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"Hints of rhizobia induced alleviation of tungsten stress in *Glycine max* via increased levels of primary and secondary metabolites"

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Abstract

The coordination chemistries of the transition metal tungsten (W) and the essential plant micro nutrient molybdenum (Mo) are similar in structural and functional aspects; for this reason, W is proposed to inhibit enzymatic activity of molybdoenzymes such as nitrate reductase, by replacing Mo in the molybdopterin co-factor (Mo-Co). This would severely effect N-assimilation, causing N-deficiency. A few studies suggest that another enzyme, the nitrogenase of symbiotically plant living rhizobacteria, which presents a different Fe-Mo co-factor for N_2 fixation, can retain its functionality after W exposure. Moreover, there is some evidence that roots and nodules of symbiotically grown leguminous plants exhibit higher levels of proteins involved in hormone and flavonoid biosynthesis, compounds involved in stress regulation, in the presence of high concentrations of W. The aim of my thesis was to develop an integrative analytical method for comprehensive primary and secondary metabolite detection in order to clarify if and how symbiotic N_2 fixation changes metabolite abundances during tungsten exposure, compared to non-symbiotic plants and if this relates to enhanced tolerance against heavy metal toxicity.

Soybean plants inoculated with *Bradyrhizobium japonicum* (Nfix) and a non-symbiotic control supplied with nitrate (Nfed – 10 mM KNO_3) were grown in a semi hydroponic setup. After three weeks, when symbiosis was fully established, in order to investigate the W and Mo specific response, plants were exposed to W (0.5 mM Na_2WO_4) or high Mo (0.5 mM Na_2MoO_4) for two weeks and subsequently harvested for metabolomic analysis applying a novel sequential extraction procedure.

My results showed that Nfix plants exhibit a stronger metabolic response compared to their non-symbiotic counterparts in presence of both W and Mo. While a decrease in biomass was observed in both Nfix and Nfed treatments when exposed to tungsten, symbiotically grown soy beans were

able to retain shoot growth, healthier roots, and nodule mass/count. I found an increase in phenolic compounds, flavonoids, and soluble sugars in Nfix roots and leaves exposed to tungsten which resulted in a higher antioxidant capacity in comparison to Nfed plants. Furthermore, I could show an increase in organic acids, polyamines (i.e. putrescine, spermidine) and amino acids (i.e. Proline, Alanine) in Nfix plants in response to 0.5 mM W. Plants exposed to molybdenum retained their biomass to control level, however, roots of Nfix plants exhibited higher values in amino acids, soluble sugars, phenols and flavonoids. My results suggest a symbiont induced alleviation of tungsten stress via enhanced osmo-protective, radical-scavenging and metal-chelating capacity.

Abstrakt

Die Koordinationschemie des Übergangsmetalls Wolfram (W) und des essentiellen pflanzlichen Mikronährstoffs Molybdän (Mo) sind in struktureller und funktioneller Hinsicht ähnlich. Aus diesem Grund liegt es nahe, daß W die enzymatische Aktivität von Molybdoenzymen wie der pflanzlichen Nitratreduktase hemmt, indem Mo im Molybdopterin-Co-Faktor (Mo-Co) ersetzt wird. Dies würde die N-Assimilation stark beeinträchtigen und einen N-Mangel bei Pflanzen verursachen. Einige Studien legen nahe, dass ein anderes Enzym, die Nitrogenase von symbiotisch lebenden Wurzelbakterien, die einen Fe-Mo-Co-Faktor für die N_2-Fixierung aufweist, ihre Funktionalität nach W-Exposition beibehalten kann, da sie von einer Symbiosomenmembran geschütz werden. Das würde die N-Versorgung der Pflanzen sichern. Darüber hinaus gibt es Hinweise darauf, dass Wurzeln und Knöllchen von symbiotisch gewachsenen Hülsenfrüchten in Gegenwart hoher Konzentrationen von W einen höheren Anteil an Proteinen aufweisen, die an der Hormon- und Flavonoid-Biosynthese beteiligt sind, also Verbindungen, die an der Stressregulation beteiligt sind. Um zu klären, ob und wie die symbiotische N_2-Fixierung die Metabolitenhäufigkeit während der W-Exposition im Vergleich zu nicht-symbiotischen Pflanzen verändert und ob dies mit einer erhöhten Toleranz gegenüber Schwermetalltoxizität zusammenhängt, habe ich eine integrative Analysemethode (Sequentielle Extraktion) zum Nachweis von Primär- und Sekundärmetaboliten ausgearbeited und angewendet.

Mit *Bradyrhizobium japonicum* (Nfix) oder Nitrat (Nfed; 10 mM KNO_3) vertilisierte Sojabohnenpflanzen wurden in einem halbhydroponischen Aufbau angezogen. Drei Wochen nach vollständiger Ausbildung der Symbiose, wurden die Pflanzen zur Untersuchung der W- und Mo-spezifischen Reaktion zwei Wochen lang hohen Konzentrationen an W (0,5 mM Na_2WO_4) oder Mo (0,5 mM Na_2MoO_4) ausgesetzt und anschließend zur metabolischen Analyse geerntet.

Meine Ergebnisse zeigten, dass Nfix- im Vergleich zu Nfed-Pflanzen, sowohl in Gegenwart von W als auch Mo eine stärkere metabolische Reaktion aufwiesen. Während bei beiden, Nfix- und Nfed-Pflanzen, nach W-Verabreichung eine Abnahme der Sproßbiomasse beobachtet wurde, wiesen symbiotisch gewachsene Sojabohnen eine höhere Wurzel- und Knöllchenbiomasse / -zahl auf. Ich fand zudem eine Zunahme von Phenolverbindungen, Flavonoiden und löslichen Zuckern in Nfix-Wurzeln und Blättern, was auf eine höhere Antioxidationskapazität im Vergleich zu Nfed-Pflanzen hindeutet. Darüber hinaus konnte ich in Nfix-Pflanzen einen Anstieg von organischen Säuren, Polyaminen (z.B. Putrescin, Spermidin) und Aminosäuren (z.B. Prolin, Alanin) als Reaktion auf 0,5 mM W nachweisen. Im Vergleich dazu behielten Molybdän-exponierte Pflanzen ihre Biomasse auf Kontrollniveau. Dennoch zeigten Wurzeln von Nfix-Pflanzen hier höhere Werte in Aminosäuren, löslichen Zuckern, Phenolen und Flavonoiden. Meine Ergebnisse deuten auf eine Linderung des Wolframstresses durch verbesserten Osmoseschutz, sowie erhöhte radikalfangende und metallchelatisierende Kapazität bei symbiotischen Pflanzen hin.

1. Introduction

Heavy metals are important environmental pollutants, especially in areas strongly impacted by anthropogenic activities. Their presence in the soil can be deleterious for plants, therefore it is necessary to understand how plants are affected by these pollutants and how they cope with their presence.

Tungsten (W) is a rare transition element that belongs in the Group VI-B of the periodic table of elements, along with chromium, molybdenum and seaborgium. It presents unique physical and chemical properties, making it suitable for a wide range of industrial, civil and military applications (Koutsospyros et al., 2006). Due to its widespread use, tungsten has become increasingly present in environmental systems, however, knowledge of its toxicological effects in organisms is relatively limited compared with other heavy metals.

Tungsten is the twin element of the essential micronutrient molybdenum (Mo). They represent the only bio elements in their respective transition row and they share similar coordination chemistries in structural and functional aspects. The majority of lifeforms, including bacteria and eukarya, utilize molybdenum enzymes for essential bioprocesses. The enzymes nitrate reductase and nitrogenase possess a molybdopterin molybdenum cofactor (Moco) (McMaster and Enemark, 1998) and an iron-molybdenum cofactor (FeMoco) (Allen et al., 1994) respectively, where molybdenum participates as a transition metal in the catalytic center. (Mendel and Hansch, 2002). Aside from nitrate reductase, other plant enzymes interact with Moco: xanthine dehydrogenase, aldehyde oxidase and sulfite oxidase. The only life forms that do not require molybdenum enzymes use tungsten instead, although the occurrence of tungsten enzymes appears to be restricted to archaea and some bacteria (Bevers et al., 2009). Eukaryotes have not

developed a functional use of tungsten; on the contrary, its intake may affect their physiological processes.

Cells acquire molybdenum as the highly soluble oxoanion molybdate (MoO_4^{2-}), which they internalize by means of an active transmembrane importer for subsequent conversion into the enzyme cofactor Moco and FeMoco in nitrogen fixers (Hagen, 2011). Molybdate and tungstate (WO_4^{2-}) are very similar in geometry and charge, to the extent that in E.coli it has been observed that ABC (adenosine triphosphate binding cassette) transporter family proteins with a molybdate-specific binding protein (ModA) bind molybdate and tungstate with a similar affinity and do not discriminate between the two oxyanions (Imperial et al., 1998). Thus, after entering the cytoplasm in form of tungstate, tungsten is likely to take similar pathways as molybdenum in eukaryotes and bacteria (Bevers et al., 2009).

Once in the cytoplasm, tungsten antagonizes effectively and may substitute molybdenum in the molybdenum cofactor (MoCo) of the aforementioned molybdoenzymes (Xiong et al., 2012); but in spite of the similarity in both the atomic radii and chemistry, the interchange of these metals between proteins usually yields inactive forms of the enzymes (Deng et al., 1989; McMaster and Enemark, 1998; Bevers et al., 2009; Xiong et al., 2012).

There are studies confirming that, in nitrate reductase, when molybdenum is replaced with tungsten in Moco, the activity of the enzyme is inhibited but not its production (Harper and Nicholas, 1978; Deng et al., 1989; Zimmer and Mendel, 1999; Bevers et al., 2009, Preiner et al., 2019). Interestingly, a few studies showed that nitrogenase of symbiotic rhizobacteria, which presents a different Fe-Mo co-factor for N_2 fixation, can retain its functionality after tungsten exposure (Kletzin and Adams, 1996; Schwarz et al., 2007; Bevers et al., 2009). However, there is

also some evidence that tungsten may negatively affect the fixation activity of N_2 fixing bacteria (Siemann et al., 2003).

It is clear that molybdenum is central to nitrogen metabolism, and its substitution with tungsten is likely to affect it. Nitrogen is a component of proteins, amino acids, lipids, pyrrole rings of chlorophyll and it is involved in the central intermediate metabolism with carbon metabolism and photorespiration. It represents by far the most critical nutrient for plant development and growth; in fact, nitrogen deficiency in plants is known to cause a decrease in photosynthetic structural compounds, such as chlorophyll and ribulose bisphosphate carboxylase (rubisco), which leads to a loss of photosynthetic capacity and carboxylation efficiency (Delgado et al., 1994).

In addition to its impact on the functionality of molybdoenzymes, tungsten presents toxic attributes similar with those of other heavy metals. These include hindering of seedling growth, reduction of root and shoot biomass, ultrastructural malformations of cell components, aberration of cell cycle, disruption of the cytoskeleton, and deregulation of gene expression related with programmed cell death (PCD) (Adamakis et al., 2008; Adamakis et al., 2011, Adamakis et al., 2012).

To the best of my knowledge, no investigations about potential molybdenum toxicity in plants have been carried out; however, in vitro data show that, under some conditions, it can participate in the Fenton reaction that results in the production of harmful radical oxygen species (Popivker, Zilbermann, Maimon, Cohen, & Meyerstein, 2013).

I wanted to assess if the metabolic response of non-symbiotically and symbiotically grown plants exposed to the same concentrations of tungsten and molybdenum differed. Soybean (*Glycine max*. Primus), due to the symbiosis with *Bradyrhizobium japonicum*, was chosen as a model for

this study. Moreover it has been observed that symbiotically grown soybeans exhibit enhanced flavonoid biosynthesis (chalcone synthase, chalcone isomerase, dihydroflavonol 4-reductase and isoflavone reductase) (Preiner et al., 2019), which are known to exert a radical-scavenging activity, beneficial in case of heavy metal-induced oxidative stress. Therefore, I wanted to elucidate if nitrogen fixation may provide a specific metabolic response that would result in enhanced tolerance against molybdenum and tungsten toxicity. In order to do that, I adapted and further developed a sequential extraction protocol (Shankar L. Laware 2015) in order to analyze primary and secondary metabolite levels from the same sample extract by means of photometry and GC-MS analyses.

2. Materials and methods

2.1 Chemicals

All chemicals, if not stated otherwise, come from Sigma Aldrich.

