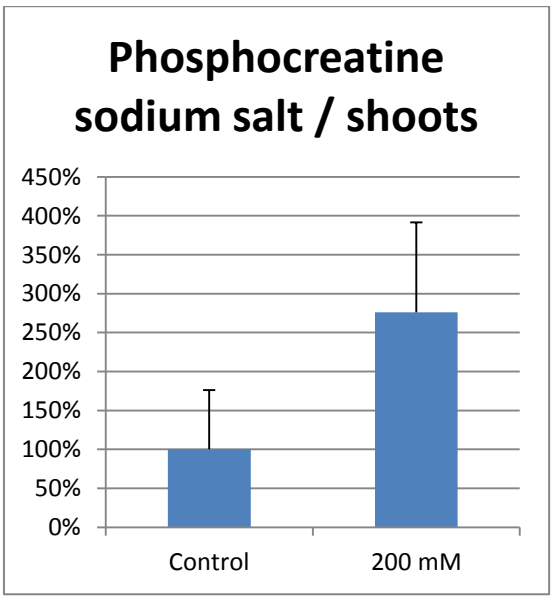


Figure 11 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 200 mM NaCl in shoots

Roots

Oxalic acid dihydrate / roots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	200 mM	Control	200 mM
Reference value	57	20	10	8
Change	100%	34%	18%	14%

2-Oxoglutaric acid / roots **T-Test = 0.03**

	Mean		Standard deviation	
	Control	200 mM	Control	200 mM
Conc [nmol/l]	71	119	12	1
Change	100%	166%	16%	1%

Alanine / roots **T-Test = 0.03**

	Mean		Standard deviation	
	Control	200 mM	Control	200 mM
Conc [nmol/l]	166	315	46	40
Change	100%	190%	28%	24%

Glutamic acid / roots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	200 mM	Control	200 mM
Conc [nmol/l]	431	1076	143	148
Change	100%	249%	33%	34%

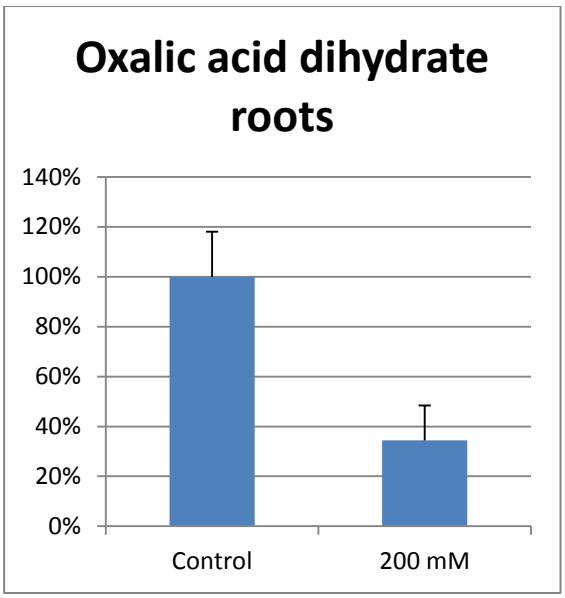
Succinic acid / roots **T-Test = 0.01**

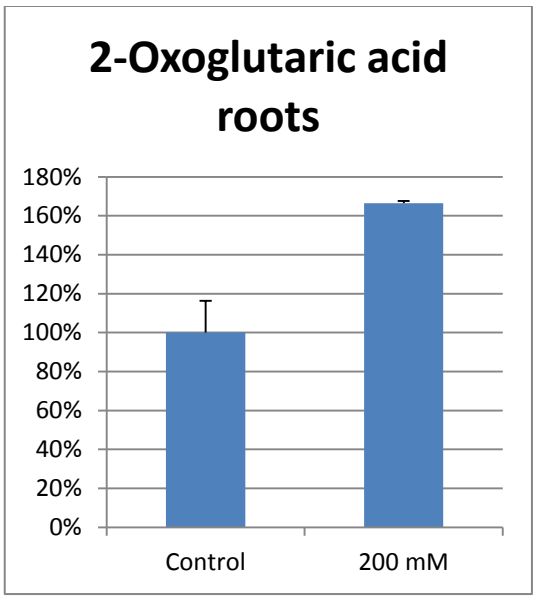
	Mean		Standard Deviation	
	Control	200 mM	Control	200 mM
Conc [nmol/l]	140	238	23	12
Change	100%	169%	17%	9%

Threonine / roots **T-Test = 0.04**

	Mean		Standard deviation	
	Control	200 mM	Control	200 mM
Conc [nmol/l]	113	145	10	4
Change	100%	128%	9%	4%

Table 13 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 200 mM NaCl in roots





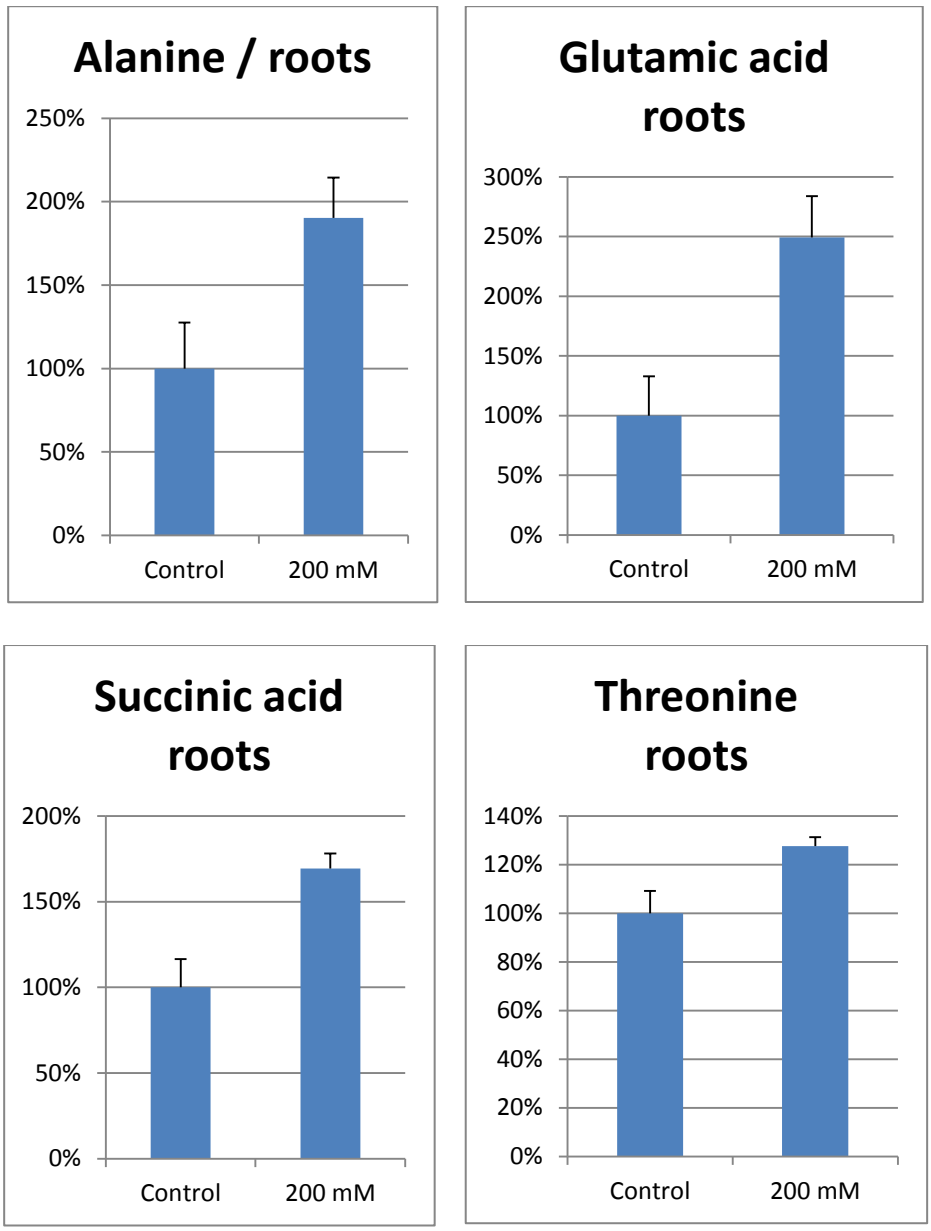


Figure 12 Significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days treatment with 200 mM NaCl in roots

3.1.2.3 Plants - 2 days after drought treatment

M. truncatula / Rhizobium - 2 days dry	effect
Shoots	
Roots	
Glycerol	⇧
Arabitol	⇩
Adonitol	⇩
Tagatose	⇩
Valine	⇩

Table 14 Overview of significant metabolite changes of *M. truncatula* in symbiosis with RHIZOBIUM after 2 days drought treatment

Roots

Glycerol / roots **T-Test = 0.03**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	114	198	161	158
Change	100%	175%	141%	139%

Arabitol / roots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	75	29	8	17
Change	100%	39%	11%	23%

Adonitol / roots **T-Test = 0.03**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	13	2	4	3
Change	100%	18%	32%	26%

Tagatose / roots **T-Test = 0.02**

	Mean		Standard Deviation	
	Control	Dry	Control	Dry
Reference value	61	32	1	4
Change	100%	52%	2%	7%

Valine / roots **T-Test = 0.03**

	Mean		Standard deviation	
	Control	Dry	Control	Dry
Conc [nmol/l]	93	48	12	23
Change	100%	52%	13%	25%

Table 15 Significant metabolite changes of *M. truncatula* in symbiosis with *rhizobium* after 2 days drought treatment

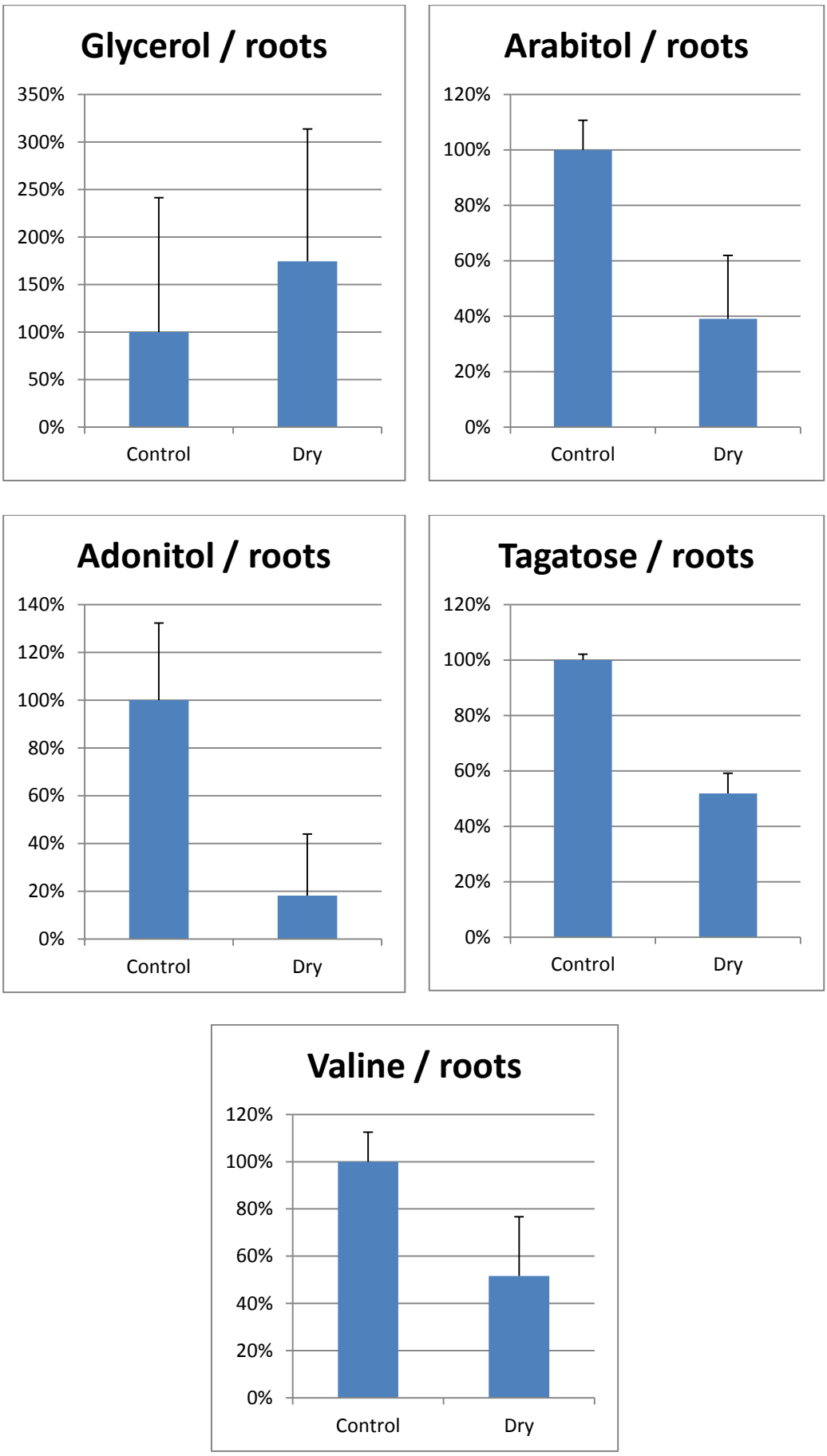


Figure 13 Significant metabolite changes of *M. truncatula* in symbiosis with *rhizobium* after 2 days drought treatment

