BIOMASS PRODUCTION AND BIOCHEMICAL PARAMETERS ARE AFFECTED BY SULFUR DEFICIENCY IN *TRIGONELLA FOENUM-GRAECUM*

Mariame Houhou[1,2], Khalid A. Joutei[2], Said Louahlia[1]

Abstract— The reduction in atmospheric inputs of S due to stricter emission regulations caused serious decrease in sulfur deposition and, as result, affected markedly growth and quality of numerous economically important plants. *Trigonella foenum-graecum* (Fenugreek) is one of the most widely aromatic and medicinal cultivated plants used in the world. To explore the response of Fenugreek towards poor sulfur nutrition, we studied the effect of S-starvation on plants of *Trigonella foenum-graecum* grown in nutrient solution containing less sulfur. S-deprivation decreased the total dry matter of the plant by 36%, specially the aerial part. The chlorophyll content was reduced by 60% and the concentration of soluble sugars also decreased in both shoot and root by 50% and 56%, respectively. The measurement of *in vivo* nitrate reductase activity in the root tissues showed that sulfur deprivation reduced the capacity of plant to assimilate nitrogen by 68%.

Keywords — Sulfur deficiency, *Trigonella foenum-graecum,* soluble sugars, nitrate reductase activity.

1 INTRODUCTION

The relevance of sulfur (S) in plant nutrition is well known, since S is essential for the synthesis of cysteine, methionine, glutathione, proteins, thiamine, Coenzyme-A, iron-sulfur centers, phytochelatines, and glucosinolates and serves as a precursor for a variety of further reduced sulfur-containing compounds [1]. Also, S is required for the synthesis of chlorophyll since S is a vital part of the ferredoxins, Fe-S protein in the chloroplasts [1]. Sulfur is involved in numerous biochemical pathways in plants, such as biosynthesis and regulation of enzymes activities, photosynthesis, respiration and cells antioxidant protection, [2], [3], [4]. Sulfur plays also an important role in nitrogen fixation, quality and yield of legume crops [5], [6].

In recent years S-deficiency has become an increasing problem for agriculture resulting in decreased crop quality parameters and yield [7]. Several studies showed a serious deficiency of sulfur content of agronomical crops [5], [8], [9] associated to the decrease of the S concentrations in the atmosphere. This is a result of the intensive production of higher yielding crops, higher use of fertilizers containing little or no sulfur and the decrease of the sulfur deposition because of the reduction in atmospheric inputs and stricter emission regulations. Klimont et al. [10] reported a decrease of atmospheric S concentration about 20TG between 1990 and 2011 in Eastern Europe, Central Asia, Canada and US. This adverse situation affected markedly growth and quality of numerous economically important plants. Several studies have reported that many biochemical and physiological responses were initiated upon exposure of plants to S-deficiency, leading to a general reduction in metabolic activity including decrease in biomass and increased root/shoot ratio [2], [11].

Sulfur deficiency also leads to a large decrease in amino acids pool and chlorophylls content when compared with situations where S is in adequate supply, especially in young leaves [2], [11], [12]. Plants submitted to S-deficiency can also modify their root morphological traits to maximize the acquisition of nutrients under nutrient-deficient conditions [13].

Fenugreek (*Trigonella foenum-graecum*) belongs to Fabaceae family and it is one of Mediterranean aromatic and medicinal plants, very cultivated in India, Egypt and Morocco [14]. Trigonella foenum-graecum is characterized by its powerful antioxidant and anti-radical activity [15] it is used in traditional medicine for the treatment of wounds, abscesses, arthritis, bronchitis, ulcer and digestive problems [14]. Our investigations were focused on the effect of the inorganic sulfur starvation on the metabolism and biochemistry of Trigonella foenum-graecum by analyzing the effects of S-deficiency on growth, mineral elements, chlorophyll, water soluble sugars, amino acids contents and nitrate reductase activity.

2 MATERIAL AND METHODS

2.1 Culture conditions

The seeds of fenugreek (*Trigonella foenum-graecum*) were put to germinate on filter paper soaked with 10 ml of distilled water at temperature of 25°C. The seedlings were then transplanted into pots containing 1 kg of sand and vermiculite mixture (3:1) and placed in a greenhouse at between October and April, under natural light and photoperiod. The pots were divided into two batches of five and watered once per week for six weeks with 100 ml of nutrient solution (Hoagland and Arnon 1950) described in Table 1. The control plants received the complete nutrient solution containing 1 mol.m^{-3} S, while the S-deficient

1. Laboratory of Natural Resources and Environment, Polydisciplinary Faculty of Taza, USMBA, Fez, Morocco.
2. Laboratory of Bioactive Molecules, Faculty of Science and Technology, USMBA, Fez, Morocco

plants received the same solution but containing only 0.05 mol.m^{-3} S. The sampling was performed before the appearance of the flower. Harvested plants were thoroughly washed with tap water and then with distilled water to clean and remove surface filth. They were separated into leaves, stems and roots and then dried in the oven at 80°C for 24 hours to measure dry weight.