2.2 Plant Culture and Sampling

The plants were germinated and handled according to Preiner, Wienkoop, Weckwerth, & Oburger, (2019), with few changes. Glycine max cv. Primus obtained from Die SAAT Austria seeds were soaked in diluted industrial Radicin inoculant (*B. japonicum*). In order to test the effects of different nitrogen regimes (nitrogen fertilization, nitrogen fixation) on tungsten and molybdenum uptake by the plants, control, 0.5 mM tungsten (Na_2WO_4) and 0.5 mM molybdenum (Na_2MoO_4) solutions were administered for 14 days when plants reached 3 weeks, at fully established symbiosis. One half of the plants (Nfed) was supplied with 10 mM KNO_3 from week two onwards, the other half (Nfix) received 0.25 mM KNO_3 for 2 weeks and then were only watered with N-free nutrient solution. For each treatment 8 biological replicates were prepared. The plants were grown under controlled conditions with a 12/12 light/dark cycle at 29/21◦C and an approximate photosynthetic radiation of 400 µmol m−1s−1 at canopy level. The plants were harvested at 5 weeks, the shoots and the roots were separated and weighted, the nodules where removed and counted, when present. One half of the plants was put to exsiccate, the other half was rapidly frozen with LN_2 and stored at -80°C.

Metal treatment (for 14 days)	Nitrogen regime: Nfed (10 mM KNO_3)	Nitrogen regime: Nfix (0.25 mM KNO_3 (for two weeks))
Control	**CN**	**CZ**
Tungsten (0.5 mM Na_2WO_4)	**WN**	**WZ**
Molybdenum (0.5 mM Na_2MoO_4)	**MoN**	**MoZ**

Table 2.2.1 Experimental setup and treatments naming. Nfed = not fixing, N-fertilized; Nfix = nitrogen fixing.

2.3 Analytical procedures

Sequential extraction protocol

A sequential extraction protocol (Shankar L. Laware 2015) was adapted and further developed in order to analyze primary and secondary metabolite levels from the same sample extract by means of photometry and GC-MS analyses.

Step 1: Extraction of photosynthetic pigments and lipids

Approximately 50 mg of ground frozen plant material was weighted in 2 ml Eppendorf tubes. In the first step of the extraction, 1 ml of pre-chilled 80% acetone was added to the plant material, the tube was vortexed thoroughly and centrifuged at 10.000 x g for 10 minutes. The supernatant was collected in a new tube while the pellet was extracted twice in 0.5 ml of 80% acetone and centrifuged at 10.000 x g for 10 minutes. Subsequently, 200 µl of the extract were transferred in a new tube and the volume was made up to 1 ml with 80% acetone. Absorbance of diluted samples (1:5) was measured at 663, 646 and 470 nm on a spectrophotometer for estimation of pigments according to Lichtenthaler & Wellburn (1983).

Step 2: Extraction of soluble sugars, reducing sugars, phenols, flavonoids and tannins.

The pellet obtained in the first step was re-extracted in 1 ml 80% methanol, vortexed and incubated at 95°C for 30 minutes. The content was cooled on ice, centrifuged at 20.000 x g for 10 minutes and the supernatant collected in a new tube. The pellet so obtained was re-extracted in 1 ml 80% ethanol and incubated according to the previous step. The content was cooled on ice, centrifuged at 20.000 x g for 10 minutes and the supernatant was pooled with the methanol extract. The pellets were left open at room temperature for approximately ten minutes in order to let residual solvents evaporate and stored at -80°C for further analyses.

In order to perform GC measurements, 1 ml of the extract obtained in the first step and 1 ml of the one from second step were pooled in a tube, 5 µl of the internal standards PE and PGP were added and the samples were actively dried under a nitrogen flow. The dried extracts were then stored at -80°C. The extract necessary for the photometric assays was prepared pooling 1 ml of extract from the acetone phase and 1 ml of the ethanol-methanol phase. Part of it was used immediately, while the rest was stored at -20°C.

Estimation of Soluble Carbohydrates

The assay was performed according to (Hansen & Møller, 1975). Anthrone reagent was prepared daily by dissolving 2 g l^{-1} of Anthrone in 72% H_2SO_4. A mixture of 100 µl of sample extract/standard/blank and 200 µl of pre-chilled 72% H_2SO_4 solution was prepared and vortexed in a 1.5 ml tube. 400 µl of Anthrone reagent was added to each sample and subsequently vortexed and heated at 95°C for 15 minutes. In order to obtain background absorbances of the samples, method blanks were prepared reacting of 100 µl sample with 600 µl of 72% H_2SO_4 solution (without Anthrone reagent). A glucose standard curve with the following concentrations was prepared: 0 (blank), 9, 90.075, 180.15, 360.3 and 540.45 µg/ml. Following a cooling period on ice, the absorbance of two 250µl replicates at 630 nm on a spectrophotometer within 10 minutes after heating. Carbohydrates are dehydrated with concentrated H_2SO_4 to form furfural, which condenses with Anthrone to form a green color complex

Estimation of Reducing Sugars

The assay was performed according to by Dubois et al. (1951), Dubois et al. (1956), Masuko et al. (2005). 20 µl of sample extract/standard/blank were aliquoted in respective 1.5 ml tubes. 5 µl of acetic acid were added to each tube and subsequently mixed in a thermal shaker at 80°C for 5 minutes. Following heating, the tubes were centrifuged at 17.000 rpm for 10 minutes. The

samples were transferred on ice and 600 µl of sulfuric acid was added followed immediately by the addition of 200 µL of 5% phenol. The resulting mixture was incubated at 95°C for five minutes and afterwards cooled to room temperature in a water bath. A method blank was prepared adding 20 µl of sample extract to 5 µl of acetic acid and 800 µl of sulfuric acid. A glucose standard curve with the following concentrations was prepared: 0 (blank), 180.15, 360.3, 540.45, 720.6 and 900.75 µg/ml. The absorbance of two 250 µl replicates was recorded at 490nm. Simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl ethers with free or potentially free reducing groups, when treated with phenol and concentrated sulfuric acid, give an orange-yellow color that can be measured photometrically.

Estimation of total Phenols

The assay was performed according to per method given by Biju et al. (2014). 100 µl of sample extract/standard/blank were added to 700 µl of distilled water in 2 ml Eppendorf tubes. 80 µl of Folin-Ciocalteu phenol reagent was added to the extract mixture and shaken to mix. After five minutes 800 µl of 7% Na_2CO_3 solution was added and the resulting mixture was incubated for 90 minutes at room temperature. A method blank was prepared adding 100 µl of sample extract to 880 µl of distilled water. A gallic acid standard curve with the following concentrations was prepared: 0 (blank), 10, 20, 40, 60, 80 and 100 µg/ml. 250 µl of each sample, with two replicates, was loaded into microtiter plate wells and absorbance was measured at 550 nm. The total phenolic compounds content was expressed as mg Gallic acid Equivalents (GAE). This method relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds.

Estimation of total Flavonoids

The assay was performed according to Chang et al. (2002), changed (Biju et al. 2014). 200 µl of extract/standard/blank were added to 2 ml Eppendorf tubes containing 800 ml of distilled water. 60 µl of 5% $NaNO_2$ were added to the mixture and, after five minutes, 60 µl of 10% $AlCl_3$ were added. Following another five-minute wait, 400 µl of 1M NaOH were added. A method blank was prepared adding 200 µl of sample extract to 1.42 ml of distilled water. A quercetin standard curve with the following concentrations was prepared: 0 (blank), 10, 20, 40, 60 and 80 µg/ml. The solution was mixed and 250 µl of each sample were pipetted in 2 replicates in a microtiter plate and the absorbance was read at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents (QE). $AlCl_3$ forms acid stable complexes with the C4 keto groups and either the C3 or C5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. The complex is forming a pink color that is measured by the photometer.

Estimation of total condensed Tannin contents

The assay was performed according to by . For the samples, 200 µl of extracts were added to a 2 ml tube containing 750 µL of a 4% vanillin solution in methanol and 350 µl of hydrochloric acid. For the standards, instead of 200 µl of stock, 100 µl were added and the volume was made up to 200 µl. A blank method was prepared by adding 200 µl of extracts to a 2 ml tube containing 750 µL of methanol and 350 µl of hydrochloric acid. A catechin standard curve with the following concentrations was prepared: 0 (blank), 1, 2, 4, 6, 8 and 10 µg/ml. After 15 min of incubation at RT, 250 µl of the samples were pipetted in 2 replicates in a microtiter plate and the absorbance was read at 500 nm. Tannins, unlike most natural phenolic compounds, react with vanillin in acidic medium to yield a red colored product.

GC MS measurements

A QC was prepared pipetting 5, 10, 20, 30, 40, 60, 80 and 100 µl of QC stock solutions in 2 ml tubes and making up the volume to 100 µl with MQ water. A blank was also prepared. 5 µl of the internal standards PE and PGP were added and the samples were dried in a Speed-vac for approximately an hour and stored at -80°C. The dried samples obtained during the step 2 were taken out of the -80°C freezer and left to thaw at RT for 20 minutes. In the meantime, a 40 g/L methoxyaminhydrochlorid CH_3ONH_2*HCl solution was prepared using pyridine as solvent. 20 µL of methoxyaminhydrochlorid solution were added in the tubes, followed by incubation on thermoshaker at 30°C for 90 minutes. Subsequently, a solution of 30 µL of alkanes in 1 mL N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was prepared, and 80 µL of it was added in each sample. After a 30 minutes incubation on thermoshaker at 37°C, the tubes were centrifuged for 2 minutes with 14000 g. 70 µL of the supernatant were transferred to a MS vial and caps with septum were applied. The samples, the blanks and the QC were then measured with GC-MS. Most metabolites were measured in split rate 5, except sugars, pinitol, proline and asparagine that were measured in split rate 100, due to overloading of the detector in split rate 5. Peak searching, retention time indexing and peak annotation was performed using the LECO-ChromaTOF software. The metabolites were relatively quantified by means of the internal standard and normalized with the sample fresh weight.

Starch assay

The assay was performed according to Nagler et al., (2015). A pH 4.8 standard acetate buffer was prepared mixing 100 ml of acetic acid and 50 ml of NaOH and making the volume up to 500 ml with MQ water. A pH 7 Tris-glycerin buffer was prepared dissolving 6.1 g of Tris in 8.5 ml of 5 N HCl. The volume was made up to 100 ml with MQ water and 660 ml of glycerin were added. The glucoseoxidase reagent was prepared dissolving 15 mg of glucoseoxidase, 1.5 mg of peroxidase and 5 mg of o-Dianisidine hydrochloride in 50 ml pH 7 Tris-glycerin buffer. The amyloglucosidase reagent was prepared dissolving 22 mg of amyloglucosidase in 22 ml of pH 4.8 standard acetate buffer.

Dry plant material was ground to a fine powder using a Retsch mill. Nearly 10 mg of dry, ground plant material were weighted in 2 ml tubes. Subsequently, 500 µl of 80% ethanol were added and the tubes were boiled for 30 minutes at 80°C. After cooling, the samples were centrifuged at 20°C with 10.000 rpm for 10 minutes, the supernatant was transferred in a new set of tubes and the pellet was boiled for 30 minutes at 80°C in 500 µl of 80% ethanol for a second time. The samples were then re-centrifuged at 20°C with 10.000 rpm for 10 minutes. The supernatant was transferred in the new set of tubes, 500 µl of 0.5 N NaOH were added to the pellet and shaken with 1400 rpm for 15 minutes. The solubilized pellets were incubated for 30 minutes at 95°C. Following incubation, 500 µl of 1 N acetic acid were added and the samples were centrifuged with 13.000 rpm for 5 minutes. Subsequently, 300 µl of supernatant were transferred in a new tube, 300 µl of amyloglucosidase reagent were added and the samples were incubated at 55°C for 2 hours. After the incubation, 25 µl of the extracts were diluted with 175 µl MQ water, 400 µl of glucoseoxidase were added and the tubes were incubated for 1 hour at 30°C. The reaction was stopped by adding 800 µl of 5 N ice cold HCl, the absorbances were read at the wavelength

of 540 nm. A glucose standard with the following concentrations was prepared: 0 (blank), 125, 250 and 500 μM. The calibration curve was processed in the same way of the samples.

2.4 Statistical analysis

Statistical analysis of biomass and metabolites was performed with Infostat software (InfoStat, RRID:SCR_014310) (Di Rienzo et al., 2018). Biomass and metabolites data were analyzed by one-way ANOVA using DGC post hoc test.