3.1.3 PCA Analysis

To evaluate if similar treated plants are clustering, principle component analysis (PCA) has been carried out. The data set that has been investigated consist of all 48 samples. Per sample only those metabolites were put into the data set which have been significantly changed according to t-test (<= 0.05). Further settings were defined for PCA:

- Missing value fill: prior distribution
- Log transformation
- 2 principle components analysed

The PCA plot looks as follows:

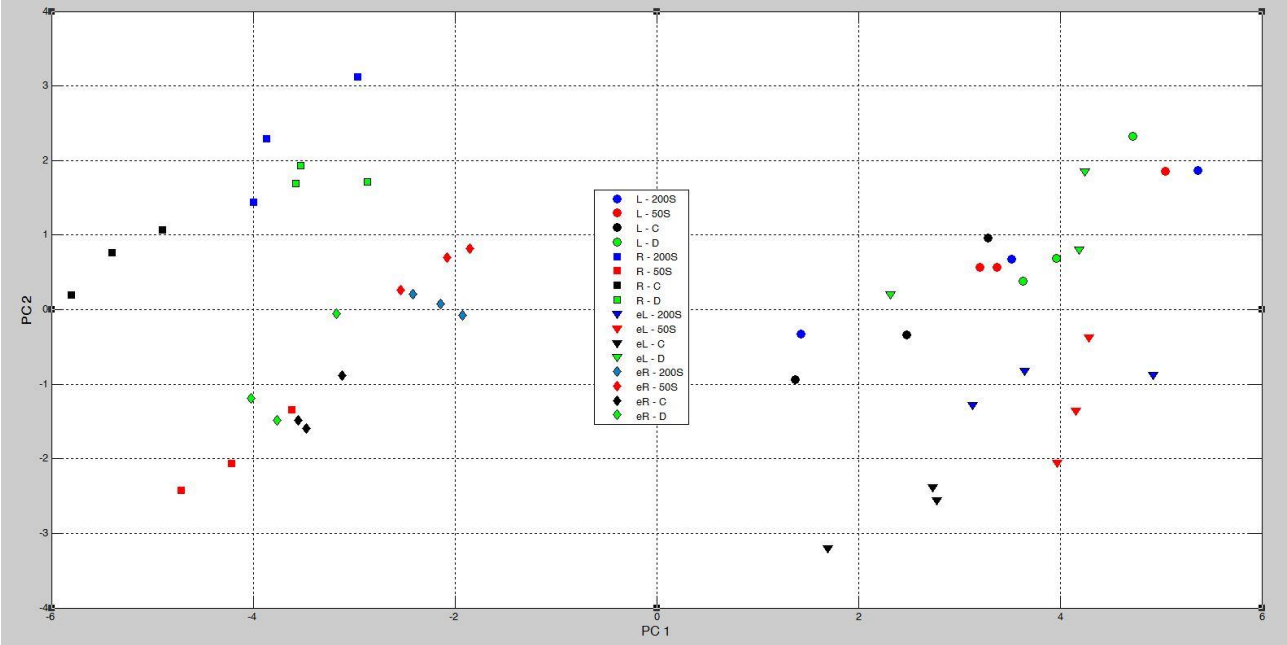


Figure 14 PCA plot for all plants of the significantly changed metabolites in comparison to the respective control group

3.1.4 MapMan Analysis

To have a better overview, which metabolites are affected in the metabolite pathway, a MapMan data visualisation analysis was carried out. The existing mapping file “MappingMetabolites”, which was created by Staudinger (Staudinger, 2012) was selected. Before loading the measurement data into MapMan, it must be checked for recognition purposes if the metabolite names are identical with the

MapMan substance names. For those samples that show at least 4 significant metabolites (according to t-test <= 0.05) a respective MapMan diagram has been created.

3.1.4.1 Shoots of nitrogen-fixing plants - 2 days with 50mM NaCl

Metabolite	Change (control = 100%)
2-Piperidinecarboxylic acid	355%
Alanine, beta-	168%
DL-Pyroglutamic acid	213%
Asparagine	59%

Table 16 Significant metabolites in shoots of plants with *rhizobium* symbiont treated with 50 mM NaCl for 2 days

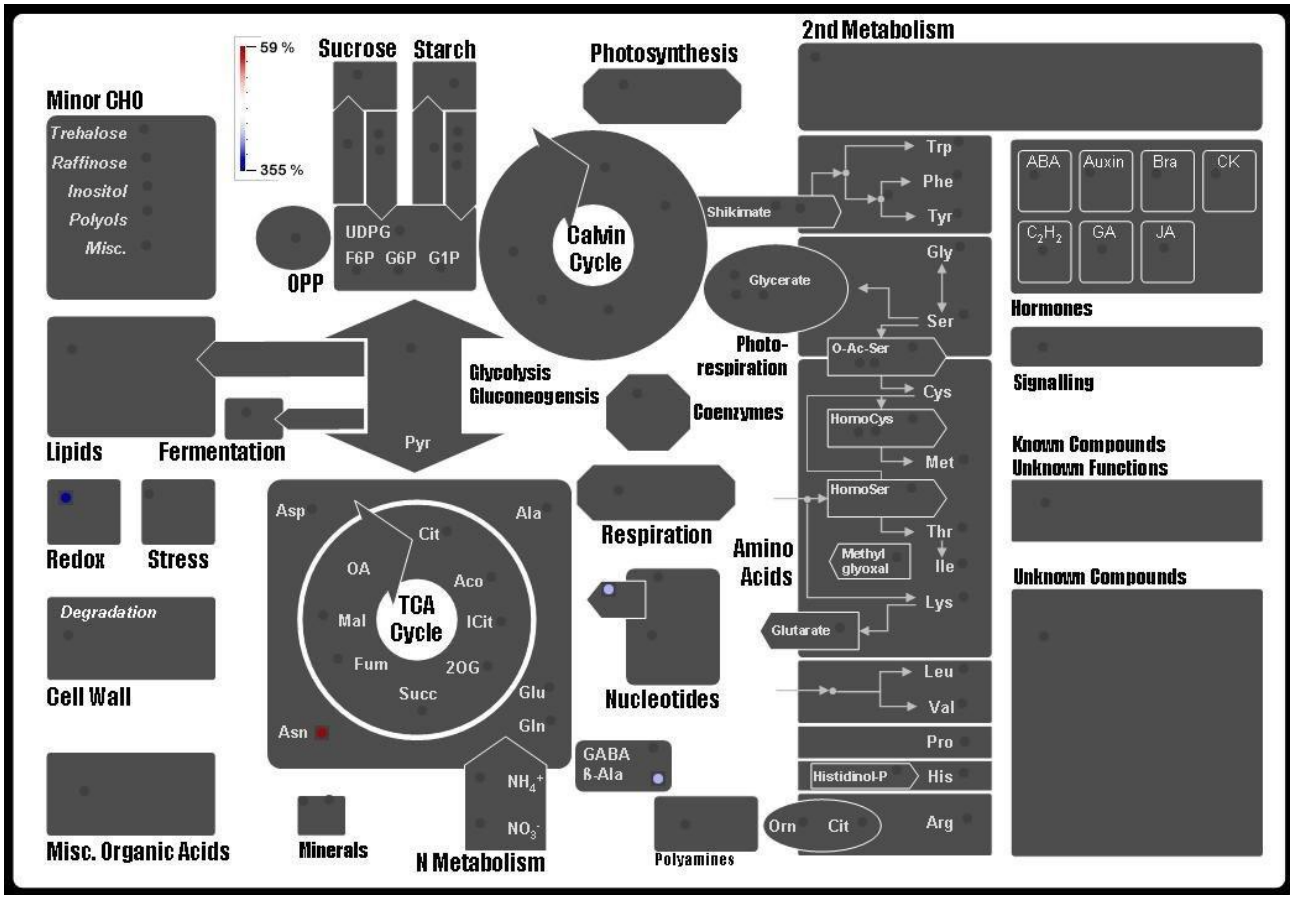


Figure 15 MapMan plot shows significant metabolites in shoots of plants with *rhizobium* symbiont treated with 50 mM NaCl for 2 days

3.1.4.2 Roots of fertilized plants - 2 days dry

Metabolite	Change (control = 100%)
Aspartic acid	258%
Glutamic acid	258%
Succinic acid	219%
Phosphoric acid monomethyl ester	169%

Table 17 Significant metabolites in roots of plants without *rhizobium* symbiont kept dry for 2 days

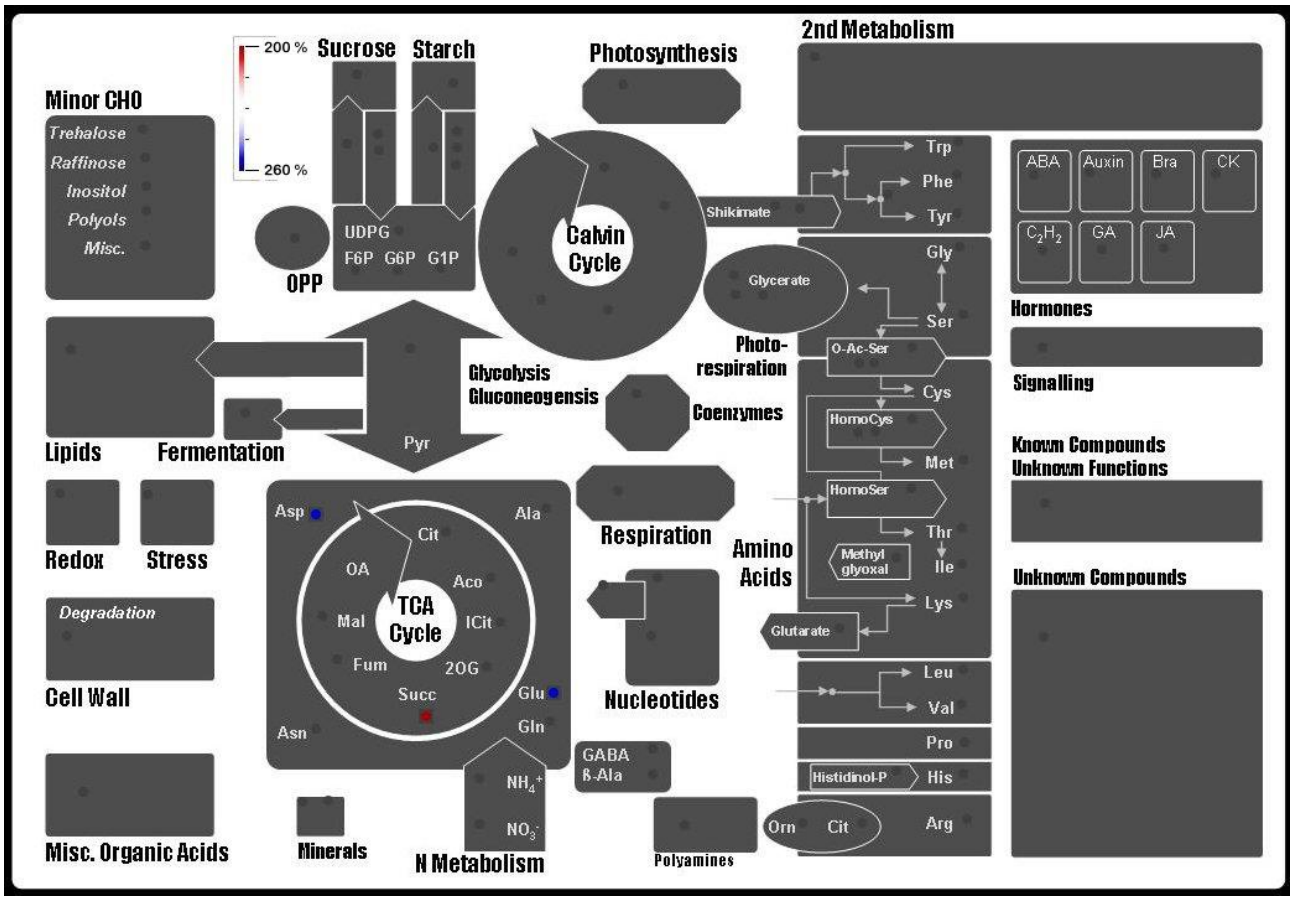


Figure 16 MapMan plot shows significant metabolites in roots of plants without *rhizobium* symbiont kept dry for 2 days