2.2 Mineral elements determination

Sulfate ions in leaves were extracted by boiling fresh tissue in water whereas total sulfur content was determined by digestion of 50 mg of dry matter of leaves in nitric acid (HNO_3) and perchloric acid ($HClO_4$) in the ratio of 85:15, for 4h at 120°C. The digested solution was adjusted to 10 ml with distilled water. The sulfate concentration in the extracts was determined turbidimetrically [17], [18]. Sulfate ion in the extract was converted to a barium sulfate suspension and the resulting turbidity was measured by reading the optical density at 430 nm (VWR UV-6300PC spectrophotometer). The standard curve was prepared by using different concentrations of sodium sulfate solutions.

The other mineral elements: calcium (Ca), potassium (K), Magnesium (Mg), and Molybdenum (Mo) were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). The mineralization of 1g dry matter was performed in a mixture of nitric acid and of perchloric acid (4:1, v/v) during 4h at 120°C.

2.3 Chlorophyll Content determination

The chlorophyll was extracted from 30 mg of fresh young expanded leaves with dimethyl sulfoxide (DMSO) in the dark at 65°C for 30 min. After centrifugation the absorbance of extract was measured by using a spectrophotometer (VWR UV-6300PC spectrophotometer) at 663 and 645 nm. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated according the following formulae [19], [20]:

Chl a (g.l^{-1}) = 0.0127 *A663 – 0.00269*A645
Chl b (g.l^{-1}) = 0.0229 *A645 – 0.00468*A663
Total Chl (g.l^{-1}) = 0.0202 *A645 + 0.00802 *A663

2.4 Total soluble sugars determination

The total soluble sugars concentration was determined following the method described by Grandy et al. [21]. 100 mg of fresh materiel were ground in 4 ml of ethanol (80%), the mixture was placed in a water bath at 80°C for 30 min. After centrifugation at 4500 rpm for 10 min, total soluble sugars were measured by using anthrone reagent (freshly prepared 0.2% (w/v) anthrone in sulfuric acid). The absorbance was immediately read at 625 nm by using a spectrophotometer (VWR UV-6300PC spectrophotometer), and then converted into glucose equivalent (mg.g^{-1}) using a calibration curve of glucose standards.

2.5 Total amino acids determination

The Extraction of amino acids was adapted from Barber et al. [22] with some modifications. Briefly, 100 mg of fresh material were grinded in 2 ml of sodium phosphate buffer (pH 7.5) followed by successive hydro alcoholic extractions (80% and 50%) by incubation in water bath at 80°C. The mixture was then centrifuged at 5000 rpm for 10 min, the supernatant was evaporated and the dry residue was resumed in 500 µl of distilled water. The amino acid assay was carried out by adding 1 ml of the ninhydrin reagent prepared extemporaneously [23] to 100 µl of the diluted extract. Test tubes were then incubated for 20 min in a boiling water bath and the reaction was stopped by rapid cooling into cold water. The absorbance was then read spectrophotometerically at 570 nm (VWR UV-6300PC spectrophotometer).

2.6 Measurement of the Nitrate Reductase Activity (NRA)

NRA was assayed *in vivo* according to the method of Jaworski [24]. 200 mg of plant material were cut into 4 mm fragments and then incubated in test vial containing a reaction phosphate buffer (100 mM, pH 7.5 with 20 mM KNO_3 and 1% isopropanol). Test tubes were incubated at 30°C in the dark and the amount of nitrite produced by action of the enzyme was measured after addition of 1% sulfanilamide and 0.02% α-naphtyl-ethylenediamine. In presence of nitrite, a pink coloration appears and absorbance was measured by spectrophotometry at 540 nm. A standard curve was prepared in the same way as the samples with standard solutions of sodium nitrite ($NaNO_2$).

2.7 Statistical analysis

Statistical analysis was performed using SYSTAT 12. Data were subjected to one-way analysis of variance (ANOVA) in order to determine significant differences among the treatments. The results were considered significant at $P<0.05$.

3. RESULTS

3.1 Effects of sulfur deficiency on biomass production

Sulfur deficiency influenced significantly the global growth of *Trigonella foenum-graecum;* the dry matter of the whole plant reached 420 mg.plant^{-1}. The low concentration of sulfur in the medium provoked a very significant decrease in total dry matter. When compared to the control, S-deficient plants exhibited a decrease of total biomass by 36% ($p < 0.05$). The analysis of different organs of plants showed significant differences between the control and S-deficient plants both in shoot and roots. In deficient plants, significant decreases were recorded in leaves, stem and roots of 61%, 21% and 22%, respectively ($p<0.05$) (Fig.1).

3.2 Effect of S-deficiency on chlorophyll and mineral elements contents

In the S-sufficient plants when the conditions of culture were optimal the concentration of total chlorophyll was 9.6 mg.g^{-1} DW. The limitation of sulfur supply decreased the total chlorophyll content in leaves by 60%, it has fallen from 9.6 mg.g^{-1} DW in the control to 3.6 mg.g^{-1} DW in S-deficient plants. This decrease was recorded both in the concentrations of chlorophyll a and chlorophyll b. Under control conditions the concentrations of chlorophylls a and b was 6.3 mg.g^{-1} DW and 3.3 mg.g^{-1} DW, respectively. When plants were submitted to poor nutrition of sulfur, the corresponding concentrations of chlorophylls decreased significantly ($p<0.05$) by 60% and 56%, respectively (Fig. 2).