3. Results

3.1 Biomass

A significant decrease in the shoot biomass expressed as dry weight was observed in the WZ treatment when compared to its Nfed counterpart and to all the other Nfix treatments. The MoZ treatment presented significantly higher biomass than the MoN. Root biomass shown an increase in the CZ treatment when compared to CN, but no significant differences were observed between the others; however, the W treatments showed the lowest values. Intrestingly, the WN roots appeared more oxidized and overall less healthy than the WZ ones (figure 3.1 2). No significant differences were observed between treatments in shoot length, however, the WZ plants presented higher values than the WN ones (figure 3.1 1). In root length, no significant differences were found between treatments. The exposure to the metals did not seem to affect the number of nodules grown on the Nfix plants.

Figure 3.1 1 Shoots of control (CZ and CN) , 0.5mM Mo (MoZ and MoN) and 0.5mM W (WZ and WN) Nfix and Nfed plants after 14 days of treatment .

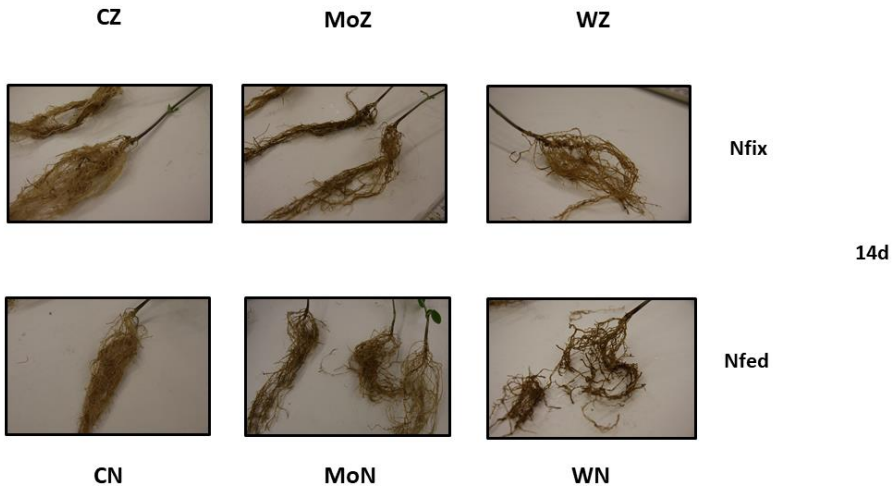


Figure 3.1 2 Roots of control (CZ and CN) , 0.5mM Mo (MoZ and MoN) and 0.5mM W (WZ and WN) Nfix and Nfed plants after 14 days of treatment .

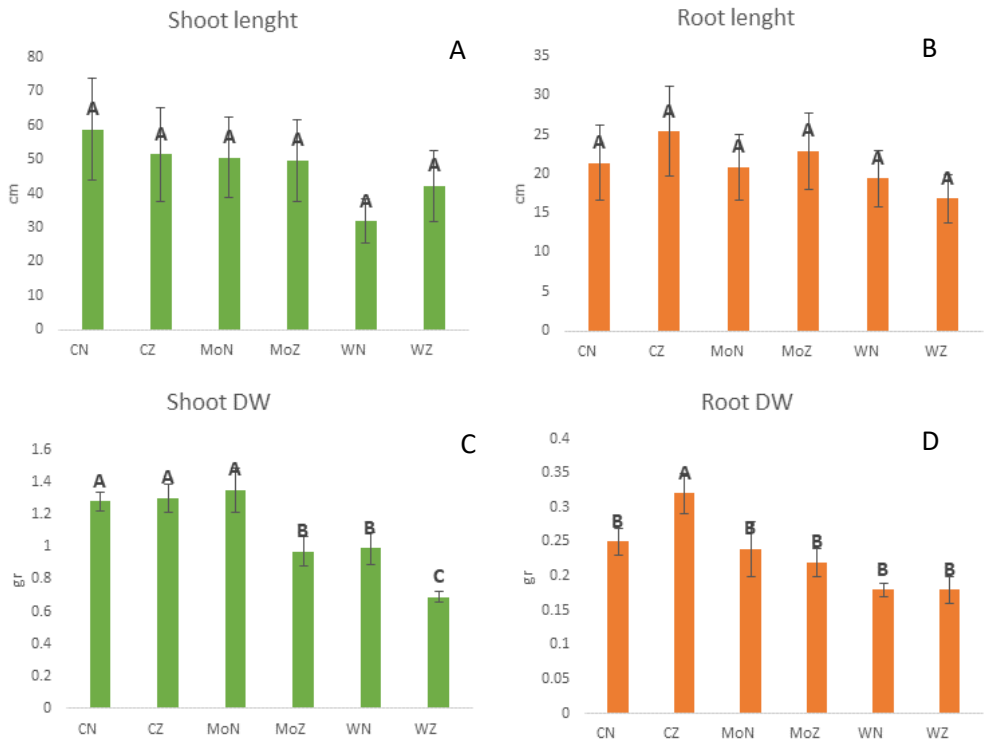


Figure 3.1 3 A Effect of the treatments on mean shoot length (±S.E.); **B** effect of the treatments on mean root length (±S.E); **C** effect of the treatments on mean shoot dry weight; **D** effect of the treatments on mean root dry weight. Letters indicate significant difference at p≤.05.

3.2 Photosynthetic pigments

No significant difference between the treatments was observed in chlorophyll a and b and carotenoid levels.

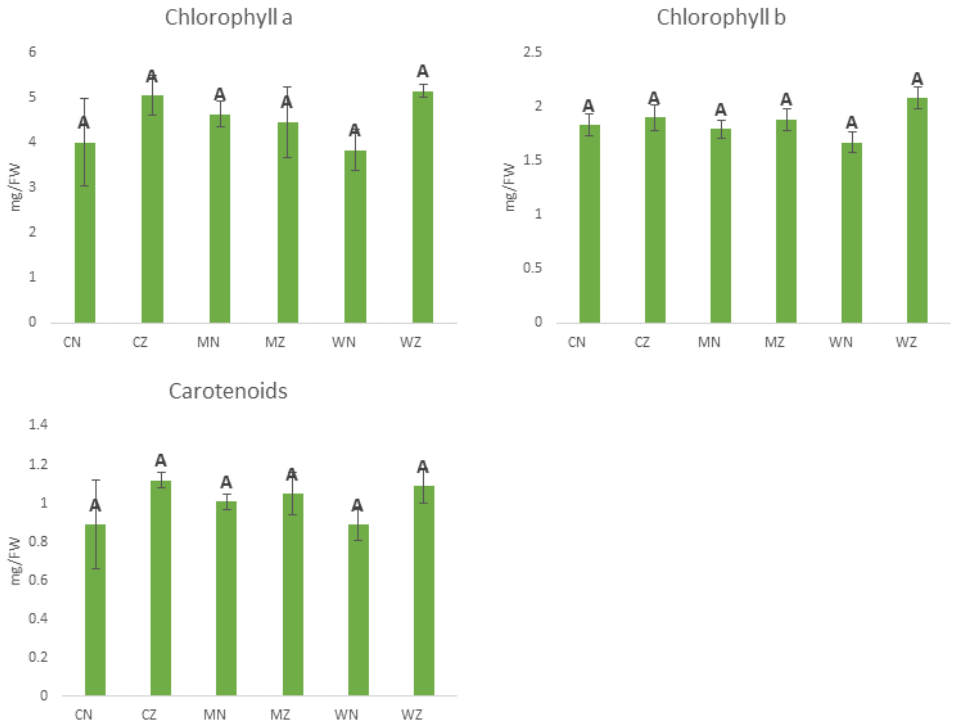


Figure 3.2 1 Effect of the treatments on chlorophyll a, b and carotenoids content (±S.E.). Letters indicate significant difference at p≤.05.

3.3 Phenols, flavonoids and tannins: photometric assays

In leaves, the WZ treatment showed a significant increase in phenols when compared to all the other treatments. Moreover, the Nfix treatments always showed slightly higher values of phenols than their Nfed counterparts. In root tissue, no significant differences between treatments were detected, although the MoZ treatment showed the highest phenol concentrations.

In leaves, the WZ treatment showed a significant increase of flavonoids when compared to all the other treatments. On the other hand, the WN treatment presented a significantly lower concentration of flavonoids than all the others. In root tissue, the MoZ treatment showed a significant increase in flavonoid concentrations when compared to all the treatments. No significant differences in tannin levels were observed between the treatments in leaf or root tissue.

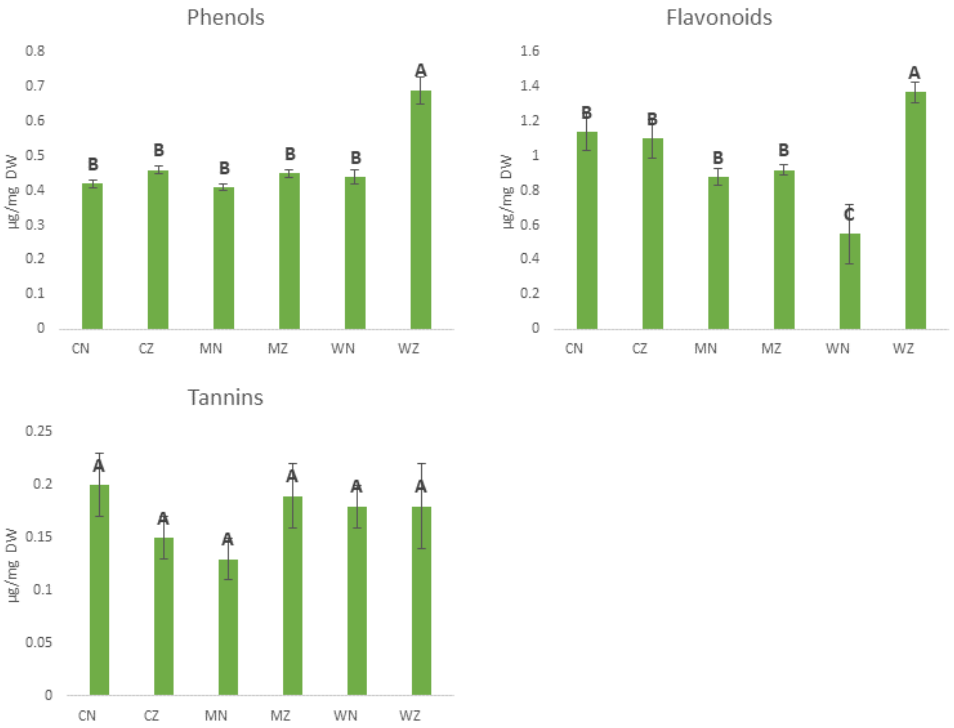


Figure 3.3 1 Effect of the treatments on phenols, flavonoids and tannins content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

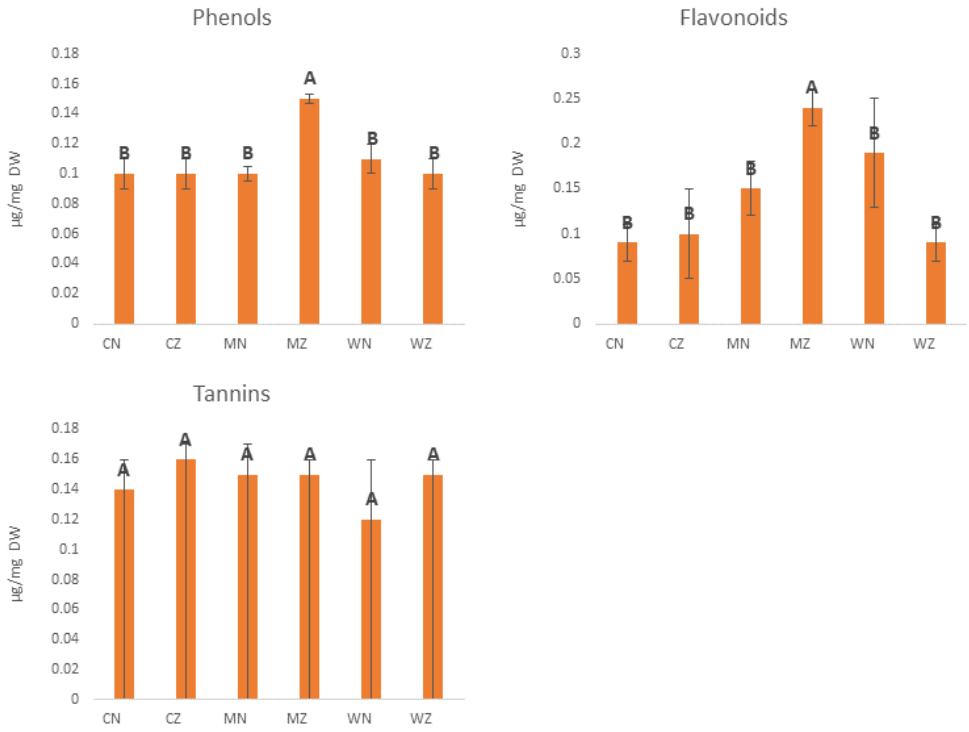


Figure 3.3 2 Effect of the treatments on phenols, flavonoids and tannins content in roots (±S.E.). Letters indicate significant difference at p≤.05.