3.1.4.3 Roots of nitrogen-fixing plants - 2 days with 200mM NaCl

Metabolite	Change (control = 100%)
2-Oxoglutarate	166%
Alanine	190%
Glutamic acid	249%
Succinic acid	169%
Threonine	128%

Table 18 Significant metabolites in roots of plants with *rhizobium* symbiont treated with 200 mM NaCl for 2 days

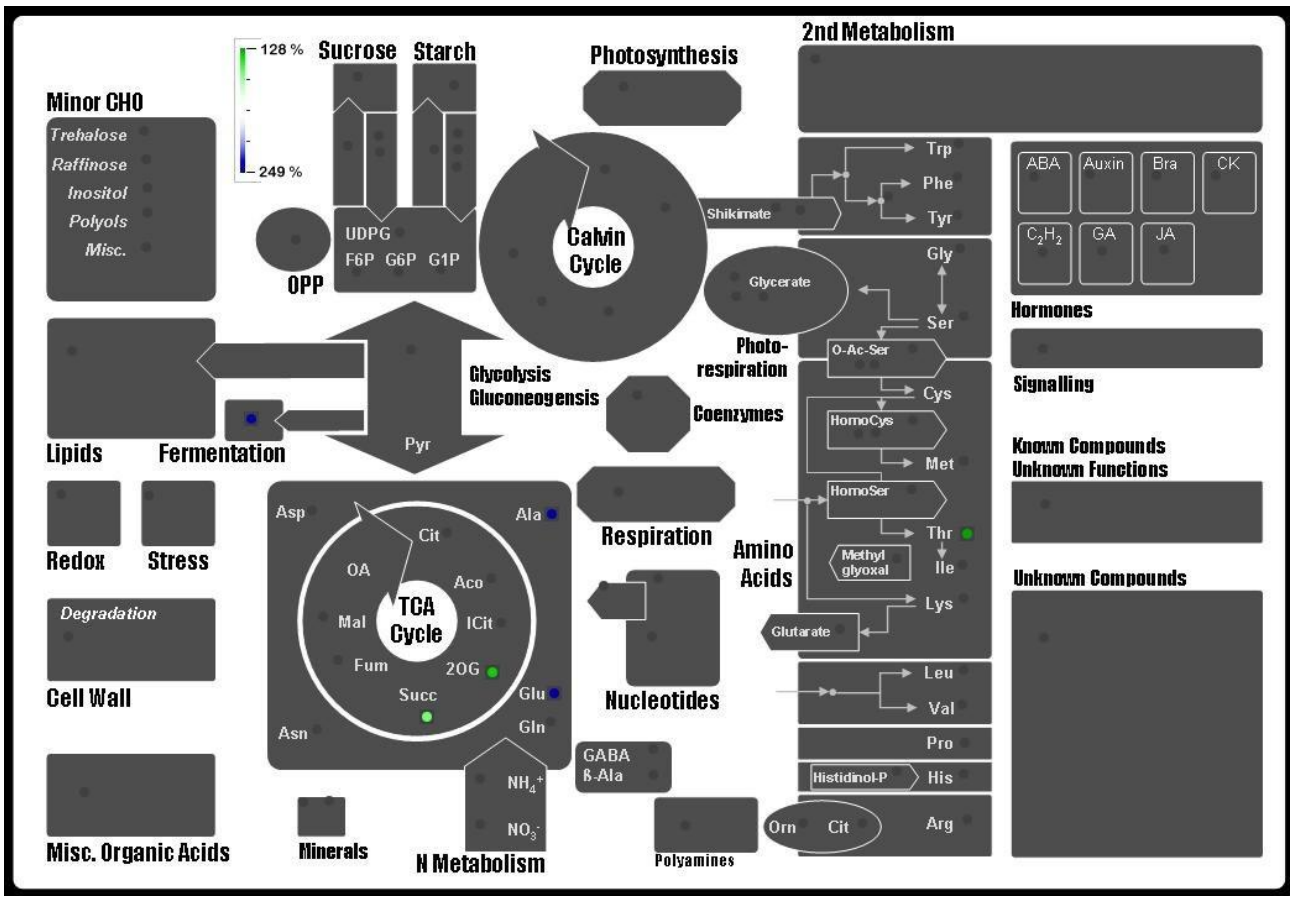


Figure 17 MapMan plot shows significant metabolites in roots of plants with *rhizobium* symbiont treated with 200 mM NaCl for 2 days

3.2 Comparison between nitrogen-fixing plants with fertilized plants

Within this series we compare plants with nitrogen-fixing bacteria with plants without any bacteria for nitrogen-fixing but with the same treatment (normal, salt stress or drought stress).

3.2.1 Plants of control group

M. truncatula - control group	effect
Shoots	
Alanine, beta-	⇧
Methionine methylsulfonium chloride	⇧
Tagatose	⇩
Asparagine	⇧
Serine	⇧
Roots	
Phosphoric acid monomethyl ester	⇩
Maleic acid	⇧
Argininosuccinic acid	⇧
Oxalic acid dihydrate	⇧
Methionine methylsulfonium chloride	⇧
Pyroglutamic acid	⇧
Butanoic acid, 4-amino-	⇧
Glycine	⇧

Table 19 Overview of significant metabolite changes of *M. truncatula* with rhizobium symbionts in comparison to plants without symbionts in control groups

Shoots

Alanine, beta- / control **T-Test = 0.04**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	26	61	14	19
Change	100%	237%	53%	74%

Methionine methylsulfonium chloride / control **T-Test = 0.01**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	23	53	6	10
Change	100%	230%	24%	44%

Tagatose / control **T-Test = 0.01**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	234	54	59	39
Change	100%	23%	25%	17%

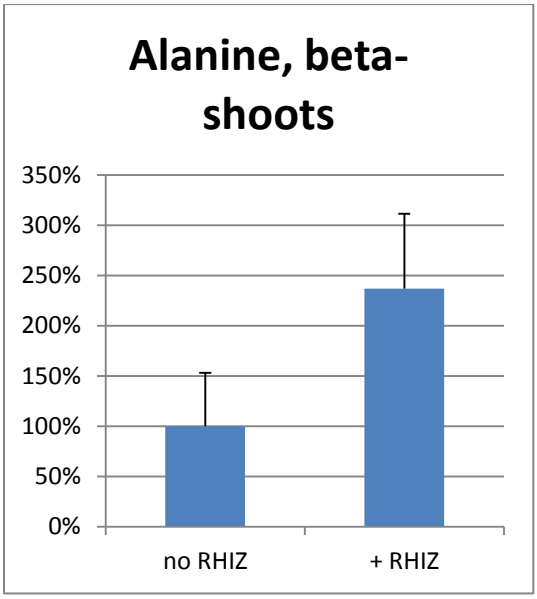
Asparagine / control **T-Test = 0.05**

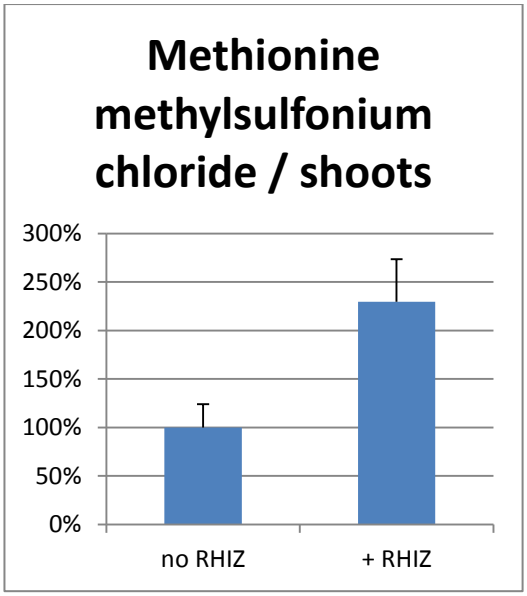
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	147	15517	54	4899
Change	100%	10588%	37%	3343%

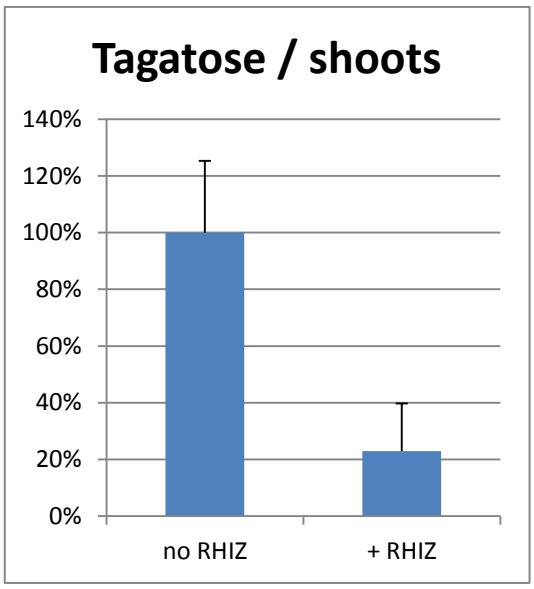
Serine / control **T-Test = 0.01**

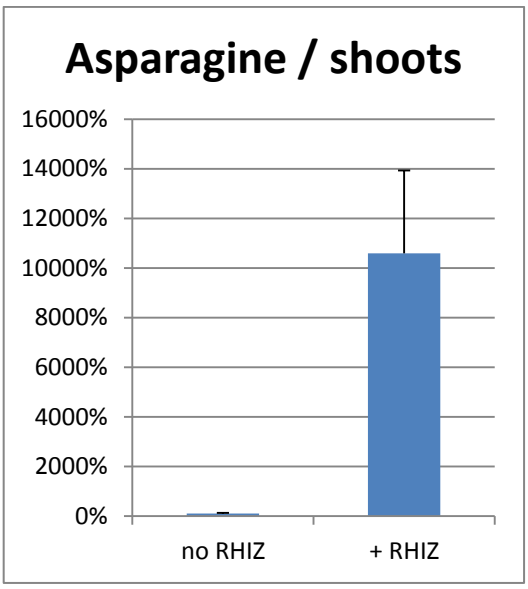
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	130	384	37	69
Change	100%	294%	29%	53%

Table 20 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts in control groups