In control plants the mineral elements were distributed equally between the leaves and the roots (Table 2). Ca and K represented the highest mineral content of the plant, while Mo was the lowest content. In the case of sulfur deficiency, there was an increase in the contents of all mineral elements in leaves

except for Ca (P> 0.05). Significant increases in order of 11%, 17% and 19% were recorded in K, Mg, and Mo, respectively (p <0.05). By contrast, no significant difference in the mineral composition in roots was observed, except for K which exhibited a significant increase when plants were submitted to S deficiency.

Under optimal conditions of growth the concentration of total sulfur in leaves was 1.5 $mg.g^{-1}$ DW. Reduction in the sulfur supply caused an important decrease of 68%, the concentrations of sulfur dropped from 1.5$mg.g^{-1}$ in control plants to 0.5 $mg.g^{-1}$ in deficient ones (Fig.3a). The same reduction was observed in sulfate concentrations in S deficient leaves, by 55% when compared to the control ones (P <0.005). It has fallen from 67 $\mu g.g^{-1}$ DW to 30 $\mu g.g^{-1}$ DW (Fig.3b).

3.3 Effect of S-deficiency on amino acids contents

The level of amino acid pool in *Trigonella foenum-graecum* was markedly affected by the low sulfur supply. The decrease of amino acids content was by 56% and 40% in leaves and roots respectively, when compared with control plants (p<0.05), Amino acids level has fallen from 1.3 $mg.g^{-1}$ DW and 0.9 $mg.g^{-1}$ DW in control plants to 0.6 $mg.g^{-1}$ DW and 0.5$mg.g^{-1}$ DW in S-deficient ones in leaves and roots, respectively (Fig.4).

3.4 Effect of S deficiency on total soluble sugars content

The reduction of sulfur nutrition of *Trigonella foenum-graecum* caused an important decrease in the concentration of soluble sugars in both shoot and roots. In control plants, the soluble sugars content was 58.2 $mg.g^{-1}$ and 30.6 $mg.g^{-1}$ DW in leaves and roots, respectively. After exposure to sulfur deficiency the concentration of soluble sugar decreased by 56% in leaves and by 50% in roots (p<0.05). The content of soluble sugars dropped to 28.5 $mg.g^{-1}$ DW and 13.5 $mg.g^{-1}$ DW in leaves and roots, respectively (Fig.5).

3.5 Effect of S deficiency on nitrate reductase activity (NRA)

The *in vivo* NRA in roots of *Trigonella foenum-graecum* expressed in the amount of nitrite produced per hour was estimated in our experiment conditions. The result showed serious impact of S starvation on the first step of nitrate assimilation. In control plants the NRA was estimated at 7.1 µg of $NO_2^-.g^{-1}$ $FW.h^{-1}$ but after the application of limited sulfur nutrition, the *in vivo* NRA dropped to 2.1 µg of $NO_2^-.g^{-1}FW.h^{-1}$. The significant NRA decrease of 68% was recorded (P<0.05) (Fig.6).

4. DISCUSSION

In control plants when *Trigonella foenum-graecum* received sufficient sulfur nutrition, the total S content in leaves was 0.15% and 4.3 % of total S was accumulated as sulfate. In response to sulfur deficiency, the total sulfur content in *Trigonella foenum-graecum* leaves decreased markedly (0.05%). This result shows clearly the S-deficient status of *Trigonella foenum-graecum* under our conditions of culture. The critical value of sulfur under which the plant is supposed S-deficient varies among plant species and depends on several parameters (growth conditions, stage of sampling, analyzed part of plant and the S-compound species used for sulfur measurement).

Generally, in S-deficient plants, the concentration of total sulfur must be less than 1.7 $mg.g^{-1}$ and in the case of non-brassica vegetables including *Trigonella foenum-graecum* this value may be lower than 0.94 $mg.g^{-1}$ [25]. Because of the interaction of sulfur and nitrogen metabolism, the N/S ratio in S-sufficient plants has to be maintained within the range of 20/1 [26]. However, in S-deficient plants maintained with optimal N nutrition as the case in our conditions of growth, the N/S ratio increases. This was reported in tomato leaves [27], sugar beet shoots [28], spinach leaves [29], Arabidopsis leaves [11], [30] and bean plants [31] submitted to S starvation.