3.4 Carbon metabolism and partitioning: soluble carbohydrates, reducing sugars and starch

Photometric assays

A significantly higher concentration of soluble carbohydrates was observed in leaves between the WZ and MoZ treatments when compared to their Nfed counterparts and to the controls. In root tissue, the MoZ treatment showed higher values when compared to all the others. In leaves, the WZ treatment showed higher concentrations of reducing sugars than all the others. No significant differences between treatments were observed in roots.

Regarding the results of the starch experiment, in leaves, the differences observed between treatments did not result to be significant, however, the WN presented the highest concentrations of starch. Similar results were obtained in the stems. All the Nfix plants exposed to W or Mo showed, overall, lower starch concentrations than the Nfed ones, exception made for the MoZ treatment in stems.

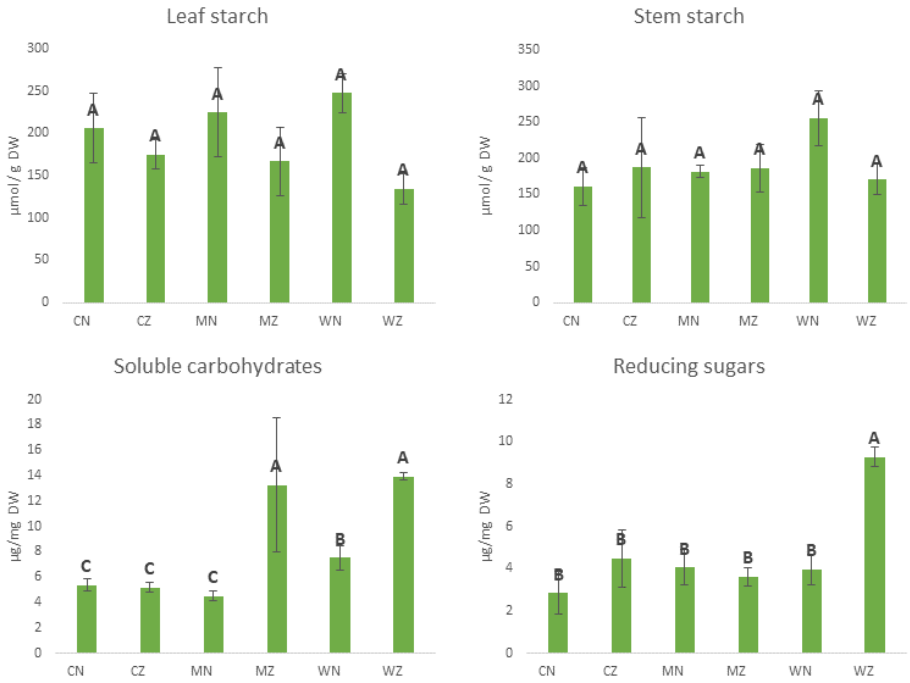


Figure 3.4 1 Effect of the treatments on leaf starch content, stem starch content, soluble carbohydrates and reducing sugars content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

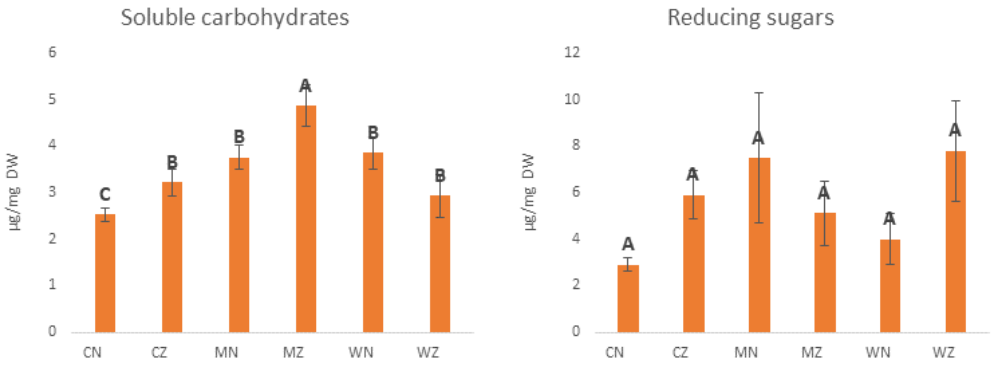


Figure 3.4 2 Effect of the treatments on soluble carbohydrates and reducing sugars content in roots (±S.E.). Letters indicate significant difference at p≤.05.

GC-MS analysis

Leaves

A total of 50 metabolites were identified. The PCA shows that the WZ treatment clearly separates from all the others through PC1, which was responsible for 40,43% of the variance, while the WN treatment separates through PC2, that contributed 21,98% to the variance. The controls and Mo treatments do not seem to be separating significantly, regardless of the nitrogen regime. In order to identify the metabolites responsible for this separation, a threshold for metabolites with the largest negative and positive loadings was set at −0.05 and 0.05. The PCA revealed that amino acids, polyamines and sugars were the metabolites that contributed most to PC1 and thus to a differentiation between the different metal treatments.

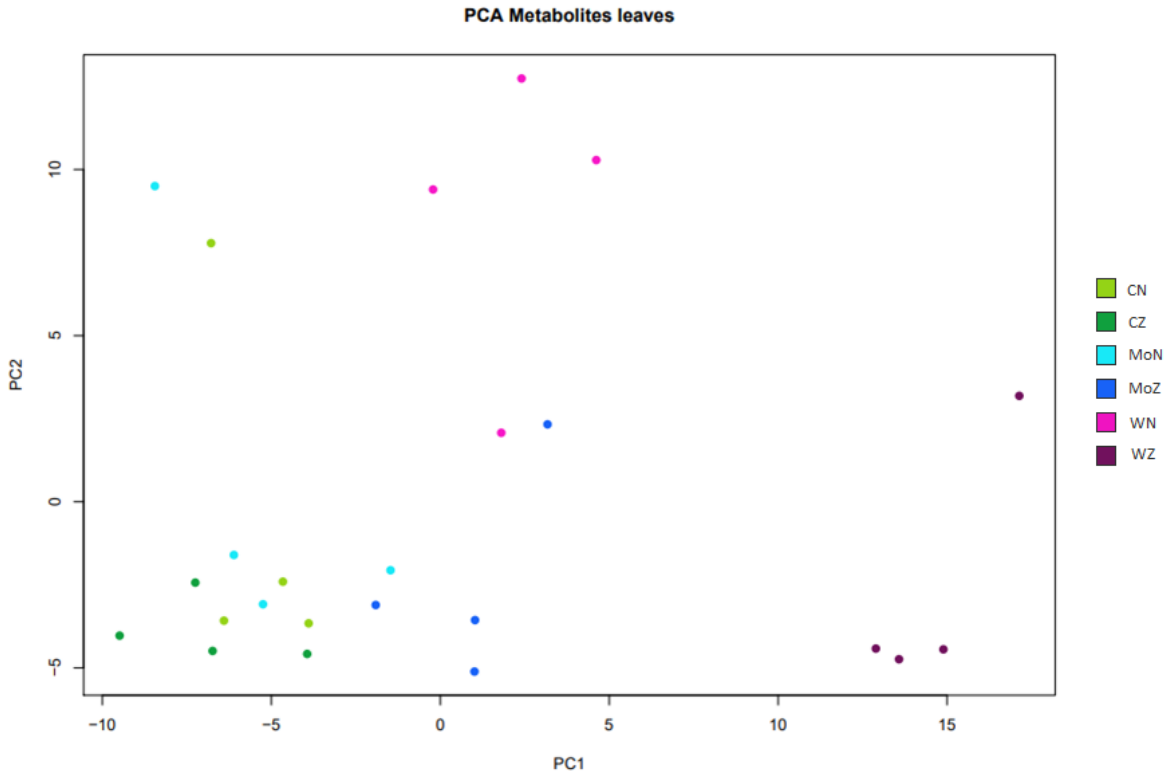


Figure 3.4 3 PCA of log-transformed significantly changed metabolites in leaves.

Metabolite	PC1
Malonic.acid	-0,14
Citric.acid	-0,1
Fumaric.acid	-0,08
Sucrose	0,05
Stigmasterol	0,05
Sitosterol beta	0,05
Threonine	0,07
Galactinol	0,08
Lysine	0,08
Gluconic.acid	0,1
Lupeol	0,1
Raffinose	0,1
Ornithine	0,12
Allantoic.acid	0,12
Phenylalanine	0,14
Asparagine	0,15
Spermidine	0,15
Glycine	0,16
Methionine	0,17
Putrescine	0,2
Tyrosine	0,22
Fructose	0,26
Leucine	0,27
Isoleucine	0,27
Valine	0,28
Proline	0,3
Galactose	0,31
Glucose	0,33

Table 3.4 1 List of the metabolites included in the threshold with the largest negative and positive loadings at −0.05 and 0.05 in leaves.

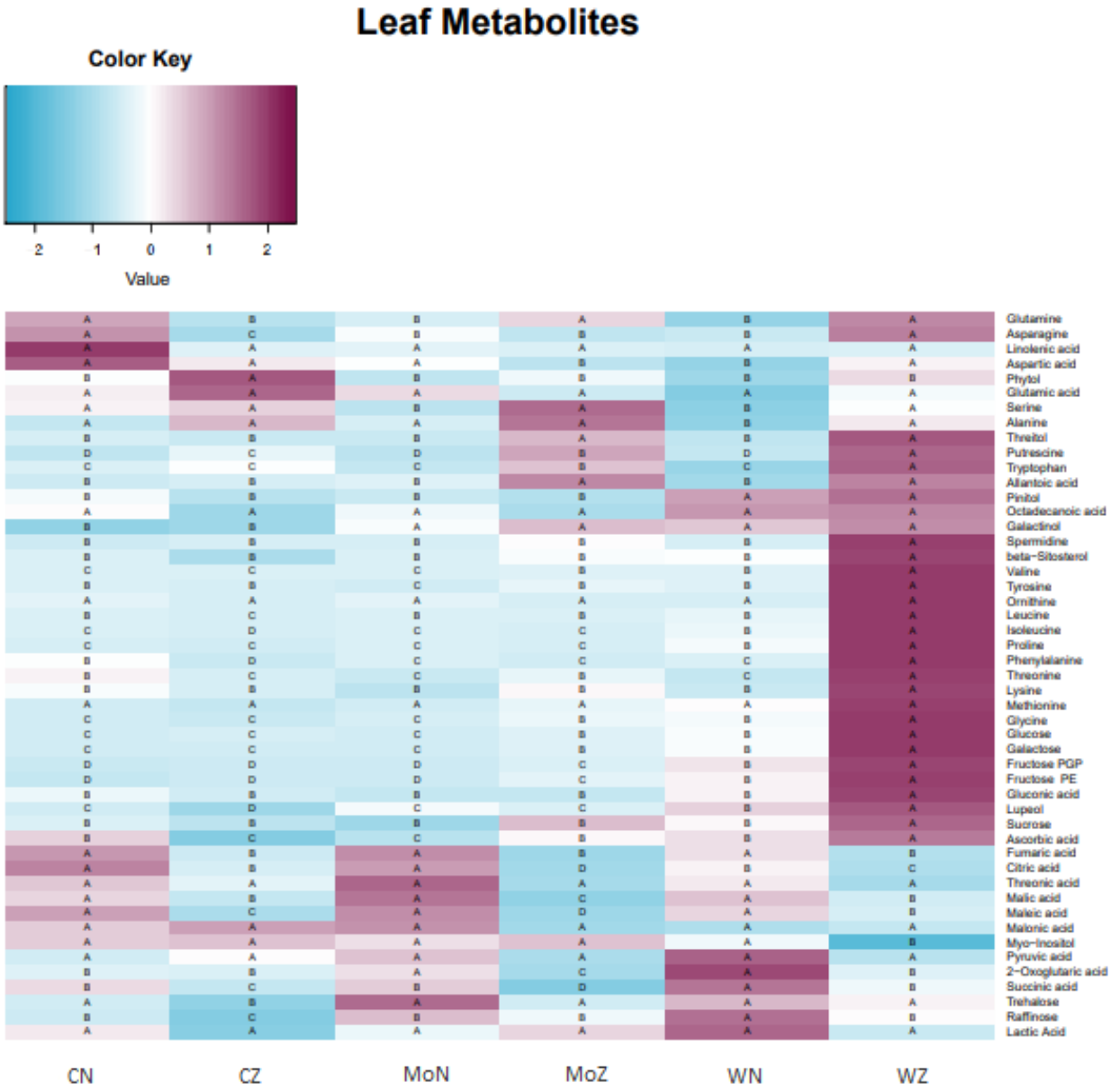


Figure 3.4 4 Heatmap of means of z-transformed metabolites concentrations in leaves. Letters indicate statistical significance (p≤.05).

