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Biomass Production, Chlorophyll Content and Morphorogical Parameters Are Affected by Sulfur Deficiency in *Eruca sativa* L.

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ABSTRACT:

Eruca sativa is one of the most cultivated brassica belonging to medicinal and aromatic species, The effect of sulphur deprivation on the growth and metabolism of *Eruca sativa* was investigated by measuring dry weights of the plant, chlorophyll content, total sulphur, leaf area, root/shoot ratio (R/S), Leaf Area Ratio (LAR), Specific Leaf Area (SLA) and leaf weight ratio (LWR). Significant decrease was observed in total dry weight of the overall plant. Both total sulphur and sulphate concentrations were markedly reduced in response to sulphur deficiency, significant decrease was recorded in chlorophyll concentration. Decreases were also observed in Leaf area and R/S ratio, while, a significant increase was recorded in LAR and SLA.

Key Words: Chlorophyll; *Eruca sativa*;, Leaf area; SLA; LAR; Sulfur Deficiency.

INTRODUCTION

Sulfur (S) is one of the most essential elements for plants growth and development, it plays crucial role of catalyst and regulator more than structural element, because it is less abundant than the other macronutrients (Marshner 2005). The essential of sulfur is taken up from the soil by the roots from which it is transported to the leaves to be reduced and incorporated in the synthesis of organic compounds (Hawkesford 2000). Cysteine is the first S-compound product after the assimilation of sulfate, then it served to the production of other compound such as methionine, S-adenosylmethionine, glutathione, thiamine, coenzyme-A, iron-sulfur centers, phytochelatines and glucosinolates (Leustek and al. 2000, Lewandowska and Sirko 2008). The sulfur is involved in many biochemical progress of the plant namely the biosynthesis and regulation of enzyme activities, the antioxidant protection of cells, the photosynthesis and respiration (Davidian and Kopriva 2010, Nikiforova et al. 2004, Rausch and Wachter 2005).

In the last decade, sulfur became a limiting factor of plant growth around the world (Scherer 2001). Several studies supported the occurrence of serious deficiency of sulfur content in agronomical crops. This is associated to 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 atmospheric pollution in industrialized areas to improve the quality of the air (Ceccotti 1995, Haneklaus et al. 2007, Scherer 2001). The concentration of SO_2 has been reported to decrease by 20tg between 1990 and 2011 (Klimont et al. 2013). The low availability of sulfur will undoubtedly influence the quality of cultivated plants and especially those belonging to the family of the Brassicaceae because of their high requirement of sulfur.

Several studies have reported that many biochemical and physiological responses were initiated upon exposure of plants to sulfur restriction, leading to a general reduction in metabolic activity including a decrease in biomass and increased root/shoot ratio especially in *Brassica napus* (Blake-Kalff et al. 1998), *Arabidopsis thaliana* (Akiko et al. 2005, Kutz et al. 2002), *Trifolium repens L* (Varin et al. 2010) and *Medicago truncatula* (Casieri et al. 2012, Gao et al. 2015). Sulfur deficiency also leads to a large decrease in chlorophylls content (Nikiforova et al. 2004 2005) compared with situations where S is in adequate supply. Plants submitted to S-deficiency can also modify their root morphological traits to maximize the acquisition of nutrients under nutrient-deficient conditions (Lopez-Bucio et al. 2003).

Eruca sativa belongs to the Brassicaceae family native to the Mediterranean area very rich in sulfur compounds as glucosinolates (GSLs) and isothiocyanates (ITCs) (Bennet et al. 2002, Kim, et al. 2004, Martinez-Sanchez et al. 2006), which are the origin of the anti-carcinogenic activity of *Eruca sativa* tested on mammals tissues (Lynn et al. 2006). The low availability of sulfur in the environment will affect certainly the biochemical quality and morphological parameters of *Eruca sativa*. In the present study, we highlight the effect of sulfur deficiency on the development of *Eruca sativa*, by analyzing the impact on biomass production, plant morphology and chlorophyll content in S deficient plants. These parameters changes would reflect a general disturbance in plant metabolism.

MATERIAL AND METHODS

Plant Material

The seeds of Rocket (*Eruca sativa*) 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 March and May, under natural light and photoperiod. The pots were divided into two batches of five and watered once per week for four weeks with 100 ml of modified nutrient solution described by Hoagland and Arnon (1950). The control plants received the complete nutrient solution containing 1 $mol.m^{-3}$ SO_4, while the S-deficient plants received the same solution but containing only 0.05 $mol.m^{-3}$ SO_4 (Table 1). The sampling was performed before the appearance of the flower (50 days). Harvested plants were thoroughly washed with tap water and then with distilled water to clean and remove surface filth.