Sulfate is often the first metabolite to change in response to sulfur deficiency, and its uptake and distribution are closely regulated. We showed in this study that the S-deficiency decreased the sulfate concentration in leaves by 68%, this result is in agreement with those of Juszczuk et Ostaszewska [31] in bean plants and Blake-Kalff et al. [32] in oilseed rape submitted to sulfur starvation. This reduction of sulfate concentration in leaves could be explained in one hand by the release of SO_4^{2-} ions from vacuoles of mesophyll cells under prolonged S stress [33], [34], [35] and in the other hand most of newly absorbed sulfur is probably kept preferentially in roots. In *Brassica napus* Abdallah et al. [36] showed that only 23% of uptaken S were translocated to leaves when plants were submitted to S deprivation, whereas when S supply was adequate the corresponding value was 55% [37]. The reduction in the level of sulfate had significant impact on the overall plant development. Biomass of both shoot and roots decreased in response to S-deficiency but the main relative decrease was recorded in leaves (-61%), the roots biomass was less affected by sulfur starvation. This result is in agreement with several works that have shown the negative effect of S-deficiency on the growth of the plant in *Arabidopsis thaliana* [38], [39], *Medicago truncatula* [40], [41] and *Trifolium repens* [42]. Several other works suggested that more metabolites are allocated from leaves to roots under S starvation [6], [41], which could explain the more pronounced decline of growth in shoot when compared to roots. The same morphological modification was observed in plants submitted to N, P or Mg starvation and could be associated to maintain the ability of roots to acquire mineral nutrient [38], [13], [30], [43], [44]. Moreover, in plants grown in S-defficient medium Juszczuk and Ostaszewska [31] observed higher decrease of leaves dry matter and showed that ATP production decreased by 48% in leaves and only by 20% in roots.

The growth inhibition induced by the poor sulfur supply, is mainly associated to the crucial role that sulfur plays in the biochemical pathway. Several studies showed that proteins, sugars, amino acids, chlorophyll contents and S/C/N ratio were unbalanced because of the S-deficiency and this is undoubtedly associated to general reduction in metabolic activity [2], [11], [28], [31], [35], [41]. In our investigations a significant decrease in the order of 60% was recorded in total chlorophyll concentration in S-deficient plants. This reduction was exhibited both in chlorophyll *a* and chlorophyll *b* concentrations in leaf tissue and could be associated to the decrease in content of numerous S compounds such as cystein, methionine and S-adenosylmethionine (SAM) [29], [30], [45]. SAM is a methionine derivate that is involved in the chlorophyll synthesis pathway as a methyl group donor [42], [45]. Two rich amino acids, cysteine and methionine act as structural and functional element of chloroplast targeted proteins [31], [46]. Lunde et al.

[47] reported that in plants submitted to sulfur deficiency the *Rubisco* level decreased six fold, chlorophyll content reduced by 48%, the PSII efficiency was 31% lower and the ability of PSI to produce $NADP^+$ decreased by 61%. These observations together with the occurrence of chloroplast degradation in response to S deficiency [31] suggest a potential disturbance in photosynthetic electron transport chain reactions and CO_2 fixation which may explain the lower dry matter production and the observed decrease in soluble sugars content when plants were submitted to S deficiency.

The reduction in soluble sugars under sulfur deficiency was also reported by Varechon [48] in Groundnut crops, [49] in *zea mays* and [50] in *Medicago truncatula* when grown in poor sulfur medium.

Several other works showed a serious disturbance in mineral composition under sulfur starvation and in same line with our investigations, Kassem et al. [51] noted an increase in potassium content in tomatoes submitted to sulfur deficiency. However, Gao et al. [41] showed a decrease in potassium levels in *Medicago sativa* crops grown in a sulfur-deficient environment while Gunes et al. [52] proved that there was no change in potassium concentrations in S deficient *Medicago sativa*. On other hand, several studies have shown an accumulation of magnesium in plants under sulfur deficiency conditions [27], [51] [53], [54]. The antagonism between the Mg^{2+} and SO^{2-} ions is probably the cause of this accumulation [51].

It is well known that the absorption and the assimilation of sulfate are controlled by the nutritional status of the plant [55], [56], [57] and that the expression of many sulfate transporters is enhanced by limiting the sulfur nutrition in the medium [58]. The high-affinity sulfate transporters (SHST1) which are responsible for sulfate uptake from soil solution have definitively been shown to transport Mo which accumulates in plants submitted to S deficiency [55], [58], [59], [60] which explain the accumulation of Mo in the leaves of *Trigonella foenum graecum* submitted to S-starvation in our investigation.

The S-starvation caused serious and significant decrease in the levels of amino acids in both leaves and roots. In previous work, Thomas et al. [28] showed that concentrations of sulfur less than 0.07 $mol.m^{-3}$ in the medium caused significant decrease in the concentration of amino acids in sugar beets crop. This decrease in amino acids level can be due to a decrease in sulfur amino acids (Cysteine and Methionine).

In this investigation, we showed significant reduction in the assimilation of nitrate ions in roots. The decrease of NR activity under sulfur deficiency reflects the primordial role of S in nitrogen assimilation [5], [6] without neglecting the impact of low availability of soluble carbohydrates. Cheng et al. [61] and Vincentz et al. [62] reported that the transcription of NR gene is induced by carbohydrates and it was clearly shown that S deficiency impact seriously the activity and the quantity of *Rubisco* enzyme [63].

Furthermore, Foyer et al. [64] showed a direct correlation between NR activity and photosynthesis and the disturbance of NR activity recorded in *Trigonella foenum-graecum* grown in deficient-sulfur medium could be associated to low levels of chlorophyll and soluble sugars. The same result was reported by Migge et al. [65] in tobacco submitted to sulfur deprivation.