Roots

49 metabolites were identified. A higher replicate variation through PC1, which was responsible for 33,43% of the variance, was observed. However, both the W treatments separate from the others through PC2, that contributed 20,23% to the variance. The controls and Mo treatments do not seem to be separating significantly, regardless of the nitrogen regime. In order to identify the metabolites responsible for this separation, a threshold for metabolites with the largest negative and positive loadings was set at -0.05 and 0.05. The PCA revealed that amino acids, polyamines and sugars were the metabolites that contributed most to PC1 and thus to a differentiation between the different metal treatments.

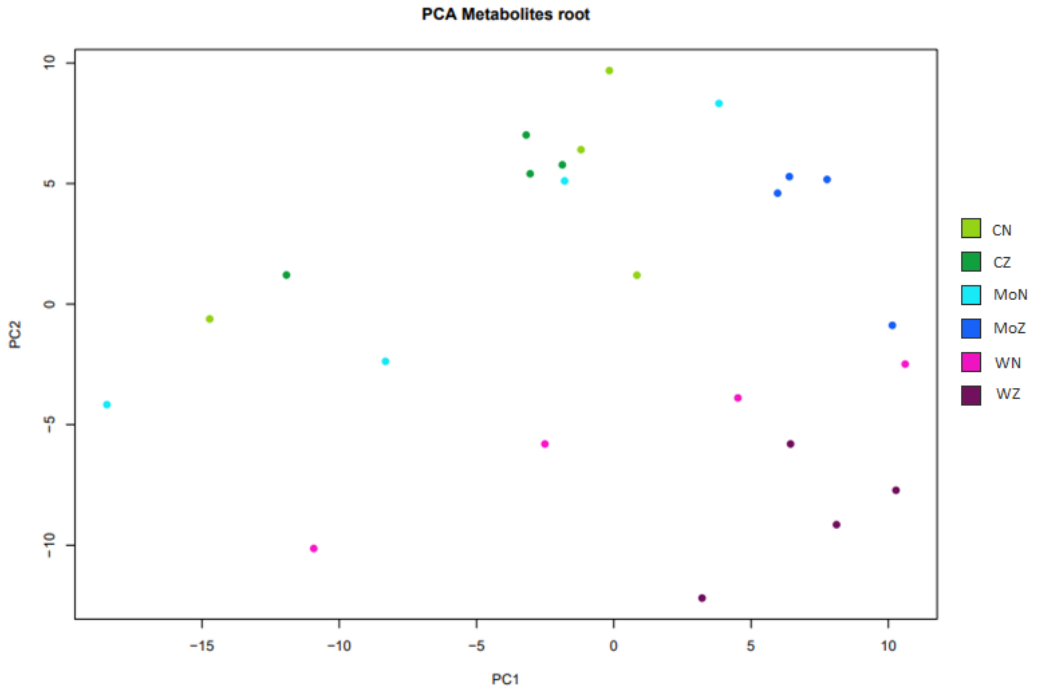


Figure 3.4 5 PCA of log-transformed significantly changed metabolites in roots.

Metabolites	PC1
Threitol	-0,06
Isoleucine	0,05
Threonine	0,05
Ornithine	0,05
Valine	0,06
Leucine	0,06
Tyrosine	0,06
Glucose	0,06
Linoleic acid	0,06
Fructose	0,07
Galactose	0,07
Malonic.acid	0,08
Pinitol	0,08
Glycine	0,09
Gluconic.acid	0,1
Oxoglutaric.acid	0,11
Ascorbic.acid	0,11
Serine	0,12
Lysine	0,13
Trehalose	0,13
Raffinose	0,14
Aspartic.acid	0,16
Puterescine	0,17
Asparagine	0,18
Methionie	0,19
Alanine	0,27
Glutamic.acid	0,27
Galactinol	0,27
Proline	0,32
Allantoic.acid	0,39
Glutamine	0,46

Table 3.4 2 List of the metabolites included in the threshold with the largest negative and positive loadings at −0.05 and 0.05 in roots.

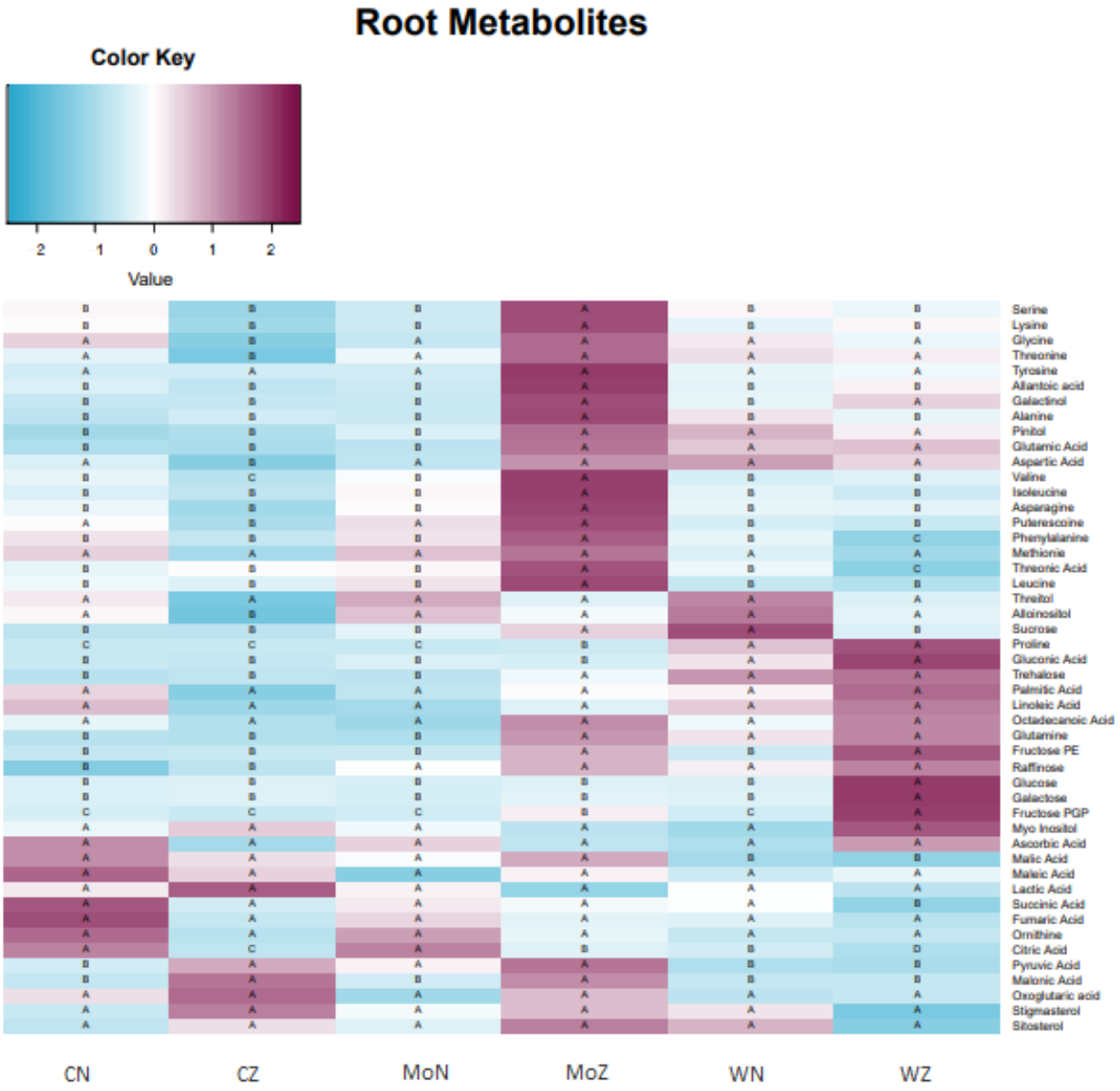


Figure 3.4 6 Heatmap of means of z-transformed metabolites concentrations in roots. Letters indicate statistical significance (p≤.05).

The data obtained from GC-MS analysis correspond very well with the results obtained in the photometric assays. The WZ treatment presented, in leaves, significantly higher concentrations of fructose, glucose, galactose and sucrose than all the other treatments. The same results were observed in roots, exception made for sucrose, which showed higher levels in the MoZ and WN treatments than in the others.

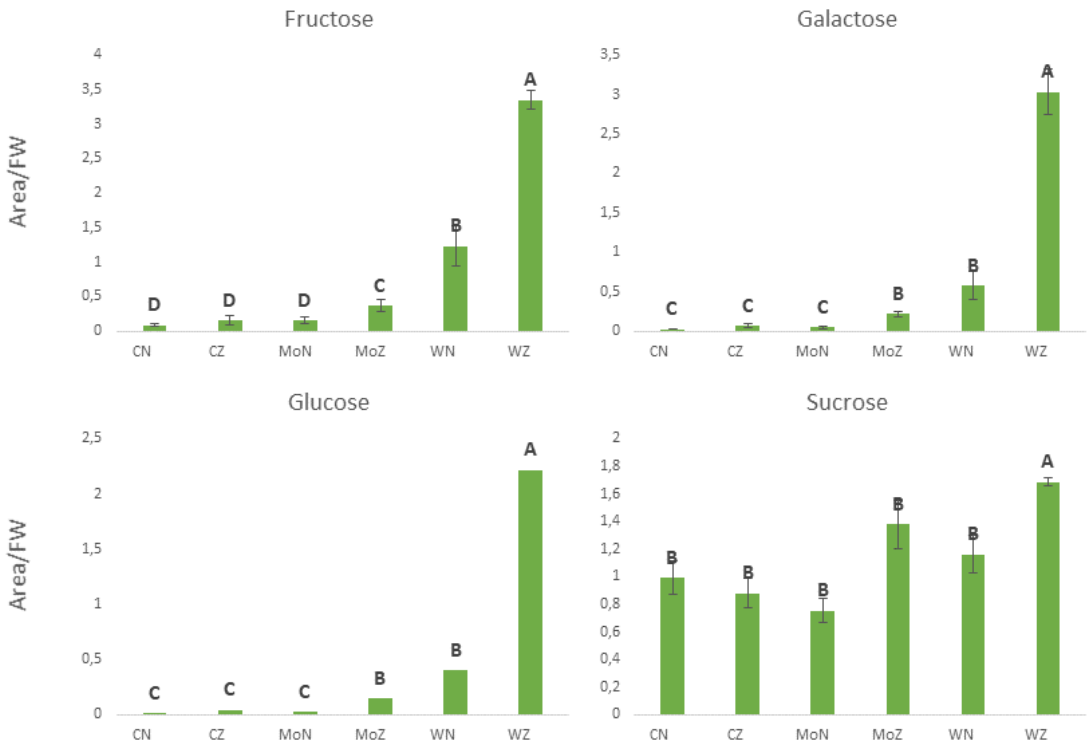


Figure 3.4 7 Effect of the treatments on fructose, galactose, glucose and sucrose content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