Biomass and Morphological Parameters

Harvested plants were separated into leaves, stems and roots. Plant dry mass was obtained after drying at 80°C for 48 h. Total leaf area was measured by using the gravimetric method (Ross and Koppel 2000). The other parameters: root-shoot ratio (R/S), Leaf Area Ratio (LAR), Specific Leaf Area (SLA) and leaf weight ratio (LWR) were calculated as follows:

R/S = root dry mass/shoot dry mass
LAR = leaf area/total plant dry mass
SLA = leaf area/leaf dry mass
LWR = leaf dry weight/total plant dry mass

Sulfate and Total Sulfur

The plant material was thoroughly washed with tap water and then with distilled water to clean and remove surface dirt. 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 (HNO3) and perchloric acid (HClO4) in the ratio of 85:15, for 4h at 120°C. The digested solution was adjusted to 10 ml with distilled water and the sulfate

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

	Nutrient elements												
	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

concentration in the extracts was determined turbidimetrically (Astolfi and Zuchi 2013, Bardsley and Lancaster 1962). 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 standards of sodium sulfate solutions

Chlorophyll Content

30 mg of fresh leaves was incubated with 7 ml of dimethylsulfoxide (DMSO) in test tubes and placed in drying oven at 65°C for 30 min. After filtration the absorbance of extract was measured by using a spectrophotometer (VWR UV-6300PC spectrophotometer) at 663 and 645 nm. The concentrations of chlorophylls a, chlorophyll b and total chlorophyll were calculated according the following formulae (Arnon 1949, Hiscox and Israelstam 1979)

$$\text{Chl a (g L}^{-1}) = 0.0127\ A_{663} - 0.00269\ A_{645}$$
$$\text{Chl b (g L}^{-1}) = 0.0229\ A_{645} - 0.00468\ A_{663}$$
$$\text{Total Chl (g L}^{-1}) = 0.0202\ A_{645} + 0.00802\ A^{663}$$

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 $P<0.05$.

RESULTS

Effect of S-Deficiency on Total Sulfur, Sulfate and Chlorophyll Content

In the S-sufficient plants, the concentration of sulfur in leaves reached 13.2 mg.g^{-1}DM. The reduction in the sulfur supply caused an important decrease of 89% in the concentrations of sulfur in leaves. When compared to the control the concentration of total sulfur in leaves dropped from 13.2 mg.g^{-1} in control to 1.4 mg.g^{-1} in S-deficient plants (Figure 1). Under control conditions the pool of sulfate constituted 12.12 % of total sulfur in leaves. In S-deficient plants the concentration of sulfate decreased drastically in leaves. It has fallen from 1.6 mg.g^{-1} in control plants to 0.7 mg.g^{-1}, in S-deficient ones (Figure 2). The decrease in both sulfur and sulfate is significant according to the statistical test ($P<0.05$).

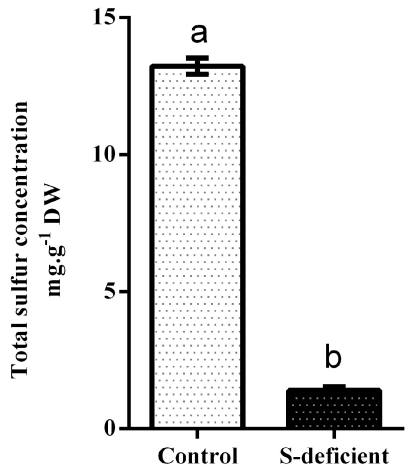

Figure 1. Total sulfur concentration in leaves of *Eruca sativa* subjected to sulfur deficiency; Control: complete nutrient solution (1,005 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Vertical bars are SE values of n=3. Different letters indicate values that are significantly different ($P < 0.05$).

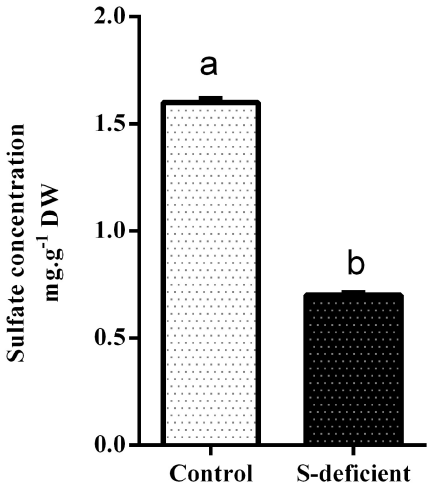

Figure 2. Sulfate concentration in leaves of *Eruca sativa* subjected to sulfur deficiency; Control: complete nutrient solution (1,005 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Vertical bars are SE values of n=3. Different letters indicate values that are significantly different ($P < 0.05$).

Under control conditions the total chlorophyll content in leaves was of 13.56 mg.g^{-1} DM. The limitation of S supply during the culture of plants showed a significant decrease in chlorophylls content (Figure 3). In the S-deficient plants the total chlorophyll content was only 4 mg.g^{-1} DM, exhibiting a decrease of 68% ($P<0.05$) when compared to control. Significant decreases ($P<0.05$) were recorded in both of chlorophyll a (-53%) and chlorophyll b (-73%) (Figure 3).