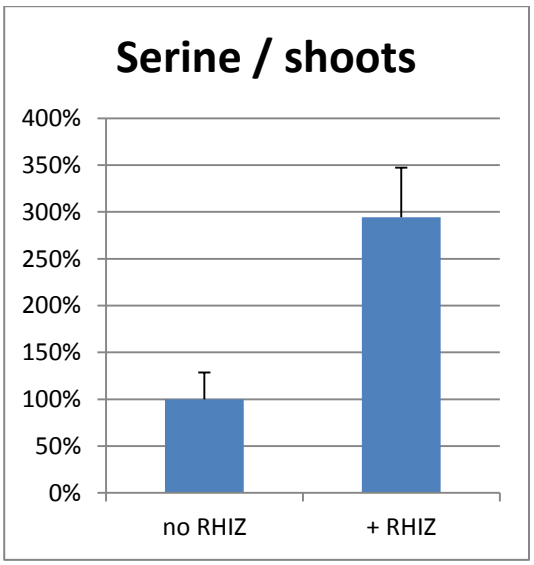


Figure 18 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts in control groups

Roots

Phosphoric acid monomethyl ester / control **T-Test = 0.03**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	47	26	4	5
Change	100%	55%	8%	12%

Maleic acid /control **T-Test = 0.04**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	238	1061	177	356
Change	100%	446%	74%	150%

Argininosuccinic acid / control **T-Test = 0.01**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	0	16	0	3
Change	---	---	---	---

Oxalic acid dihydrate / control **T-Test = 0.02**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	12	57	5	10
Change	100%	491%	41%	89%

Methionine methylsulfonium chloride / control **T-Test = 0.01**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	35	96	13	23
Change	100%	277%	38%	65%

Pyroglutamic acid / control **T-Test = 0.04**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	748	1919	480	646
Change	100%	256%	64%	86%

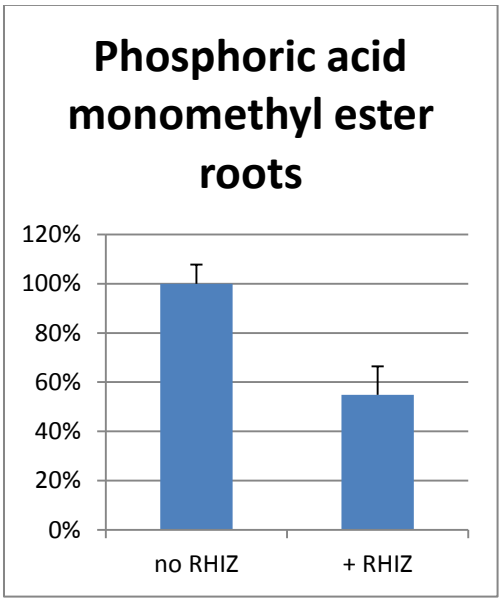
Butanoic acid, 4-amino- / control **T-Test = 0.01**

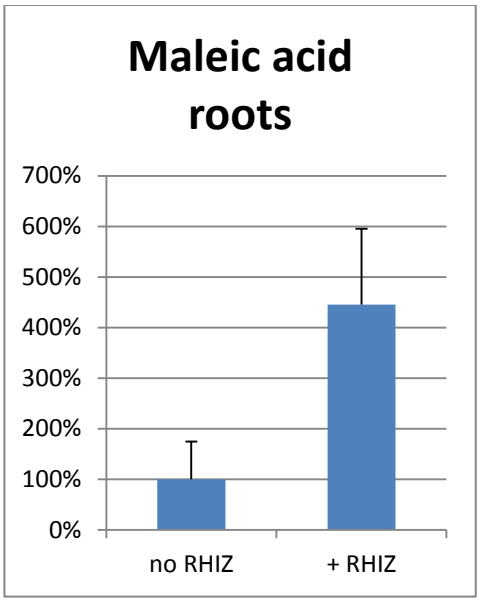
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	149	249	59	66
Change	100%	167%	40%	44%

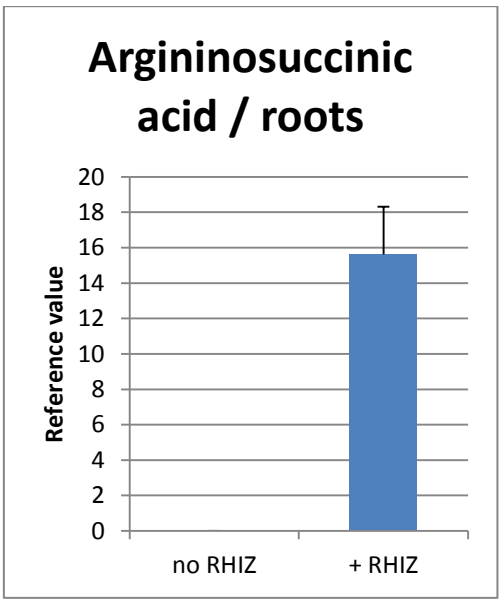
Glycine / control **T-Test = 0.01**

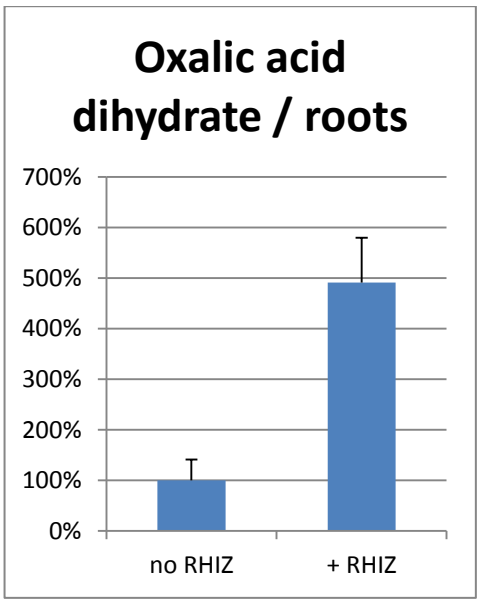
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	27	56	7	7
Change	100%	203%	26%	26%

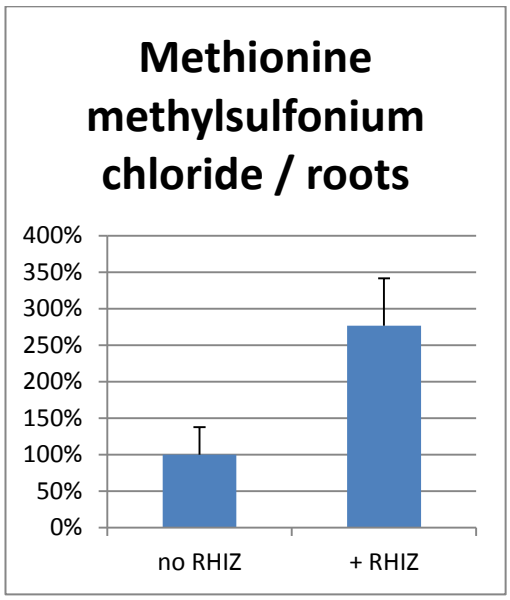
Table 21 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts in control groups

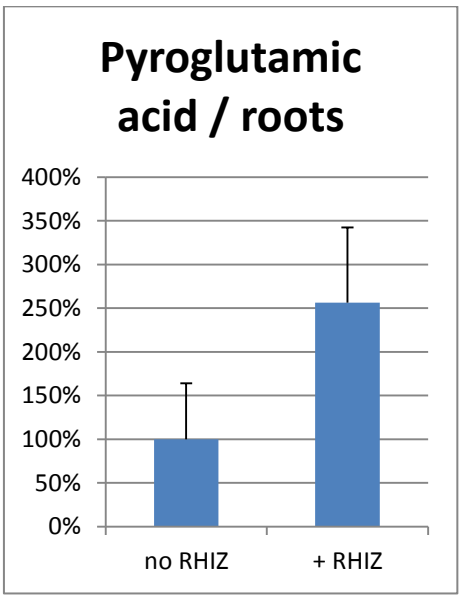





Argininosuccinic acid / roots
Reference value
20
18
16
14
12
10
8
6
4
2
0
no RHIZ
+ RHIZ


Oxalic acid dihydrate / roots
700%
600%
500%
400%
300%
200%
100%
0%
no RHIZ
+ RHIZ


Methionine methylsulfonium chloride / roots
400%
350%
300%
250%
200%
150%
100%
50%
0%
no RHIZ
+ RHIZ


Pyroglutamic acid / roots
400%
350%
300%
250%
200%
150%
100%
50%
0%
no RHIZ
+ RHIZ

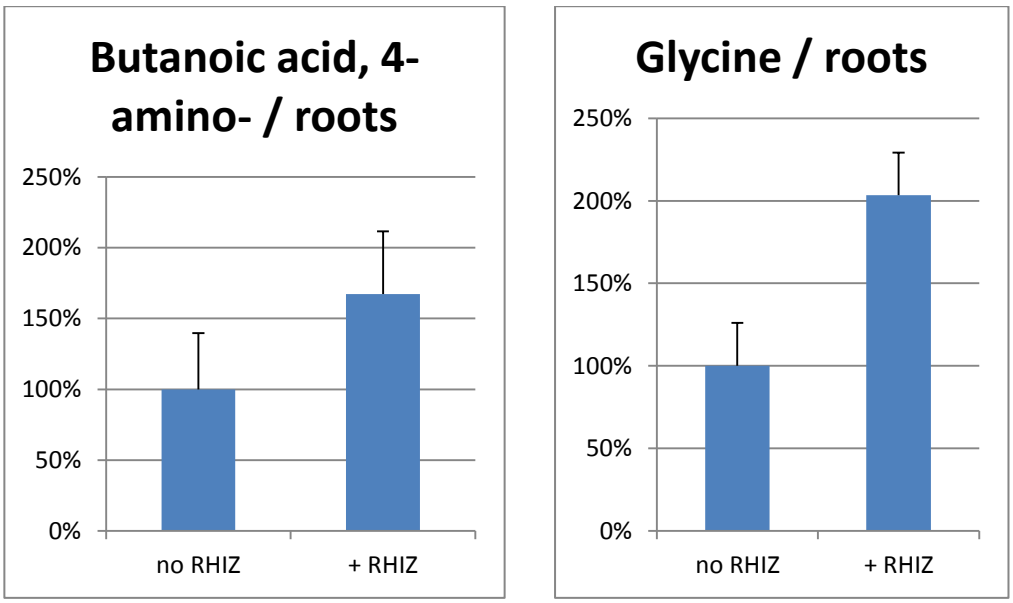


Figure 19 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts in control groups

3.2.2 Plants - 2 days with 50mM NaCl

M. truncatula - 2 days with 50mM NaCl	effect
Shoots	
Argininosuccinic acid	⇧
Roots	
Malonic acid	⇧
Phosphocreatine sodium salt	⇩
Argininosuccinic acid	⇧
Alanine, beta-	⇧
Pyroglutamic acid	⇧

Table 22 Overview of significant metabolite changes of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 50 mM NaCl

Shoots

Argininosuccinic acid / 50 mM NaCl **T-Test = 0.05**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	168	606	94	45
Change	100%	359%	56%	27%

Table 23 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 50 mM NaCl

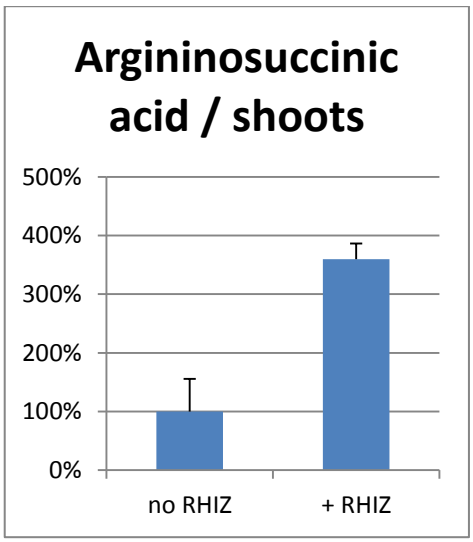


Figure 20 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 50 mM NaCl

Roots

Malonic acid / 50 mM NaCl **T-Test = 0.004**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	839	1769	323	376
Change	100%	211%	39%	45%

Phosphocreatine sodium salt / 50 mM NaCl **T-Test = 0.01**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	7651	1525	2304	1611
Change	100%	20%	30%	21%