5. CONCLUSION

This work contributes to a first understanding of the response of *Trigonella foenum-graecum* subjected to sulfur stress. When applying a low supply of sulfur, the plant reacts negatively in terms of growth and the total biomass of the plant, but the leaves remains the most affected part. Chlorophyll levels, amino acids and total soluble sugars contents were also reduced by sulfur deficiency. Moreover, the mineral composition changes significantly by the accumulation of K, Mg and Mo. Sulfur deficiency also affected the ability of the plant to assimilate nitrate ions by decelerating the nitrate reductase activity. These changes can be considered as an indicator of adaptation of *Trigonella foenum-graecum* against an environmental stress caused by low availability of sulfur in the medium

6. References

[1] Leustek T, Martin MN, Bick JA, Davies JP, 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51: 141-165.

[3] Rausch T, Wachte A, 2005. Sulfur metabolism: a versatile platform for launching defence operations. TRENDS in Plant Sci 10:10.

[2] Nikiforova VJ, Gakiere B, Kempa S, Adamik M, Willmitzer L, Hesse H, Hoefgen R, 2004. Towards dissecting nutrient metabolism in plants: a systems biology case study on sulphur metabolism. J Exp Bot 55: 1861-1870.

[4] Davidian JC, Kopriva S, 2010. Regulation of sulfate uptake and assimilation-the same or not the same?. Mol Plan 3: 314-325.

[5] Scherer HW, 2001. Sulphur in crop production. Eur J Agron 14: 81-111

[6] Scherer HW, Pacyna S, Spoth KR, Schulz M, 2008. Low levels of ferredoxin, ATP and leghemoglobin contribute to limited N_2 fixation of peas (*Pisum sativum L*.) and alfalfa (*Medicago sativa L*.) under S deficiency conditions. Biol Fertil Soils 44: 909 - 916.

[7] McGrath SP, Zhao FJ, 1996. Sulfur uptake, yield responses and the interactions between nitrogen and sulfur in winter oilseed rape (*Brassica napus*) J Agric Sci 126: 53–62.

[8] Schnug E, 1997.Significance of sulphur for the quality of domesticated plants. In: Sulphur Metabolism in higher Plants: Molecular, Ecophysiological and Nutritional Aspects; Cram WJ, De Kok LJ, Brunold C, Rennenberg H (eds). pp:109–130. Backhuys Publishers, Leiden.

[9] Hagel I, 2005. Sulfur and baking-quality of bread making wheat. Land Völk 283.

Haneklaus S, Bloem E, Schnug E, 2008. History of sulfur deficiency in crops. In:. Sulfur: A Missing Link between Soils, Crops, and Nutrition; Jez J, Madison WI, (eds). pp: 45-58. Am Soc Agr, Crop Sc Soc Am.

[10] Klimont Z, Smith SJ, Cofala J, 2011. The last decade of global anthropogenic sulfur dioxide: 2000-2011 emissions. Environ Res Lett 8:1.

[11] Nikiforova VJ, Daub CO, Hesse H, Willmitze L, Hoefgen R, 2005. Integrative gene-metabolite network with implemented causality deciphers informational fluxes of sulphur stress response. J Exp Bot 56: (417) 1887-1896.

[13] Lopez-Bucio J, Cruz-Ramırez A, Herrera-Estrella L, 2003. The role of nutrient availability in regulating root architecture. Current Opinion in Plant Biology 6: 280-287.

[14] Snehlata HS, Payal DR, 2012. Fenugreek (*Trigonella foenum-graecum L*.): An Overview. Int J Curr Pharm Res