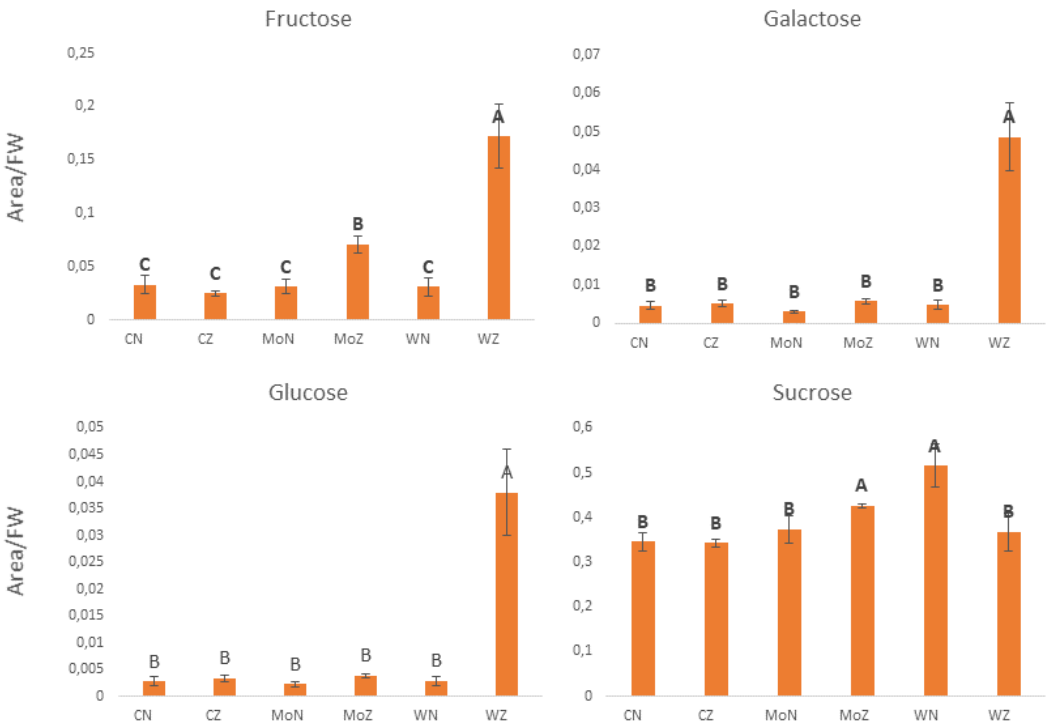
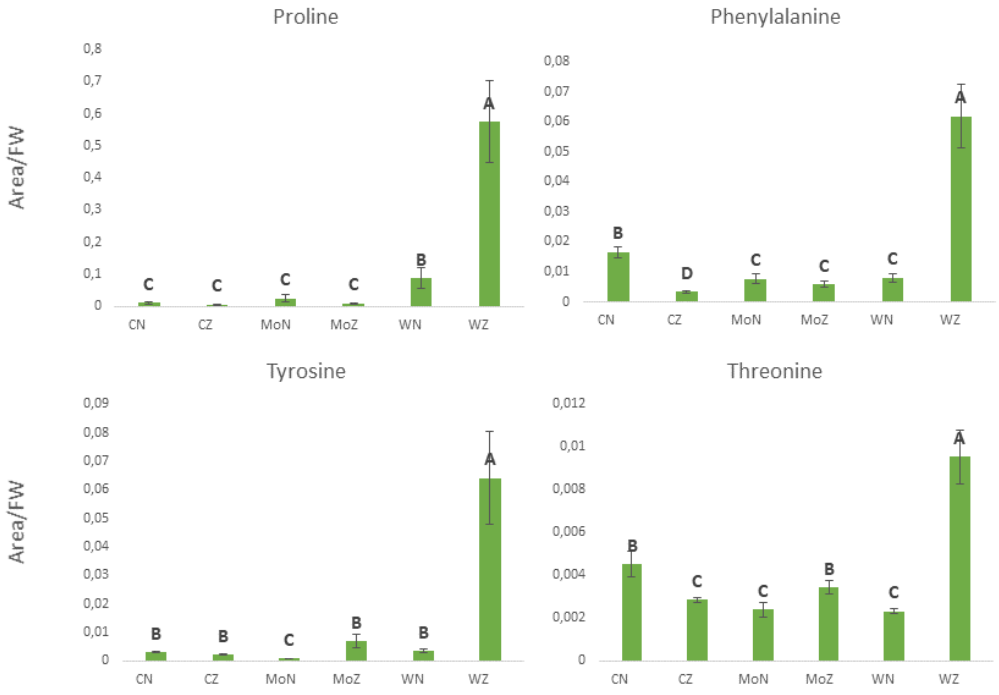


Figure 3.4 8 Effect of the treatments on fructose, galactose, glucose and sucrose content in roots (±S.E.). Letters indicate significant difference at p≤.05.

3.5 Nitrogen metabolism: amino acids and polyamines

In leaves, the amino acids valine, glycine, leucine, threonine, isoleucine, phenylalanine, proline, lysine and tryptophan presented significantly higher concentrations in the WZ treatment when compared to all the others. In comparison with the Nfix control, 119 fold-in proline, 88-fold in isoleucine, 72-fold in valine, 70-fold in leucine and 61-fold in asparagine increases were observed. The MoZ treatment presented higher concentrations of valine, glycine, serine, threonine, glutamine, tyrosine and tryptophan than its Nfed counterpart. In the WN plants, the aminoacid alanine showed a significantly lower concentration than in all the other treatments.

In roots, both the tungsten treatments showed significantly higher levels of proline than all the others, and the Nfix treatment presented significantly higher levels than the Nfed one. The MoZ treatment showed the highest levels of valine, alanine, leucine, serine, isoleucine among all the treatments. A significant increase in proline was also observed in MoZ with regards to MoN.



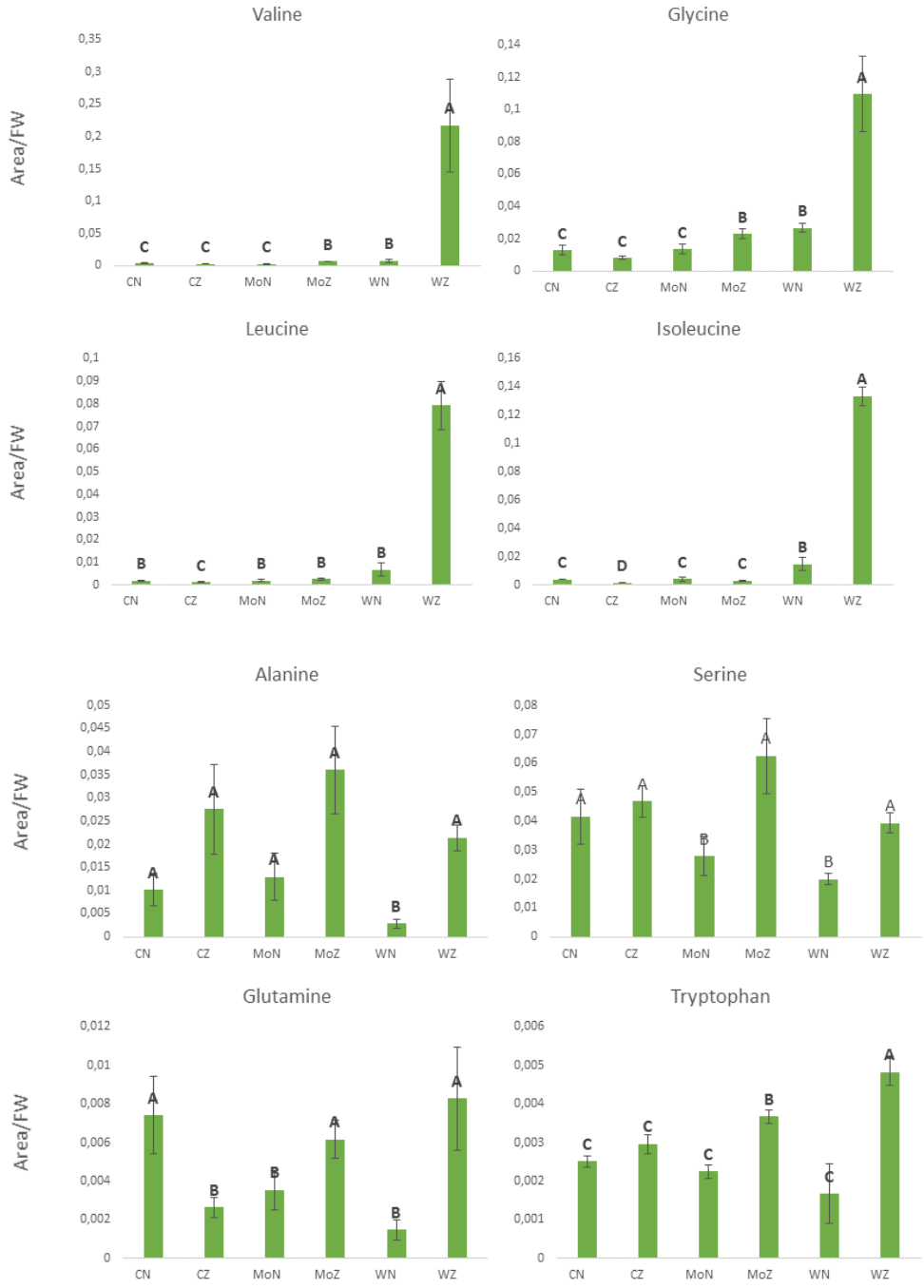


Figure 3.5 1 Effect of the treatments on amino acids content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

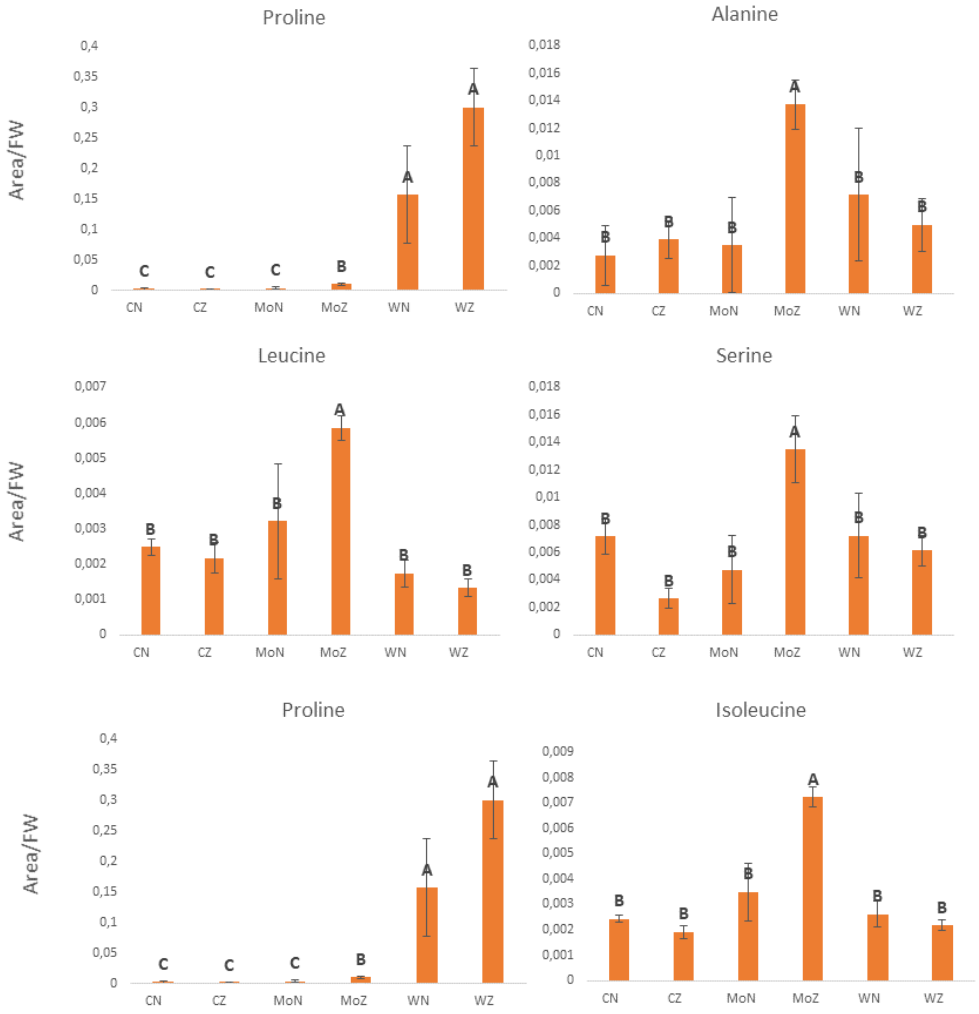


Figure 3.5 2 Effect of the treatments on amino acids content in roots (±S.E.). Letters indicate significant difference at p≤.05.