Argininosuccinic acid / 50 mM NaCl **T-Test = 0.03**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	0	30	0	7
Change	---	---	---	---

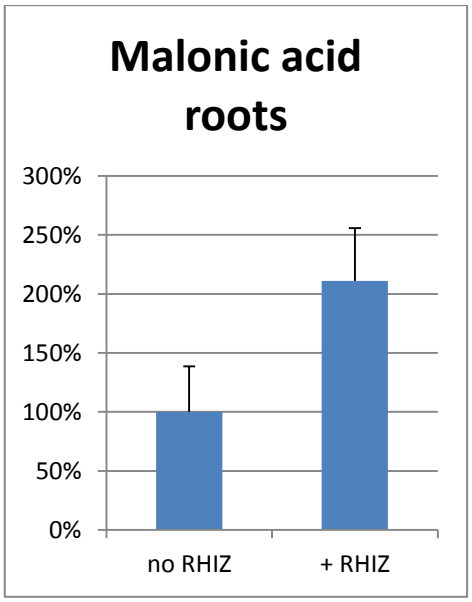
Alanine, beta- / 50 mM NaCl **T-Test = 0.02**

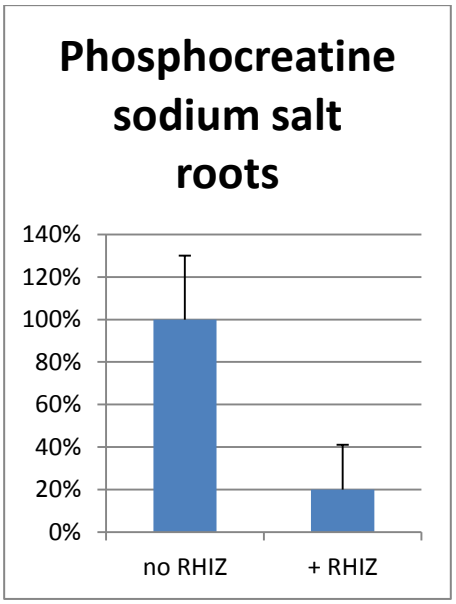
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	63	156	9	24
Change	100%	247%	14%	38%

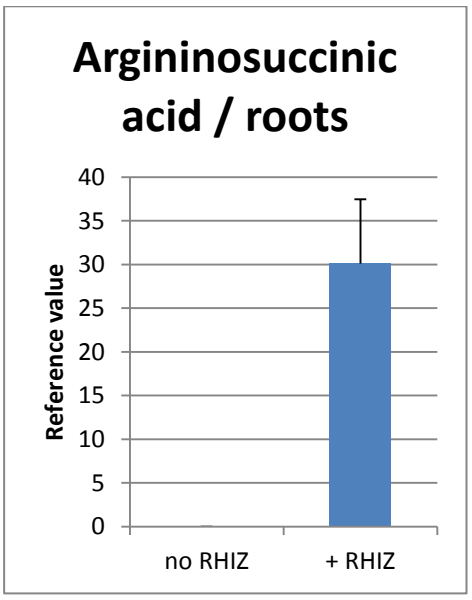
Pyroglutamic acid / 50 mM NaCl **T-Test = 0.05**

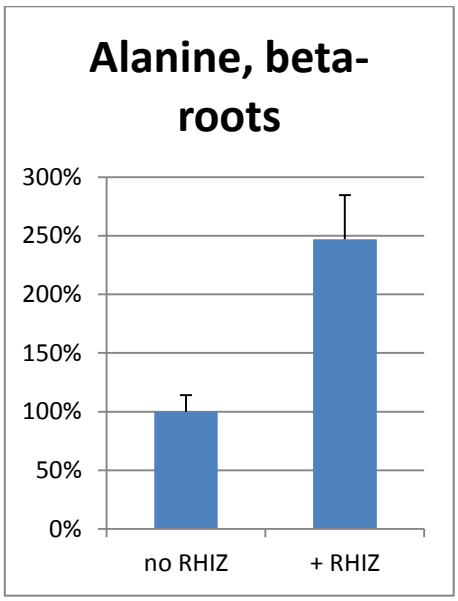
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	1035	3022	338	625
Change	100%	292%	33%	60%

Table 24 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 50 mM NaCl









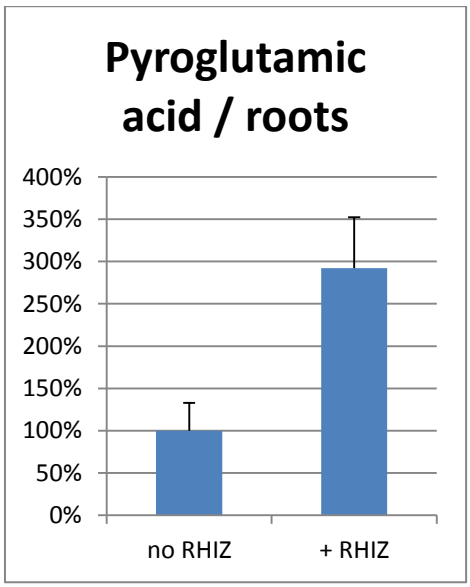


Figure 21 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 50 mM NaCl

3.2.3 Plants - 2 days with 200mM NaCl

M. truncatula - 2 days with 200mM NaCl	effect
Shoots	
Lactic acid	⇩
Malonic acid	⇧
Roots	
Glycolic acid	⇩
Maleic acid	⇩
Glyceric acid Hemicalcium salt	⇩
Butanoic acid, 2,4-dihydroxy-	⇩
Alanine	⇧
Serine	⇧

Table 25 Overview of significant metabolite changes of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 200 mM NaCl

Shoots

Lactic acid / 200 mM NaCl **T-Test = 0.03**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	1479	600	553	429
Change	100%	41%	37%	29%

Malonic acid / 200 mM NaCl **T-Test = 0.03**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	1591	2705	1227	1478
Change	100%	170%	77%	93%

Table 26 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 200 mM NaCl

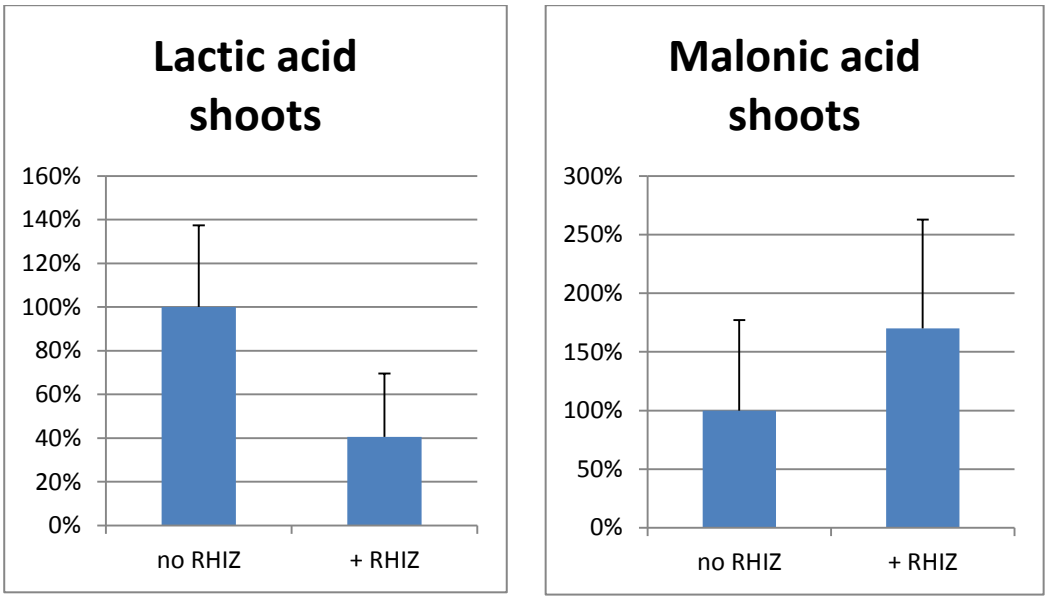


Figure 22 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 200 mM NaCl

Roots

Glycolic acid / 200 mM NaCl **T-Test = 0.03**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	44	25	8	7
Change	100%	56%	17%	16%

Maleic acid /200 mM NaCl **T-Test = 0.03**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	746	255	124	22
Change	100%	34%	17%	3%

Glyceric acid Hemicalcium salt / 200 mM NaCl **T-Test = 0.001**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	236	123	24	25
Change	100%	52%	10%	11%

Butanoic acid, 2,4-dihydroxy- / 200 mM NaCl **T-Test = 0.04**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	168	74	26	20
Change	100%	44%	16%	12%

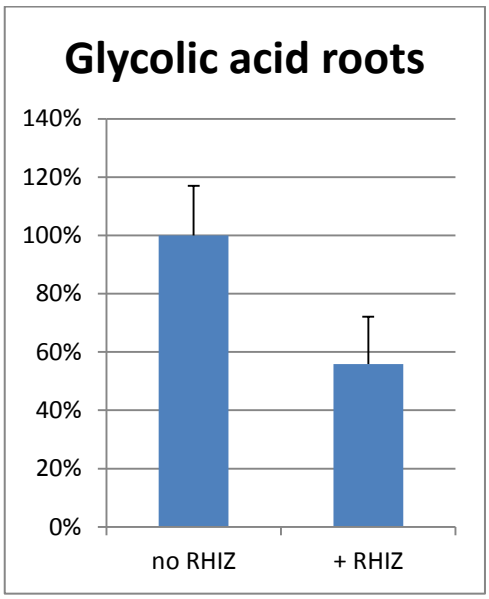
Alanine / 200 mM NaCl **T-Test = 0.02**

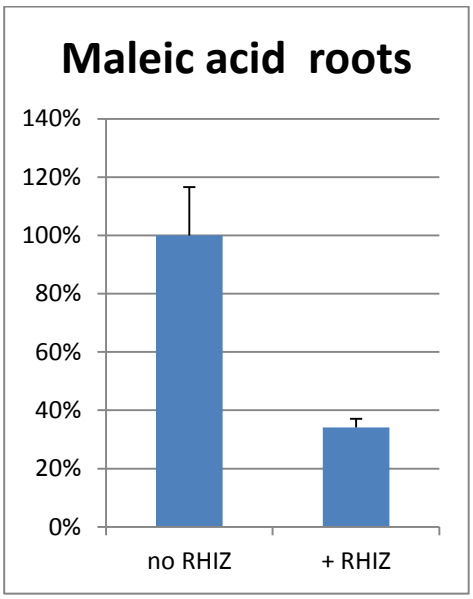
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	122	315	10	40
Change	100%	259%	9%	33%

Serine / 200 mM NaCl **T-Test = 0.02**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	164	296	29	24
Change	100%	180%	18%	15%