2: 0976-822.
[15] Kaviarasan S, Naik GH, Gangabhagirathi R, Anuradha CV, Priyadarsini KI, 2007. In vitro studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum graecum*) seeds. Food Chem 103, 31–37.
[16] Hoagland DR, Arnon DI, 1950. The water-culture method for growing plants without soil. Calif Agric Exp Sta (347).
[17] Bardsley CE, Lancaster JD, 1960. Determination of reserves sulphur and soluble sulfate in soil. Soil Sci Soc Am Proc 24: 265-268.
[18] Astolfi S, Zuchi S, 2013. Adequate S supply protects barley plants from adverse effects of salinity stress by increasing thiol contents. Acta Physiol Planta 35(1): 175-181.
[19] Arnon DI, 1949. Copper enzymes isolated chloroplasts, polyphenol oxidase in Beta vulgaris. Plant Physiol 24(1): 1-15.
[20] Hiscox J, Israelstam DGF, 1949. A method for the extraction of chlorophyll from leaf tissue without maceration, Rev. Can Bot 57: 1332-1334
[21] Grandy AS, Erich MS, Porter GA, 2000. Suitability of the anthrone-sulfuric acid reagent for determining water soluble carbohydrate in soil water extracts. Soil Biol Biochem 32: 725-727.
[22] Barber L, Joern BC, Volenec JJ, 1996. Cunningham SM. Supplemental nitrogen effects on alfalfa regrowth and nitrogen remobilization from roots. Crop Sci 36: 1217-1223.
[23] Rosen H, 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Bioph 67: 10-15
[24] Jaworski EG, 1971. Nitrate reductase assay in intact plant tissues. Biochem Biophys Res Commun 43:1274–1279.
[25] Haneklaus S, Bloem E, Schnug E, De Kok L J, Stulen I, 2007. Sulfur. In: Handbook of Plant Nutrition; Barker AV, Pilbeam DJ (eds). Group pp: 183-238. CRC Press Taylor and Francis, New York.
[26] Gram WJ, 1990. Uptake and transport of sulphate. In: Rennenberg H, ed. Sulphur Nutrition and Assimilation in Higher Plants. SPB Acadmic Publishing p: 3-11. The Netherlands
[27] Xu HL, Lopez J, Rachidi F, Tremblay N, Gauthier L, Desjardins Y, Gosselin A, 1996. Effect of sulphate on photosynthesis in greenhouse-grown tomato plants. Physiol Planta 96: 722–726.
[28] Thomas SG, Bilsborrow PE, Hocking TJ, Bennett J, 2000. Effect of sulphur deficiency on the growth and metabolism of sugar beet (Beta vulgaris cv Druid). J Sci Food Agr 80: 2057-2062.
[29] Prosser IM, Purves JV, Saker LR, 2001. Clarkson DT. Rapid disruption of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate. J Exp Bot 52: 113-121.
[30] Nikiforova VJ, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R, 2003. Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650.
[31] Juszczuk IM, Ostaszewska M. Respiratory activity, energy and redox status in sulphur-deficient bean plants. Env Exp Bot, 2011; 74: 245-254.
[32] Blake-Kalff MMA, Harrison KR , Hawkesford MJ, Zhao FJ, McGrath SP, 1998. Distribution of Sulfur within Oilseed Rape Leaves in Response to Sulfur Deficiency during Vegetative Growth. Plant Physiol 118 (4): 1337-1344
[33] Clarkson DT, Smith FW, Vanden BPJ, 1983. Regulation of sulfate transport in a tropical legume, Macroptilium atropurpureum, cv. Sirato. J Exp Bot 34: 1463 – 1483.
[34] Christopher I. Bell13, David T. Clarkson2 and W. John Cram. 1995 Partitioning and redistribution of sulphur during S-stress in Macroptilium atropurpureum cv. Siratro; Journal of Experimental Botany, Vol. 46, No. 282, pp.
[35] Zhao FJ, Wood AP, McGrath SP, 1999. Effects of sulphur nutrition on growth and nitrogen fixation of pea (Pisum sativum L.). Plant Soil 212: 207–217.
[36] Abdallah M, Dubousset L, Meuriot M, Etienne P, Avice JC, Ourry A, 2010. Effect of mineral sulfur availability on nitrogen and sulfur uptake and remobilization during the vegetative growth of Brassica napus L. J Exp Bot 61(10): 2635-2646.
[37] Granth C, Hawkesford MJ, 2015. Sulfur. In: Handbook of Plant Nutrition, Second Edition; Barker AV, Pilbeam DJ (eds). pp: 261-301. CRC Press Taylor and Francis Group, New York.
[38] Kutz A, Muller P, Hennig W, Kaiser M, Piotrowski M, Weiler EW, 2002. A role for nitrilase 3 in the regulation of root morphology in sulphur-starving Arabidopsis thaliana. Plant J 30: 95-106.
[39] Akiko MN, Yumiko N, Akiko WT, Eri I, Tomoyuki Y, Hideki T, 2005. Identification of a novel cis acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J 42: 305–314.
[40] Casieri L, Gallardo K , Wipf D, 2012. Transcriptional response of Medicago truncatula sulfate transporters to arbuscular mycorrhizal symbiosis with and without sulphur stress. Planta 235: 1431- 1447.
[41] Gao Y, Li X, Tiana QY, Wanga BL, Zhanga WH, 2015. Sulfur Deficiency had Different Effects on Medicago Truncatula Ecotypes A17 and R108 in Terms of Growth, Root Morphology and Nutrient Contents. J Plant Nutr 39: 301-314.
[42] Varin S, Cliquet JB, Personeni E, Avice JC, Lemauviel-Lavenant S, 2010. How does sulphur availability modify N acquisition of white clover (Trifolium repens L.)? . J Exp Bot 61: 225–234.
[43] Christian Hermans, John P. Hammond, Philip J. 2006; White and Nathalie Verbruggen. How do plants respond to nutrient shortage by biomass allocation?; TRENDS in Plant Science Vol.11 No.12
[44] Dan H, Yang G, Zheng Z L, 2007. A negative regulatory role for auxin in sulphate deficiency response in Arabidopsis thaliana. Plant Mol Biol 63: 221-235.
[45] Hoefgen R, Nikiforova VJ, 2008. Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiol Planta 132: 190–198.
[46] Droux M, 2004. Sulfur assimilation and the role of sulfur in plant metabolism: a survey. Photosynth Res 79 : 331-348.
[47] Lunde C, Zygadlo A, Simonsen HT, Nielsen PL, Blennow A, Haldru A, 2008. Sulfur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. Physiol Plant 134: 508–521.
[48] Varechon C, 1971. Influence de la carence en soufre sur l'évolution des glucides chez l'arachide, de la germination à la floraison. Rev gén Bot 78 :297-328.