GC-MS measurements showed that in all the Nfix plants, putrescine showed significantly higher levels than in Nfed plants, and both the Nfix molybdenum and tungsten treatments showed significantly higher values than the control. In addition, spermidine levels in the WZ treatment were significantly higher than in all the other treatments, which did not present any significant difference between each other. No clear trend in putrescine levels was observed in root tissue, and peaks of spermidine were not appreciable in the vast majority of cases.

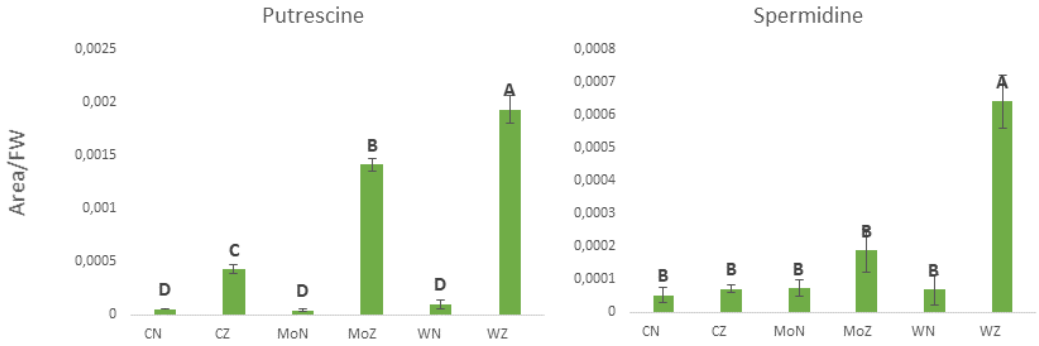


Figure 3.5 3 Effect of the treatments on putrescine and spermidine content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

3.6 Organic acids

In the context of organic acids involved in the TCA cycle, citric, malic, fumaric and succinic acid in leaf tissue of the Nfix plants showed significantly lower levels than the Nfed ones regardless of the treatment. Such a clear trend was not observed in roots, however the tungsten treatments had significantly lower concentrations of citric, malic and pyruvic acid than the others.

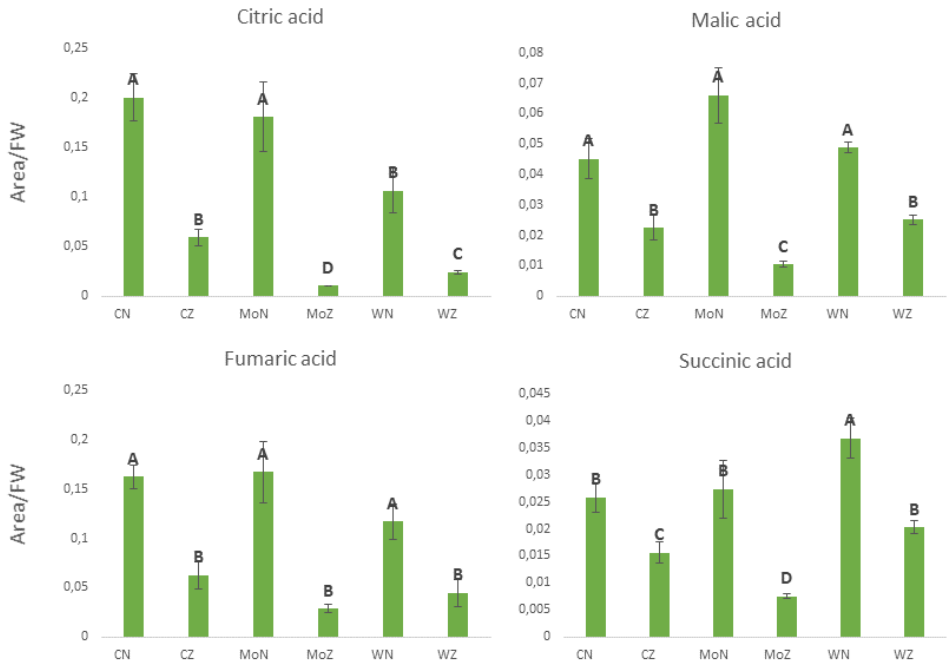


Figure 3.6 1 Effect of the treatments on citric, malic, fumaric and succinic acid content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

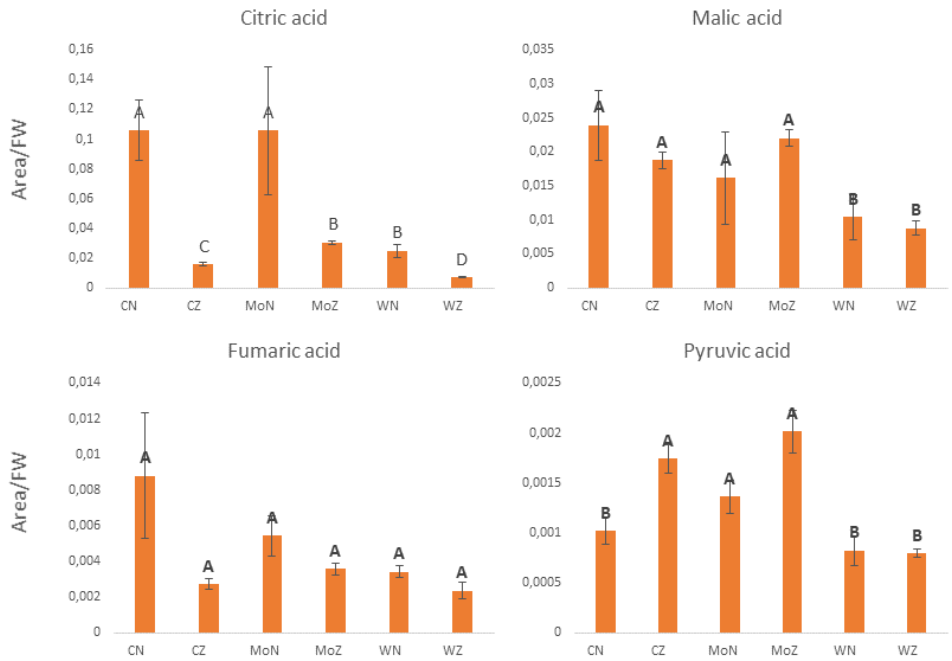


Figure 3.6 2 Effect of the treatments on citric, malic, fumaric and succinic acid content in leaves (±S.E.). Letters indicate significant difference at p≤.05.

4. Discussion

The goal of my thesis was to apply an initially developed analytical method in order to integratively investigate the metabolic effect of tungsten on Soy leaves and roots. Furthermore, I compared symbiotic (Rhizobium) with non-symbiotic plants to find out if and how this symbiosis influences primary and secondary metabolism of the plants and whether this would have an effect on the response to tungsten stress. A number of studies proved the deleterious effect of tungsten on plant biomass production (Jiang et al., 2004; Kumar et al., 2011, Adamakis et al., 2012; Kennedy et al., 2012). In particular, Adamakis et al. (2008) showed that tungstate caused inhibition of root elongation and lateral root formation, a retardation of seedling growth rate, and leaf emergence in *Pisum sativum*. In my experiment, no significant difference in shoot length was observed upon W exposure, however, in both the metal treatments (W and Mo), Nfix plants showed reduced biomass (dry weight), while Nfed plant biomass reduction was restricted to W treatment. It remains unclear, if Nfed plants kept a higher biomass because of the continually administered dose of nitrate supplied. While deleterious results in W treatments seem predictable, such effect of Mo on nitrogen fixation and biomass production does not seem as likely, because it is a micro nutrient needed by the plant. Hence it is rather possible that Mo exerted on Nfed plants a fertilizing effect in the context of nitrogen reduction and assimilation that resulted in a stable dry weight biomass. Root biomass expressed as root length and dry weight was not significantly affected by W or Mo, regardless the nitrogen regime; however, the W treatments always showed the lowest values. Overall, these results suggest that W affects negatively both nitrogen fixation and nitrogen assimilation, resulting in reduced biomass. Such an inference cannot be drawn for Mo.

The increased accumulation of phenols and flavonoids observed in leaves of the WZ (tungsten Nfix) treatment may be a consequence of the activation of the phenylalanine ammonia-lyase (PAL) enzyme, the main biosynthetic pathway of phenolic compounds. This is triggered by an increase in reactive oxygen species (ROS) concentrations, a consequence of the presence of metals. (Dai, Xiong, Huang, & Li, 2006). Due to their neighboring hydroxyl groups, phenolic compounds can act as reducing agents, hydrogen donors, and singlet oxygen (Lopes, Schulman, & Hermes-lima, 1999), hence, they can directly scavenge molecular species of active oxygen, providing an important non-enzymatic antioxidant response. Moreover, in case of heavy metal stress, phenolic compounds can act as metal chelators (Michalak, 2006). There have been many reports of induced accumulation of phenolic compounds in plants treated with high concentrations of metals (Manquián-Cerda et al., 2018; Manquián-cerda et al., 2016), those results strongly suggest that their synthesis is a protective mechanism against metal stress to avoid the deleterious effects these have on the organism. In all the treatments including the controls, the Nfix treatments presented higher phenol values than the Nfed ones, with a highly significant increase in the WZ treatment. Given the fact that the precursor for most phenolic compounds is the aromatic amino acid phenylalanine (Tzin & Galili, 2010), it can be suggested that the phenylalanine increase observed in the leaves of W-exposed Nfix plants, and in the roots of Mo exposed Nfix plants, may be connected to the increased synthesis of phenols and flavonoids observed in the respective tissues. In root tissue, the MoZ treatment showed significantly higher values than all the others in both phenols and flavonoids, suggesting that in response to molybdenum exposure, the Nfix plants can respond with production of phenols and flavonoids in the roots as a first defense against metal toxicity in a more effective way than the Nfed ones. Overall, our results correspond very well with Rauser (1978), who observed that the

content of phenolics in leaves of *Phaseolus vulgaris* exposed to heavy metals was six times greater than for roots.

A number of studies have found that flavonoids, especially anthocyanins, are produced in response to various types of stress, including metal stress (Hale et al., 2001; Winkel-shirley, 2002). In vitro studies have shown that flavonoids (e.g., quercetin, catechin, luteolin, hesperidin) can directly scavenge molecular ROS with the formation of less reactive flavonoid phenoxyl radicals (Arora et al., 1998); moreover, flavonoids are able to chelate transition metals before their presence leads to oxygen free radical formation (Afanas'ev et al., 1989). In addition, according to Arora et al., flavonoids are able to alter peroxidation kinetics by modifying the lipid packing order. They stabilize membranes by decreasing membrane fluidity and preventing the access of deleterious molecules to the hydrophobic region of the bilayer. It has also been proposed a role for flavonoids such as anthocyanins in complexation and vacuolar sequestration of molybdenum, allowing plants to separate it from vital biochemical processes in other cell compartments, reducing metal toxicity (Hale et al., 2001). The significantly higher values in flavonoid content of the WZ leaves and MoZ roots correspond well with Preiner et al., 2019, who showed that plants depending on functional symbiosis present higher flavonoid biosynthesis (chalcone synthase, chalcone isomerase, dihydroflavonol 4-reductase and isoflavone reductase) than nitrogen fertilized plants. It is reasonable to propose that the increase in flavonoid concentrations observed in the WZ leaves and MoZ roots may be explained with the enhanced flavonoid biosynthesis induced by the symbiosis, together with the activation of the biosynthetic pathways for flavonoids as a response to the exposure to W and Mo.

It has been shown that in case of heavy metals stress, the translocation of photoassimilates from the leaves to sink tissues in the plants can be impaired, leading to an accumulation of sugars and starch in the leaves (Samarakoon & Rauser, 1979). Despite the lack of significance, the Nfed

treatments always showed higher starch levels than the Nfix ones, especially in the metal treatments. It has been observed that heavy metals can inhibit proteins involved in starch breakdown such as α-amylase and β-amylases, with consequent accumulation of starch (Preiner et al., 2019; Solanki & Dhankhar, 2011). Moreover, the fact that W polymerizes at low pH in the vacuole (Johnson, Ang, Bednar, & Inouye, 2010; Oburger et al., 2018), where the heavy metals are normally sequestered, can lead to phosphorous immobilization and deficiency. Plants affected by phosphate starvation show an increase in starch and sucrose levels (Hermans, Hammond, White, & Verbruggen, 2006) because of reduced functionality of fundamental processes such as glycolysis, respiration and photosynthesis, ATP-synthesis and Calvin cycle (Preiner et al., 2019.)