Table 27 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 200 mM NaCl





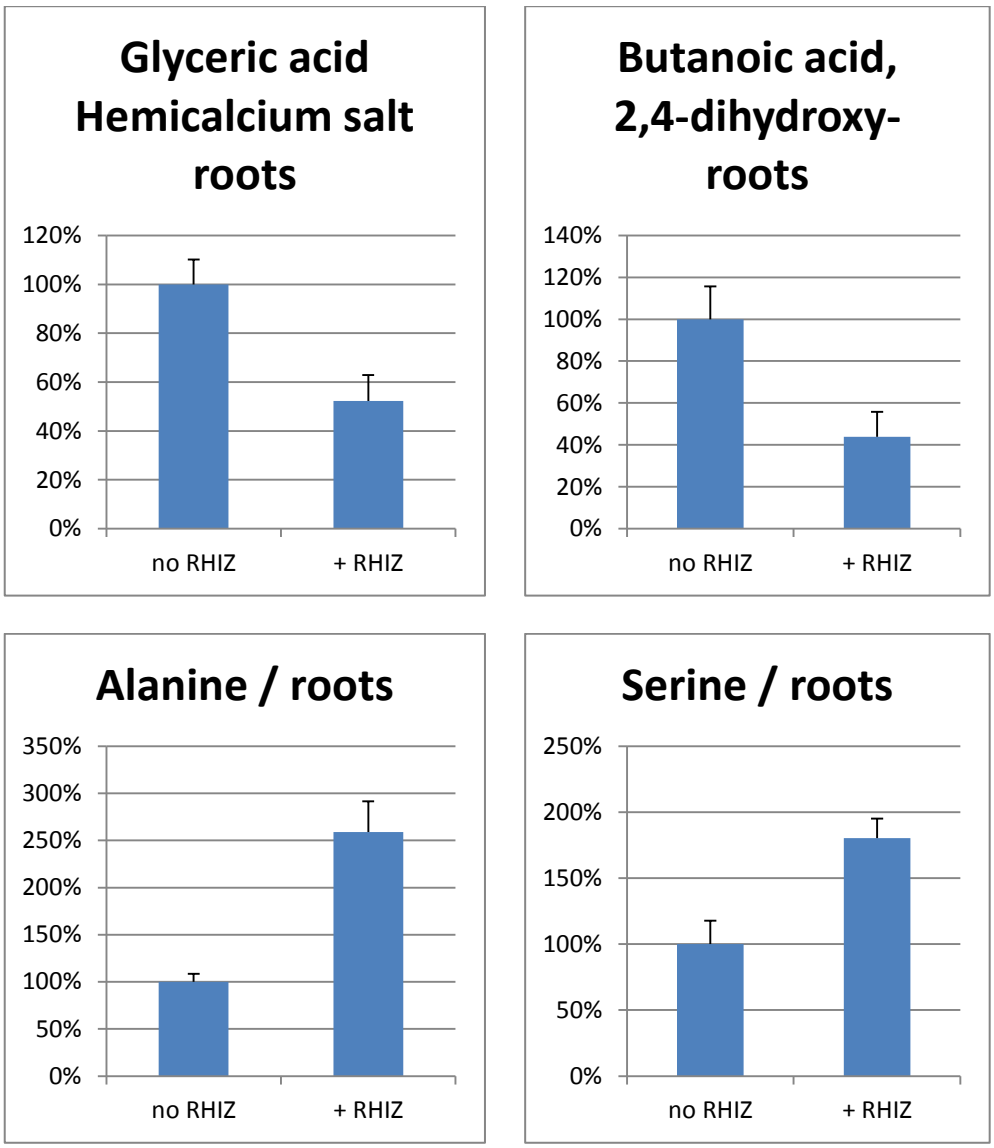


Figure 23 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated with 200 mM NaCl

3.2.4 Plants – 2 days without water

M. truncatula - 2 days dry	effect
Shoots	
Butanoic acid, 2,4-dihydroxy-	⇩
Fumaric acid	⇧
Roots	
Glyceric acid Hemicalcium salt	⇩
Pyroglutamic acid	⇩
Arabinose	⇩
Tagatose	⇩
2-Oxoglutaric acid	⇩
Succinic acid	⇩

Table 28 Overview of significant metabolite changes of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated without water

Shoots

Butanoic acid, 2,4-dihydroxy- / dry **T-Test = 0.02**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	819	640	355	350
Change	100%	78%	43%	43%

Fumaric acid / dry **T-Test = 0.05**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	357	469	184	198
Change	100%	131%	51%	55%

Table 29 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated without water

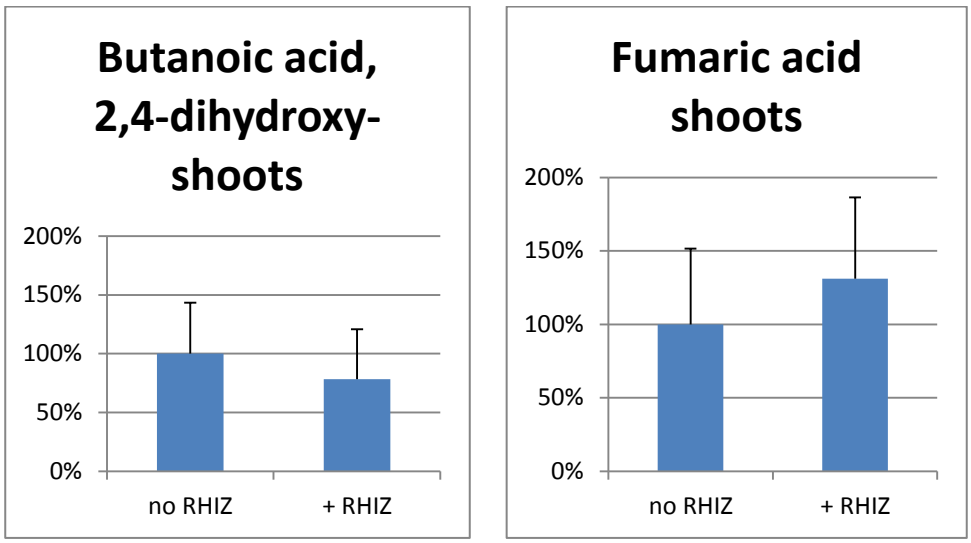


Figure 24 Significant metabolite changes in shoots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated without water

Roots

Glyceric acid Hemicalcium salt / dry **T-Test = 0.02**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	298	92	71	84
Change	100%	31%	24%	28%

Pyroglutamic acid / dry **T-Test = 0.04**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	1714	839	231	249
Change	100%	49%	13%	15%

Arabinose / dry **T-Test = 0.02**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	838	247	56	89
Change	100%	29%	7%	11%

Tagatose / dry **T-Test = 0.04**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Reference value	83	32	16	4
Change	100%	38%	19%	5%

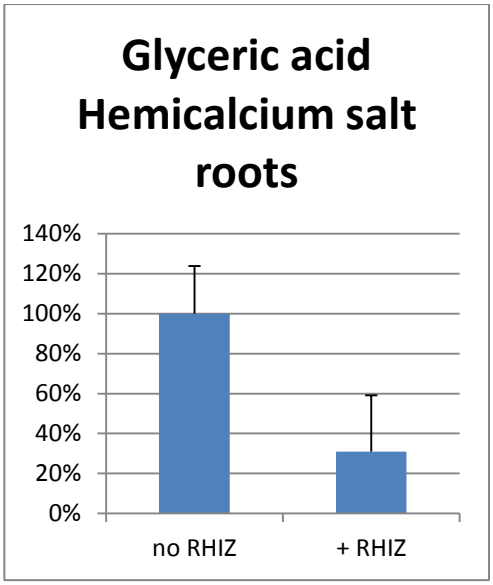
2-Oxoglutaric acid / dry **T-Test = 0.05**

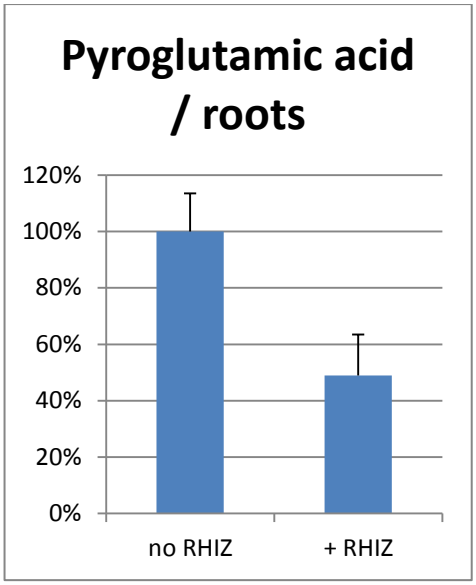
	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	167	68	26	23
Change	100%	41%	15%	14%

Succinic acid / dry **T-Test = 0.02**

	Mean		Standard Deviation	
	no RHIZOBIUM	+ RHIZOBIUM	no RHIZOBIUM	+ RHIZOBIUM
Conc [nmol/l]	365	129	85	38
Change	100%	35%	23%	10%

Table 30 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated without water





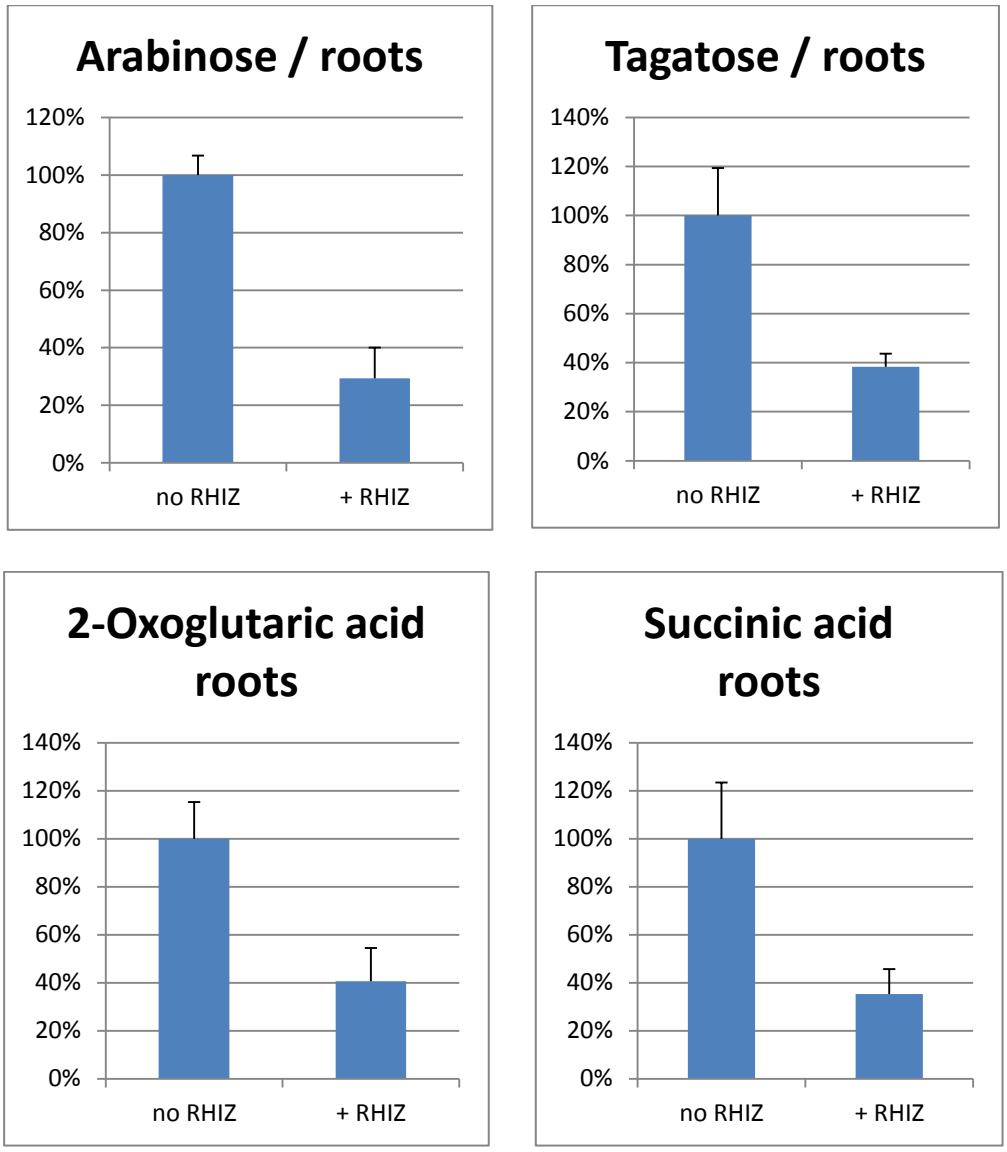


Figure 25 Significant metabolite changes in roots of *M. truncatula* with RHIZOBIUM symbionts in comparison to plants without symbionts 2 days treated without water

4 Discussion

4.1 Abiotic stress induces stronger response in roots than shoots

Independent of the kind of stress induced (salt or drought) and also independent of the strategy of nitrogen assimilation (nitrogen-fixing or nitrogen fertilization), much stronger response is observed in roots than in shoots after 2 days stress treatment (**Table 3-5**, **Table 8**, **Table 11** and **Table 14**). This effect is not very surprising because the roots are the first entry points into the plant and therefore applied stress has more time to establish responses. Nevertheless, this analysis proofs the hypothesis.