[49] Rendig VV, Oputa C, McCOMB EA, 1976. Effects of sulfur deficiency on Non-protein nitrogen, soluble sugars, and n/s ratios in young corn (*zea mays l.*) Plants. Plant Soil 44: 423-437.

[50] Zuber H, Poignavent G, Le Signor C, Aimé D, Vieren E, Tadla C, Lugan R, Belghazi M, Labas V, Santoni AL, Wipf DJ, Buitink J, Avice C, Salon C, Gallardo K, 2016. Legume adaptation to sulfur deficiency revealed by comparing nutrient allocation and seed traits in Medicago truncatula. The Plant J 76: 982-996.

[51] Kassem A, Mohammed S, Hellal FA, EL-Sayed SAA, 2015. Influence of Sulfur Deprivation on Biomass Allocation, Mineral Composition and Fruit Quality of Tomato Plants. Middle East J Agric Res (4): 42-48.

[52] Gunes, A., A. Inal, D. J. Pilbeam, and Y. K. Kadioglu. 2009. Effect of sulfur on the yield and essential and nonessential element composition of alfalfa determined by polarized energy dispersive x-ray fluorescence. Communication in Soil Science and Plant Analysis 40: 2264- 2284.

[53] Zuchi S, Cesco S, Varanini Z, Pinton R, Astolfi S, 2009. Sulphur deprivation limits Fe-deficiency responses in tomato plants. Planta 230: 85.

[54] Tewari RK, Kumar P, Sharma PN, 2010. Morphology and oxidative physiology of sulphur-deficient mulberry plants. Env Exp Bot 68: 301–308.

[55] Smith FW, Ealing PM, Hawkesford MJ, Clarkson T, 1995. Plant members of a family of sulfate transporters reveal functional subtypes. Proc Natl Acad Sci USA 92: 9373–9377.

[56] Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, van den Berg PJ, Belcher AR, Warrilow AGS, 1997. Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. The Plant J 12: 875–884.

[57] Buchner P, Takahashi HM, Hawkesford J, 2004. Plant sulphate transporters: co ordination of uptake, intracellular and long-distance transport. J Exp Bot 55: 1765-1773.

[58] Shinmachi F Buchner P, Stroud JL, Parmar S, Zhao FJ, McGrath SP. Hawkesford MJ, 2010. Influence of Sulfur Deficiency on the Expression of Specific Sulfate Transporters and the Distribution of Sulfur, Selenium, and Molybdenum in Wheat. Plant Physiol 153 : 327–336.

[59] Leggett JE, Epstein E, 1956. Kinetics of Sulfate Absorption by Barley Roots. Plant Physiol 31(3): 222–226.

[60] Fitzpatrick KL, Tyerman SD, Kaise BN, 2008. Molybdate transport through the plant sulfate transporter SHST1. FEBS Letters 582: 1508-1513.

[61] Cheng CL, Acedo GN, christinson M, Conkling MA, 1992. Sucrose mimics the light induction o Arabidopsis nitrate reductase gene transcription. Proc Natl Acad Sci USA 89:1861-1864.

[62] Vincentz M, Moureaux T, Leydecker MT, Vaucheret H, Caboche M, 1993. regulation of nitrate and nitritr reductase expression in nicotina plumbaginifolia leaves by nitrogene and carbon metabolites. Plant J 3:315-324.

[63] Gilbert SM, Clarkson DT, Cambridge M, Lambers H, Hawkesford MJ, 1997. SO_4^{2-} Deprivation Has an Early Effect on the Content of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase and Photosynthesis in Young Leaves of Wheat. Plant Physiol 115: 1231-1239

[64] Foyer CH, Valadier MH, Migge A, Becker TW, 1998. Drought-Induced Effects on Nitrate Reductase Activity and mRNA and on the Coordination of Nitrogen and Carbon Metabolism in Maize Leaves. Plant Physiol 117: 283–292.

[65] Migge AC, Bork R, Hell T, Becker H, 2000. Negative regulation of nitrate reductase gene expression by glutamine or aspargine accumulation in leaves of sulfur- deprived tobacco. Planta 211:587-595.

Figures and tables

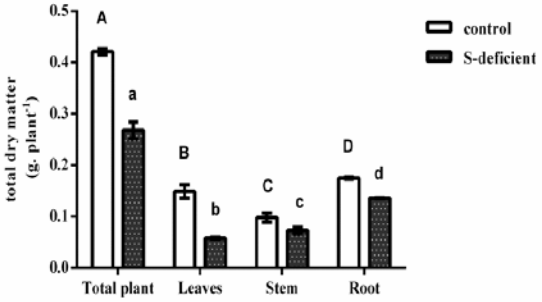

Fig. 1: Effect of sulfur deficiency on biomass production of whole plant of *Trigonella foenum graecum* submitted to sulfur deficiency. Control: complete nutrient solution (1 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Different letters shown in the error bars mean significant differences among treatments and ecotypes at $P < 0.05$.