A significant increase in soluble carbohydrates concentration was observed in the WZ and MoZ leaves, and in the MoZ roots. Moreover, WZ leaves exhibited higher concentrations of reducing sugars. An antioxidant role for sugars in plants has been demonstrated. For instance, disaccharides such as sucrose, trehalose, maltose, and lactose showed a significant free-radical quenching effect in vitro (Morelli et al., 2003). Raffinose and other raffinose family oligosaccharides, along with galactinol, were reported to successfully scavenge hydroxyl radicals in *Arabidopsis* (Nishizawa, Yabuta, & Shigeoka, 2008). An antioxidant function has also been proposed for monosaccharides, however, they are more likely to affect the antioxidative property of a plant cell indirectly, as secondary messengers to induce the expression or activity of other antioxidants (Gangola & Ramadoss, 2018). A sucrose-specific signaling pathway influencing photosynthesis and biosynthesis of anthocyanins has also been documented (Van den Ende & El-Esawe, 2014); this finding would correspond well with our results, as both soluble carbohydrates and flavonoids showed higher values in the WZ leaves.

In the Nfix tungsten treatment, the increase in soluble sugars, taken together with the increase of proline, could indicate that the tungsten toxicity caused alterations in the water relations, leading to drought stress. This hypothesis would correspond well with a number of studies about the role of heavy metals such as zinc and copper in water relations, extensively reviewed by Poschenrieder & Barcelo (1998). Drought stress symptoms associated with tungsten exposure may suggest that tungstate inhibits aldehyde oxidase activity by substitution of molybdate in MoCo, resulting in reduced abscisic acid synthesis and consequentially stomatal control loss. However, an alternative biosynthetic pathway of abscisic acid, the 2-trans-AB-ol pathway, should be functional (Jiang & Heilmeier, 2007), and heavy metals impose some degree of water deficit for other reasons as well, making speculations on the topic complicated. No definite inference can be drawn without specific experiments on water balances; for instance, on stomatal conductance. However, if our assumption would be right and tungsten had an actual role in water relations, the increase of soluble carbohydrates and reducing sugars observed in the WZ treatment would be an expected response in drought stress. A number of studies confirms the role of sugars as osmo-protectants (Slama et al., 2015), cell membrane stabilizers (Pukacka, Ratajczak, & Kalemba, 2009) and stomatal closure signalers under drought conditions (Osakabe, Yamaguchi-shinozaki, Shinozaki, & Tran, 2014). Moreover, the occurrence of lower levels of starch in Nfix plants than in Nfed plants exposed to tungsten, correspond very well with the differences in the carbon partitioning Staudinger et al. (2016) observed between Nfix and Nfed plants during drought stress.

The fact that drought and heavy metal stress share a similar metabolic response and that nitrogen fixation appears to be affecting it in a similar way, suggests that tungsten causes, to some extent, drought stress, and that nitrogen fixation is likely to provide enhanced tolerance to contrast its deleterious effects.

In the context of nitrogen metabolism, it has been observed that the accumulation and employment of specific amino acids in metal tolerance and hyper accumulation is a species-specific trait. In particular, White, Decker, & Chaney, (1981a) demonstrated that in soybean exposed to high concentrations of zinc, asparagine, isoleucine and valine represent the major amino acids in the exudate. Our results correspond quite nicely with those findings, however, the most abundant amino acid in our treated samples was proline. Asparagine has been proven to act as a ligand towards cadmium, lead and zinc in in vitro studies and models of metal complexes (Bottari & Festa, 1996; White et al., 1981b). It is possible that the amino acid increase observed in the WZ treatment had a direct function in the detoxification processes by metal chelation, or by the way of biosynthesis of chelating peptides such as glutathione and phytochelatins. In particular, proline likely had a lead role in the detoxification processes, due to its functions as an osmolyte, radical scavenger, electron sink, stabilizer of macromolecules (Matysik et al,. 2002). A number of studies, rewieved by Sharma & Dietz (2006), show significant beneficial functions for proline in plants under metal stress, with examples ranging from algae to angiosperms. Moreover, it appears that high constitutive proline contents occur in metal-tolerant populations of non-taxonomically related plants (Smirnoff and Stewart, 1987; Schat, Sharma, & Vooijs, 1997), strongly suggesting a function for proline in increased metal tolerance in these species.

As mentioned before, one of the consequences of exposure to heavy metals is the alteration of the plant–water balance (Poschenrieder & Barcelo, 1998). It is a well known fact that proline can be accumulated in considerable amounts in case of water deficit (Aspinall and Paleg, 1981), because of its role in osmoregulation; thus, it is likely that proline improves the tolerance to the drought stress caused by the exposure to heavy metals. To this purpose, it was shown that suppressing the transpiration rate at increased relative humidity (98%) by placing the plants under transparent polyethylene covers, can almost completely inhibit proline accumulation,

even at metal accumulation rates able to cause up to a 20-fold increase of leaf proline levels (Schat et al., 1997; Sharma et al., 2006). This result suggest that that proline accumulation observed under heavy metal exposure is induced by a metal-imposed water deficit stress, rather than by heavy metal toxicity, and it may be interesting to try a similar approach with W-exposed plants.

In roots, the MoZ treatment presented, when compared to all the others, significantly higher levels of valine, alanine, asparagine, lysine, leucine, serine, isoleucine and phenylalanine. Given the fact that amino acids are the metabolites that incorporate N via uptake of nitrate or ammonium (Masclaux-Daubresse et al., 2010), amino acid accumulation, in this context, could be explained with enhanced N assimilation. Since Mo is present in the Moco cofactor in nitrate reductase, MoN plants that rely on nitrate reduction for N assimilation are likely fertilized by the presence on Mo, as the overall higher (although non significantly) levels of amino acids in comparison with CN plants seem to confirm. However, Mo is also present in bacterial nitrogenase in the FeMoco cofactor. It is possible that an increased Mo supply, paired with an effective transport to the bacteria, can result in higher nitrogenase synthesis and therefore better N_2 fixation and assimilation. The fact that MoN plants, regardless the fertilizing effect of Mo, rely on a limited supply of nitrate while MoZ plants can fix atmospheric N_2, may be an explanation for the significantly higher levels in nearly all the amino acids investigated. Proline, however, showed higher levels in the W treatments when compared to all the others. As previously discussed in leaves, such increase likely represents a stress response to the exposure to W.

In the context of nitrogen compounds that bear a role in abiotic stress, polyamines deserve some attention. Our GC-MS experiment showed that in leaves of all the Nfix plants, putrescine showed significantly higher levels than in Nfed plants, and both the Nfix molybdenum and tungsten treatments showed significantly higher values than the control. In addition to that, spermidine

levels in the WZ treatment were significantly higher than in all the other treatments, which did not present any significant difference between each other. No clear trend in putrescine and spermidine levels was observed in root tissue. Polyamine functions in plants are only partially understood, however, their levels and the activities of their biosynthetic enzymes in plants have been proven to increase under environmental stresses (Evans & Malmberg, 1989). There is also some evidence that polyamine contents increase in case of exposure to heavy metals. For instance, it has been observed that putrescine levels in oat seedlings and detached oat leaves exposed to cadmium can increase up to 10 times. In the context of the same study, an increase in in spermidine and spermine levels was also observed (Weinstein et al., 1986). It is not yet possible to infer a specific role of polyamines in plants under metal stress, however, there is some indication suggesting that they can effectively stabilize and protect the membrane systems especially also against toxic effects of redox active metal ions (Sharma et al., 2006). In particular, in soybean, polyamines can inhibit lipid peroxidation when bound to the negative charges on the membrane vesicle surface (Tadolini et al.,1984). Moreover, Drolet et al. 1986, demonstrated that spermine, spermidine, putrescine and cadaverine scavenge free radicals in vitro. Thus, it is possible that the increased levels of putrescine observed in the Nfix metal treatments, and the higher values of spermidine shown in the WZ plants, represent a way of to counteract the oxidative stress imposed by metal exposure.

In nearly all the investigated organic acids involved in the TCA cycle, in leaf tissue, the Nfix treatments showed significantly lower levels than the Nfed. Leaves of MoZ plants presented significantly lower levels of succinic, malic and citric acid than all the other treatments. Functional nodules represent an important sink for organic acids, especially succinic and malic acid (Udvardi, Price, Gresshoff, & Day, 1988). Considering that Mo is crucial for nitrogen fixation, it is possible that an increased supply of Mo can sustain a higher rate of nitrogen fixation and therefore, MoZ

nodules may represent an especially strong sink for organic acids. Such trend is not visible in roots. Citric acid presented higher levels than the other acids in both leaves and roots.

In my experiment, the rhizobial symbiosis exerted a dramatic effect on the amino acid levels in leaves during W exposure, with nearly all the amino acids investigated having significantly higher values in the Nfix treatment. The same effect was observed in polyamines contents. Moreover, the rhizobial symbiosis affected the plant response to Mo: higher levels of amino acids were observed in MoZ roots with respect to the MoN ones and, over all, all the others. MoZ plants also showed higher levels of soluble carbohydrates than MoN ones in leaves and roots, and of phenols and flavonoids in roots. Interestingly, my results on nitrogen metabolism, just like afore mentioned carbon metabolism and partitioning results, match with the observations of Staudinger et al., (2016). In their study, N_2 fixing plants exposed to water deficit showed higher amino acid concentrations (including proline) and a general enhanced investment of reserves into the synthesis of osmolytes than their non-N_2 fixing counterparts. These results suggest an enhanced osmoprotective, radical-scavenging, and metal-quenching capacity in Nfix plants.

5. Conclusions

It is evident from our results that Nfed plants, in contrast with Nfix, did not synthetize the same set of metabolites that are known to have functional significance in the context of heavy metal stress tolerance, such as phenolic compounds, sugars, polyamines and specific amino acids. Clearly, both carbon and nitrogen metabolism are central to the response of plants to heavy metals and it appears that in case of W exposure, nitrogen assimilating plants that rely solely on nitrate reduction may be disadvantaged in comparison with plants whose nitrogen fixation may be impaired, but still partially functional. W is known to have a deleterious effect on nitrogen assimilation via nitrate reduction in soybean (Preiner, Wienkoop, Weckwerth, & Oburger, 2019): at concentrations of 0.5 mM W, nitrate reductase activity dropped to only 20% of the activity in the control Nfix plants, as a direct effect of W competition with molybdenum as an enzymatic co-factor and consequential inactivation. Furthermore, in Nfix plants, they observed reduced nodulation and an overall reduced nitrogenase abundance, however, despite that, N_2 fixation (nodule activity) of Nfix soybean was not affected.

In my experiment, Mo exposure did not show clear toxic effects, however, it impacted the metabolic response in different ways. The levels of amino acids in MoZ roots increased significantly, as well as soluble carbohydrates, flavonoids and phenols. However, the increase in clear stress markers such as proline, were not nearly of the same magnitude as observed in W exposed plants, making it reasonable to think that, at the same concentrations, Mo is much better tolerated than W.

It is possible that in Nfix plants exposed to W, the massive effort put into synthetizing this set of molecules that have the potential to counterbalance its deleterious effects, results in a damage

reduction on a cellular level rather than in a biomass accumulation. By means of visual inspection, it appears that WZ plants present healthier roots, in contrast with WN plants, whose roots appear severely damaged and oxidized. Shoot elongation too, overall, seems to be better in WZ plants than in WN ones. Future experiments, such as determination of lipid peroxidation and hydrogen peroxide (H_2O_2), can be a good strategy to determine if the enhanced synthesis of those metabolites results in an actual protection from the harm caused by W. Overall, our data indicates that the heavy metal response is influenced by plant–rhizobia interaction. Moreover, the differences observed in the metabolic response, such as increased levels of phenols, flavonoids, sugars, polyamines and specific amino acids, strongly hint at a possible stress alleviation mechanism in symbiotically grown plants.

It can finally be added that the novel sequential extraction protocol is working efficiently and may be applied routinely for future metabolite analyses. It allowed the quantitative analysis of primary and secondary metabolites from the same sample. Additionally, it is very likely to be applicable for various other plant species with minor adaptation. This method can also be used for protein analysis, an approach that will give further insights into metabolic adjustments in future.

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