Additionally, comparing nitrogen-fixing (N-fix) plants with nitrogen fertilized (N-fed)

plants the major differences in metabolite concentration was also detected in the roots (**Table 22**, **Table 25**, **Table 28**).

4.2 Higher metabolite concentration in unstressed nitrogen-fixing plants

Having a look at the comparing overview table between N-fix and N-fed unstressed plants (**Table 19**) it is obvious that almost all significant metabolite concentrations are higher in N-fix plants than in N-fed plants. Furthermore, it seems that the unstressed N-fix plants show an enrichment of organic acids in the roots. One of the metabolites that have a significant higher concentration in the roots of N-fix plants is oxalic acid – it is about 5 times higher as in N-fed plants (**Table 21**, **Figure 19**). As discussed in **4.6,** oxalic acid is a suitable marker for salt stress and is decreased by increasing salt concentration.

The enrichment of organic acids in the roots could be explained by a priming effect induced by rhizobium. Priming causes a higher mineralization of organic soil substrate achieved by a symbiosis of nitrogen-fixing bacteria which lead to a higher concentration of organic acids in the roots. Interestingly, this increase of organic acids in the roots is accompanied by an increase of amino acids in the shoots.

This effect is in-line with the detection that asparagine in the shoots of unstressed N-fix plants is more than 100 times higher than in the shoots of unstressed N-fed plants (**Table 20**, **Figure 18**). Asparagine plays a key role in nitrogen transport from nodules of the roots into all other parts of the plant. Moreover, the remarkable increase of asparagine and therefore a higher availability of nitrogen in the shoots could also be the reason for the higher concentration of other amino acids.

4.3 Reaction intensity in relation to salt concentration

An interesting effect could be observed after applying salt stress of different concentration for 2 days. N-fix plants show in total 9 significant changes in metabolite concentration when applied to 50mM salt stress for 2 days. However,

only in total 7 changes could be detected if the salt-stress concentration was increased to 200mM (**Table 8**, **Table 11**).

N-fix plants	Number of significant metabolites in…		
	shoots	roots	total
Salt stress 50 mM, 2 days	4	5	9
Salt stress 200 mM, 2 days	1	6	7

Table 31 Distribution of the location of significant metabolites in relation to a specific stress applied

As seen in **Table 31** this effect is taking place in the shoots of the plants but not in the roots. For that matter, this effect was also observed in unpublished data of a research group in the department of molecular biology at the University of Vienna. The reason for this effect is still unclear. There is a hypothesis that says that the plant cannot adjust to the high intensity of salt applied and its response is hampered within the short time of 2 days.

4.4 Fertilized plants seems more resistant to salt stress

N-fed plants applied to salt stress for 2 days do not show many significant changes in the concentration of metabolites (**Table 3 & 4**). Just two significant changes in metabolite concentration were detected in N-fed plants. In detail, plants treated 2 days with 50 mM NaCl show only one metabolite (pyruvic acid) in the roots which concentration decreases about by 50% (**Figure 5**). A similar behaviour of depletion of pyruvic acid of about -40% was detected in roots of *Oryza sativa* treated with 50mM NaCl (Sanchez 1, 2008) (Zuther & al, 2007). However, N-fix plants seem to be much more sensible to salt stress – about 8 significant changes in metabolite concentration were detected (**Table 8**, **Table 11**).

N-fix plants treated with 50 mM NaCl for 2 days show a significant concentration increase for all except one metabolite (asparagine is reduced to the half). The biggest increase show 2-Piperidinecarboxylic acid which is 3.5 times higher (**Figure 9**). In contrast to the decrease of asparagine in *M. truncatula* shoots, asparagine was increased in shoot tips from grapevine*, V. vinifera cv. Cabernet* exposed to salt stress

(Cramer & al, 2007). An increase of β-alanine was also determined in *Populus euphratica* (Brosche & al, 2005). Within their natural habitat *P. euphratica* have been long-term acclimated to the environment and typically exposed to environmental salt stress. A MapMan plot (**Figure 15**) was created to show the position of the significant metabolites within the metabolite pathway together with their respective concentration change.

Summarized, N-fed plants show almost no reaction to 2 days applied salt stress. This leads to the conclusion that N-fed plants can handle salt stress for 2 days much better than N-fix plants.

4.5 TCA metabolites in nitrogen-fixing plants during salt stress

The concentration of 2-oxoglutaric acid as well as succinic acid is increased in the roots of N-fix plants by about 65% when treated 2 days with 200 mM NaCl. Different studies show a decrease of 2-oxoglutaric acid as a result of salt stress in both roots and shoots of *O. sative (Sanchez 1, 2008) (Zuther & al, 2007)*. Furthermore, in contrast to the detected increase after 2 days salt treatment of about 65%, the concentration of 2-oxoglutaric acid was reduced by 70% after 6 days treatment in comparison to the control group *(Staudinger, 2012)*. The same goes for succinic acid where after two days the concentration increases of about 65% (=this study) but decreases to the half after 6 days treatment in the roots of *M. truncatula* with nitrogen-fixing rhizobium bacteria *(Staudinger, 2012)*. Again, this time for succinic acid salt stress lead to a decrease of this metabolite in both shoots and roots of *O. sative (Sanchez 1, 2008)*.

Metabolite concentration changes after treated with 200 mM NaCl			
nitrogen assimilation	**in roots of *M. truncatula*** nitrogen-fixing		**in roots and shoots of *O. sativa*** fertilized
treatment period	2 days	6 days	3 weeks
2-oxoglutaric acid	+65% ⇧	-70% ⇩	⇩
succinic acid	+65% ⇧	-50% ⇩	⇩

Table 32 Comparison of TCA metabolite changes according to salt stress

In shoot tips from grapevine, *V. vinifera cv. Cabernet* there was also a decrease of succinic acid detected applying salt stress *(Cramer & al, 2007)*. Nevertheless, succinic acid concentration was increased in *T. halophila* after applying a short-term salt shock of 150 mM NaCl *(Gong & al, 2005)*.

The MapMan plot shows the discussed metabolites in an overview picture of the metabolite pathway (**Table 18**, **Figure 17**).

4.6 Oxalic acid – a suitable marker for salt stress

Oxalic acid exhibited conserved reduction of pool sizes in response to salt-treatment in *Arabidopsis thaliana*, *Lotus japonicus* and *Oryza sativa* (Sanchez 1, 2008). Focusing on the roots of *M. truncatula*, oxalic acid is decreasing by 40% in plants with nitrogen-fixing rhizobium symbionts treated 2 days with 50 mM NaCl (**Table 10**, **Figure 10**), and further decreasing by 60% in plants treated with 200 mM NaCl (**Table 13**, **Figure 12**). Therefore, there might be a negative correlation between salt concentration and concentration of oxalic acid.

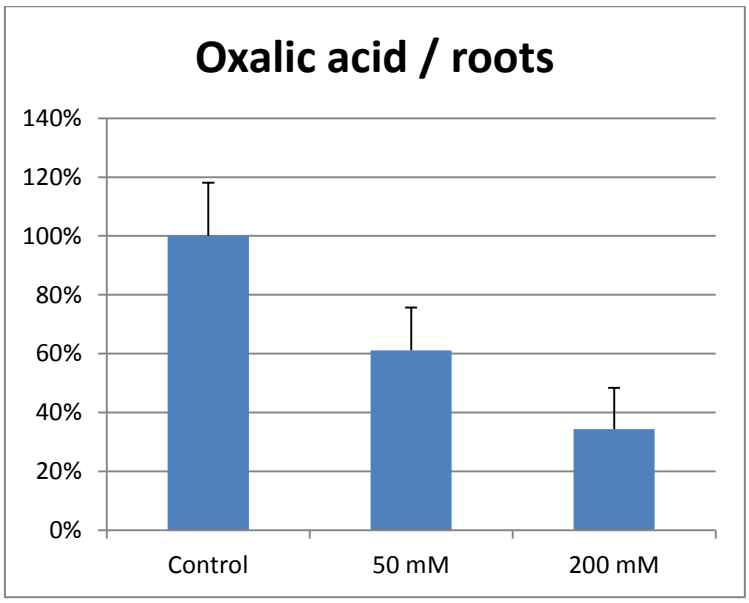


Figure 26 Oxalic acid concentration change in roots of plants with nitrogen-fixing symbionts treated with 50 mM NaCl and 200 mM NaCl

Thus, it seems that oxalic acid is a conserved marker for salt concentration not only in N-fix *Medicago* but also in other plant species. In N-fed plants, there were no significant changes of oxalic acid detected. This underlines the hypothesis that N-fed plants are not stressed after 2 days.

4.7 Influenced metabolic pathways due to drought and salt stress

The responses to salt and drought stress behave differently in N-fix plants and N-fed plants.

As mentioned before in *4.4 Fertilized plants seems more resistant to salt stress* N-fed plants show almost no significant concentration change with salt stress (**Table 3 & 4**).

However, the same plant shows in total 7 significant changes when applied to drought stress (**Table 5**). Therewith, the detected significant metabolites are not assigned to a single metabolic pathway but belong to TCA cycle, amino acids and to sugars.

As a conclusion, N-fed plants can handle salt stress much better than drought stress.

Interestingly, for N-fix plants the results look differently:

N-fix plants	Number of significant metabolites that belong to…		
	TCA	amino acids	sugars
Salt stress 50 mM, 2 days	2	3	0
Salt stress 200 mM, 2 days	2	3	0
Drought stress, 2 days	0	1	4

Table 33 Distribution of occurred metabolite types in relation to a specific stress applied

There are not only responses to drought stress as in N-fed plants but also to salt stress in N-fix plants (**Table 33**). Moreover, the stress response differs remarkably between drought and salt stress in N-fix plants and can be assigned to certain pathways or substance groups. While salt-stress seems to induce metabolites from TCA cycle and amino acid substance group, drought stress induces mostly sugars but also one metabolite that belongs to the amino acid substance group.

4.8 Difference in the drought stress response in fertilized and nitrogen-fixing plants

It is remarkable that in N-fed plants all significant changes of metabolite concentrations results in an increase of concentration during drought stress (in comparison to unstressed plants) not only in the shoots but also in the roots (**Table 5**). In contrast to that, almost all significantly changed metabolite concentrations are decreasing in N-fix plants but only in the roots (**Table 14**).

In a direct comparison of identical metabolites between N-fix plants versus N-fed plants exposed to drought stress for 2 days, an obvious decrease of metabolite concentrations in N-fix plants can be detected (**Table 28**).

Comparison of the results of similar drought stress studies

The following two tables (Table 34 and Table 35) show a comparison between different studies of different N-fed plants that were exposed to drought stress.

- *Fertilized plants*

Metabolite concentration changes as a result to drought stress				
species	*M. truncatula*		*L. japonicus*	*A. thaliana & T. halophila*
nitrogen assimilation	N-fed	N-fed	N-fed	N-fed
(Author of) study	this study	Staudinger	Sanchez	Gong
treatment period (+additional treatment)	2 days	6 days	3 weeks	drought: long - term +salt stress of 150 mM NaCl
Arabitol	+230% ⇧S		⇧ S	
Aspartic acid	+250% ⇧R		⇩ S	⇩ S
Glutamic acid	+250% ⇧ R	+200% ⇧ R	⇩ S	
Succinic acid	+200% ⇧ R		⇧ S	⇧ S

Table 34 Comparison of metabolite changes in different plants or different stress duration caused by drought stress. S depicts concentration change of metabolite detected in shoots; R depicts concentration change of metabolite in roots.

Lotus japonicus was long-term exposed (three weeks) to drought stress (Sanchez, 2012). In a different study, *Arabidopsis thaliana* und *Thellungiella halophile* were also long-term exposed to drought stress but additionally short-term exposed to salt stress of 150 mM NaCl (Gong & al, 2005). Again, the MapMan plot gives an overview of the mentioned metabolites in the metabolite pathway (**Table 17**, **Figure 16**). The investigation of N-fed *Medicago truncatula* and drought stress applied for 6 days was done by Staudinger (Staudinger, 2012).