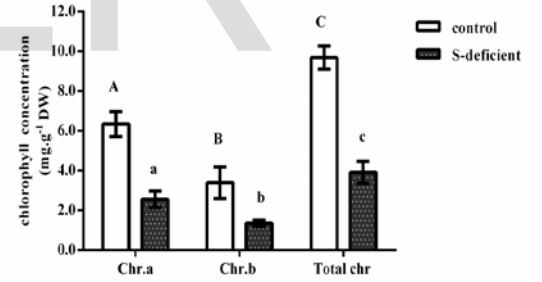

Fig. 2: Chlorophylls concentration in leaves of *Trigonella foenum graecum* submitted to sulfur deficiency. Control: complete nutrient solution (1 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Chl : Chlorophyll; Different letters shown in the error bars mean significant differences among treatments at $P < 0.05$. DW: dry weight

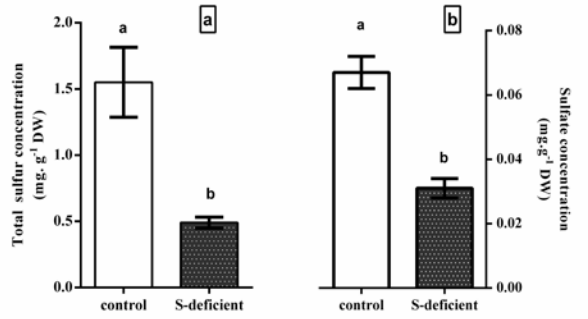

Fig. 3: (a) Total sulfur concentration, (b) Sulfates concentration in leaves of *Trigonella foenum graecum* submitted to sulfur deficiency; Control: complete nutrient solution (1 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Different letters shown in the error bars mean significant differences among treatments at $P < 0.05$. DW: dry weight

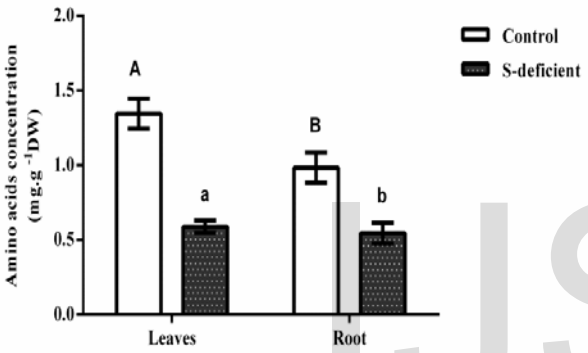

Fig. 4: Amino acids concentration in leaves and roots of *Trigonella foenum graecum* submitted to sulfur deficiency. Control: complete nutrient solution (1 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Different letters shown in the error bars mean significant differences among treatments and ecotypes at $P < 0.05$. DW: dry weight

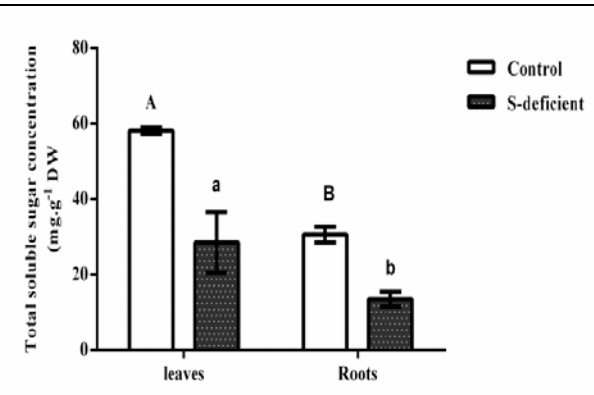

Fig. 5: Total soluble sugar concentration in leaves and roots of *Trigonella foenum graecum* submitted to sulfur deficiency. Control: complete nutrient solution (1 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Different letters shown in the error bars mean significant differences among treatments at $P < 0.05$. DW: dry weight

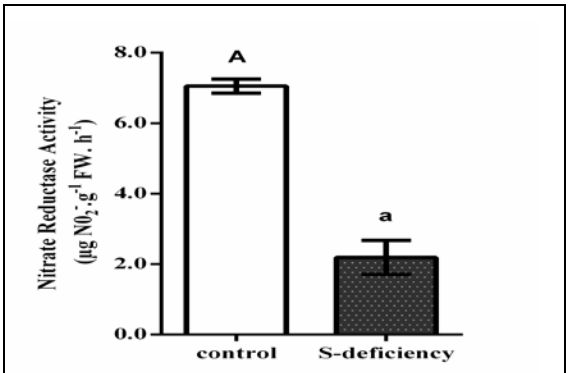

Fig. 6: Nitrate reductase activity in roots of *Trigonella foenum graecum* submitted to sulfur deficiency. Control: complete nutrient solution (1 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Different letters shown in the error bars mean significant differences among treatments and ecotypes at $P < 0.05$. FW: fresh weight.

Table 1: Concentrations of trace elements and macro elements in nutrient solutions (mol.m^{-3}). C: control plants ; -S: S-deficient plants

Nutriment element	P	K	N	S	Mg	Mn	Cu	Zn	Cl	Mo	Ca	Fe	B
C	1	4	3	1,005	1	0,003	0,0008	0,002	2,002	0,0001	1,05	0,03	0,01
-S	1	4	3	0,05	1	0,003	0,0008	0,002	2,002	0,0001	1,05	0,03	0,01