This table shows that it is very difficult to compare the results of different studies due to different conditions. Nevertheless, the increase of succinic acid seems a common response to drought stress. Specific for *Medicago truncatula*, the increase of glutamic acid in the roots was still present after 6 days.

- *Nitrogen-fixing plants*

The following table shows a comparison between different studies of different N-fix and N-fed plants that were exposed to drought stress.

Metabolite concentration changes as a result to drought stress					
species	*M. truncatula*		*L. japonicus*	*P. euphratica*	*O. sativa*
nitrogen assimilation	N-fix	N-fix	N-fed	N-fed	N-fed
(Author of) study	this study	Staudinger	Sanchez	Brosche	Sanchez 1
treatment period (+additional treatment)	2 days	6 days NO drought but 200 mM salt	3 weeks	NO drought but long-term salt stress	NO drought stress but +100mM salt stress
Glycerol	+75% ⇧R			⇧S	
Arabitol	-60% ⇩R		⇧S		
Adonitol	-80% ⇩R				
Tagatose	-50% ⇩R				
Valine	-50% ⇩R	+250% ⇧S	⇔S		⇩R

Table 35 Comparison of metabolite changes in different plants or different stress duration mainly caused by drought stress. S depicts concentration change of metabolite detected in shoots; R depicts concentration change of metabolite in roots.

Populus euphratica was grown in its natural habitat and had a long-time exposure to salt but not to drought stress (Brosche & al, 2005). *Lotus japonicus* was long-term exposed (three weeks) to drought stress (Sanchez, 2012). *Oryza sativa* was exposed to 100 mM NaCl salt stress (Sanchez 1, 2008). *Medicago truncatula* with nitrogen-fixing rhizobium symbionts were treated 6 days with 200 mM NaCl (Staudinger, 2012).

Due to the fact that most similar studies did not investigate N-fix plants, the comparison is much more difficult. Again focusing of *Medicago truncatula*, valine is interestingly decreased by 50% after 2 days drought stress but after 6 days it is increased by 250%.

4.9 Metabolite change tendencies in fertilized and nitrogen-fixing plants

The following table summarizes the significant metabolite changes when comparing N-fix plants against N-fed plants both with the same treatment.

Number and tendency of metabolite changes in N-fix versus N-fed				
treatment	unstressed	50mM	200mM	drought
Metabolite # / trend	11⇧ 2⇩	5⇧ 1⇩	3⇧ 5⇩	1⇧ 7⇩

Table 36 The table shows the number of metabolite changes together with their tendency (= metabolite increase or decrease) In direct comparison between N-fix plants and N-fed plants in relation to their treatment. E.g. 11⇧ 2⇩ means 11 metabolite concentrations are increased and 2 are decreased in comparison to N-fed plants

The difference between N-fix and N-fed plants is smaller when any kind of stress (salt or drought) is applied. Without stress, most significant metabolite concentrations are higher in N-fix plants. However, applying intensive stress as 200mM salt stress or drought stress for 2 days, significant metabolite concentrations in N-fix plants are mostly lower as in N-fed plants.

5 Summary

Unstressed nitrogen-fixing plants of *Medicago truncatula* show higher metabolite concentrations as fertilized plants of the same species. However, applying intensive stress as 200mM salt stress or drought stress for 2 days, significant metabolite concentrations in nitrogen-fixing plants are mostly lower as in fertilized plants.

Furthermore, abiotic stress induces stronger response in roots than in shoots independent of the treatment and independent if the plant was fertilized or nitrogen-fixed.

Additionally, it was detected that fertilized plants can handle salt stress for 2 days much better than nitrogen-fixing plants. In detail, fertilized plants show almost no reaction to 2 days applied salt stress. In the same context, salt stress for 2 days increases the concentration of 2 metabolites of the TCA cycle in the roots of nitrogen-fixing plants however a decrease of these metabolites were detected in a different study after applying 6 days salt stress.

It was also shown, that less salt stress (50 mM NaCl) for 2 days induces a higher response than a more intensive salt stress (200 mM NaCl). Thereby, the difference of

the number of significantly changed metabolites is located in the shoots and not in the roots. Furthermore, oxalic acid was found as a suitable marker for salt stress.

Moreover, fertilized plants can handle salt stress much better than drought stress. For nitrogen-fixing plants salt stress mainly influences amino acids and metabolites of the TCA cycle whereupon drought stress mainly influences sugars.

It is remarkable that after 2 days of drought stress all significant changes results in an increase of metabolite concentrations, not only in the shoots but also in the roots of fertilized plants. However, under the same conditions nitrogen-fixing plants react with a decrease of significant metabolite concentrations.

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8 Attachments

8.1 List of figures

8.2 List of tables

8.3 List of abbreviations

FW.....Fresh weight
DW.....Dry weight
QC.....External standard that contains about 40 metabolites with known concentration
GC.....Gas Chromatography
MS.....Mass Spectrometry
TOF-MS.....Time-Of-Flight-Mass Spectrometry
MSTFA.....n-methyl-n-dimethylsilyltrifluoroacetamide
PCA.....Principle component analysis
UV.....Ultra violet
VIS.....visible light
IR.....infra-red
ESR.....electron spin resonance
NMR.....nuclear magnetic resonance
TCA.....tricarboxylic acid (= citric acid)
N-fix.....nitrogen fixing
N-fed.....nitrogen fertilized

8.4 Zusammenfassung

In ungestressten Pflanzen von *Medicago truncatula* zeigen die Stickstoff-fixierenden Pflanzen höhere Metabolitkonzentrationen auf als die gedüngten Pflanzen. Wenn man nun 2 Tage lang hohen Salzstress von 200 mM NaCl oder Trockenstress appliziert, dann weisen Stickstoff-fixierende Pflanzen meist geringere Metabolitkonzentrationen als gedüngten Pflanzen auf. Weiters konnte festgestellt werden, dass abiotischer Stress in den Wurzeln stärkere Reaktionen hervorruft als im Spross, und dies unabhängig, ob die Pflanze Stickstoff-fixierende Bakterien besitzt oder nicht.
Zusätzlich konnte herausgefunden werden, dass gedüngte Pflanzen mit 2 Tage langem Salzstress besser umgehen können als Stickstoff-fixierende; bei gedüngten Pflanzen sieht man fast keine signifikanten Metabolitkonzentrationsänderungen. Im selben Zusammenhang konnte gezeigt werden, dass die Einwirkdauer des Salzstresses ausschlaggebend für Metabolitkonzentrationsänderungen ist: Konkret, bei der Untersuchung der Wurzeln von Stickstoff-fixierenden Pflanzen wurde in dieser Studie gezeigt, dass nach 2 Tagen Salzstress die Konzentration zweier Metabolite des TCA- Zyklus gestiegen sind, hingegen in einer anderen Studie, bei der 6 Tage lange Salzstress appliziert wurde die Konzentrationen derselben Metabolite aber gesunken sind.
Interessanterweise weisen Pflanzen, die 2 Tage lang einem geringeren Salzstress ausgesetzt waren, eine stärkere Reaktion auf als Pflanzen, die höherem Salzstress ausgesetzt waren. Dabei befinden sich die signifikanten Metabolitkonzentrationsänderungen in den Sprossen und nicht in den Wurzeln.
Weiters konnte Oxalsäure als geeigneter Marker für Salzstress identifiziert werden.
Gedüngte Pflanzen können mit Salzstress besser umgehen als mit Trockenstress. Bei Stickstoff-fixierenden Pflanzen ist es so, dass Salzstress hauptsächlich Aminosäuren und Metabolite des TCA-Zyklus beeinflussen, wogegen Trockenstress hauptsächlich Veränderungen von Zuckerkonzentrationen bedingt.
Eine weitere Auffälligkeit ist, dass in gedüngten Pflanzen nach 2 Tagen Trockenstress die Metabolitkonzentrationen in Wurzeln als auch im Spross erhöht sind, hingegen in Stickstoff-fixierenden Pflanzen bei gleicher Behandlung die entsprechenden Konzentrationen abgenommen haben.

Metabolite	Shoots / N-fertilized				Shoots / N-fixing			
	Control	50 mM NaCl	200 mM NaCl	Drought	Control	50 mM NaCl	200 mM NaCl	Drought
2-Oxoglutaric acid	102	424	227	339	140	214	246	352
Alanine	155	446	350	616	400	569	1429	497
Asparagine	147	798	786	3125	15517	9156	22148	18627
Aspartic acid	451	3130	1783	3229	659	1275	1003	2643
Fumaric acid	131	349	381	357	312	337	519	469
Glutamic acid	3162	16347	12660	19786	3256	6805	4957	11646
Glycine	18	58	41	53	56	55	97	81
Leucine	205	808	962	1989	60	212	102	612
Methionine	56	194	219	214	54	89	85	163
Phenylalanine	26	383	402	636	74	130	125	328
Proline	270	3105	1774	2336	208	768	673	1378
Putrescine	4	7	4	12	11	9	11	14
Serine	130	458	353	532	384	461	905	670
Succinic acid	294	841	841	888	234	463	576	618
Threonine	1358	5908	5937	9898	120	2359	279	4584
Valine	210	881	1129	2176	111	357	203	790

Metabolite concentration [nmol / g fresh weight]

Metabolite	Roots / N-fertilized				Roots / N-fixing			
	Control	50 mM NaCl	200 mM NaCl	Drought	Control	50 mM NaCl	200 mM NaCl	Drought
2-Oxoglutaric acid	80	168	96	167	71	151	119	68
Alanine	102	235	122	269	166	436	315	179
Asparagine	583	1856	507	4955	3426	7756	5992	3623
Aspartic acid	152	176	231	393	151	711	350	118
Fumaric acid	106	255	171	219	133	251	132	98
Glutamic acid	367	467	527	947	431	2227	1076	469
Glycine	27	104	53	82	56	81	64	42
Leucine	144	132	168	424	54	268	57	27
Methionine	53	132	91	123	63	125	68	37
Phenylalanine	59	133	91	136	57	124	70	34
Proline	175	167	293	708	114	941	260	89
Putrescine	5	14	4	14	9	14	8	7
Serine	127	335	164	438	188	400	296	154
Succinic acid	167	304	269	365	140	395	238	129
Threonine	1262	245	1413	2439	113	2298	145	232
Valine	189	184	229	502	93	492	83	48

Metabolite concentration [nmol / g fresh weight]

L e b e n s l a u f

Persönliche Daten

Name:	Dr. Thomas Kolber
Geburtsdatum:	21.12.1970 in Wien
Familienstand:	ledig
Staatsbürgerschaft:	Österreich

Ausbildung

1985 - 1990	HTL in Mödling - Abteilung Nachrichtentechnik und Elektronik
06/1990	Matura mit gutem Erfolg absolviert
10/1990 – 03/1997	Studium der Technischen Chemie/Fachrichtung Biochemie an der Technischen Universität in Wien
02/1998 – 06/2000	Doktoratsstudium an der Technischen Universität in Wien Thema: Methodenentwicklung zur Analyse superharter Schichtsysteme
10/2012 – laufend	Lehramtsstudium: Chemie und Biologie an der Technischen Universität/Universität Wien

Grundwehrdienst / Auslandseinsatz

06/1997 – 01/1998	ABC-Abwehrschule in Wien, eingesetzt als militärwissenschaftlicher Experte (MilwEx)
07/1998 – 11/1998	*Chemiewaffen-Inspektor* sowie Netzwerkbetreuer bei UNSCOM (United Nations Special Commission) im Irak

Berufspraxis

	Hewlett-Packard Ges.m.b.H., Wien
2000 - 2002	Netzwerktechnik-Consultant
2002 - 2012	Internationaler IT-Programmanager
2010 - 2012	Teamleiter Netzwerkconsulting Gruppe (6 Consultants)

Weitere Tätigkeiten

2013 – laufend	Führungen im Botanischen Garten für Schulklassen
2014 – laufend	Betreuer im chemischen Mitmachlabor der TU für Schulklassen
2015 – laufend	Ausbildung zum Tutor am Vienna Open Lab (molekularbiologisches Mitmachlabor)