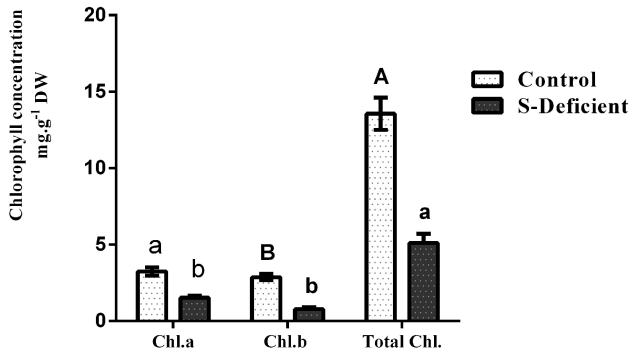

Figure 3. Chlorophylls concentration in leaves of *Eruca sativa* submitted to sulfur deficiency. Control: complete nutrient solution (1.005 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Vertical bars are the SE values of n=3 Chl: Chlorophyll. Different letters indicate values that are significantly different (P < 0.05).

Effects of Sulfur Deficiency on Biomass

The plants grown in S-sufficient medium present the highest biomass (249 mg.plant^{-1}) and the application of low-sulfur nutrition decreased the total dry weight by 83%. Significant drop ($p<0.05$) was recorded from 249 mg.plant^{-1} in control plants to 136 mg.plant^{-1} in S-deficient ones (Figure 4A). The analysis of different plants organs showed that S-starvation affected markedly both of roots and shoot. When compared to roots, the effect of S-deficiency was relatively more pronounced in leaves. In comparison with the control, S-starvation exhibited a decrease of 54.5% and 52% ($p<0.05$) in leaves and roots, respectively (Figure 4B and 4C). The effect of sulfur starvation on stem wasn't significant according to the statistical test ($p>0.05$), (Figure 4D).

The low availability of sulfur in the medium had remarkable effect on both lateral roots and taproot, when compared to control plants ($p<0.05$). The lateral roots were relatively more affected than the taproot with a decrease of 77.7% and 72.7%, respectively (Figure 4E and 4F).

Effect of Sulfur Deficiency on Morphology

The reduction of sulfur nutrition in *Eruca sativa* plants caused a small but a significant decrease in leaf area. The leaf area in S-deficient plants decreased by 16%, when compared to the control (Figure 5A). When expressed on leaf dry weight basis the results showed that specific leaf area (SLA) was higher in S-deficient plants. SLA has passed from 683.203 cm^2.g^{-1} in control plants to 1160.870 cm^2.g^{-1} in S-deficient ones ($p<0.05$) (Fig.5B). Under control conditions, the recorded LAR and LWR were 186.26 g.g^{-1} and 0.272 g.g^{-1}, respectively. In S-starvation conditions, the plants showed a small but significant decrease in LWR values of 11.7%, but the LAR increased by 33.5% ($p<0.05$), (Figure 5C and 5D). S-deficiency had also a significant effect on Root/Soot ratio, when compared with control plants ($p<0.05$). R/S decreased by 13% when the supply of S in the medium was limited (Figure 5E).

DISCUSSION

In response to reduced sulfur supply, both of sulfate and total sulfur concentrations in the leaves decreased markedly in S-deficient *Eruca sativa* plants. In S-sufficient plants the concentration of total sulfur was about 13.2 mg.g^{-1} and at low availability of S in the medium the sulfur pool in leaves decreased by 89%, the S concentration dropped to 1.4 mg.g^{-1}. This indicates the S-deficient state of *Eruca sativa* plants in our conditions of treatment. The critical value of sulfur under which the plant is supposed S-deficient varies among plant species and depends on several parameters: i) growth conditions, ii) development stage of sampling, iii) analyzed part of plant and iv) the S-compound species used for sulfur measurement. An overview of literature show that generally an insufficient sulfur supply is indicated by total sulfur concentration less than 1.7 mg.g^{-1} but in Brassicaceae vegetables, such as in the present study, plants are supposed S-deficient when this value is less than 7.5 mg.g^{-1} (Haneklaus et al. 2007). Several studies reported that low sulfur concentration in the medium decreased the concentration of internal S in leaves. That has been shown in tomato leaves (Xu et al. 1996), sugar beet shoots (Thomas et al. 2000), spinach leaves (Prosser et al. 2001), Arabidopsis leaves (Nikiforova et al. 2003 2005) and bean plants (Juszczuk and Ostaszewska 2011). S-sulfate is often the first metabolite to change in response to sulfur demand and its uptake and distribution to the leaves are closely regulated. In our investigation the reduction in sulfate supply (0.05 mM) exhibited a decrease by 52% in the sulfate content of leaves. This result is in agreement with those reported by Juszczuk et al. (2011) in bean plants and Blake-Kalff et al. (1998) in Oilseed Rape submitted to sulfur starvation. The reduction of SO_4 concentration in leaves in S-deficient plants could be explained by the release of sulfate ions

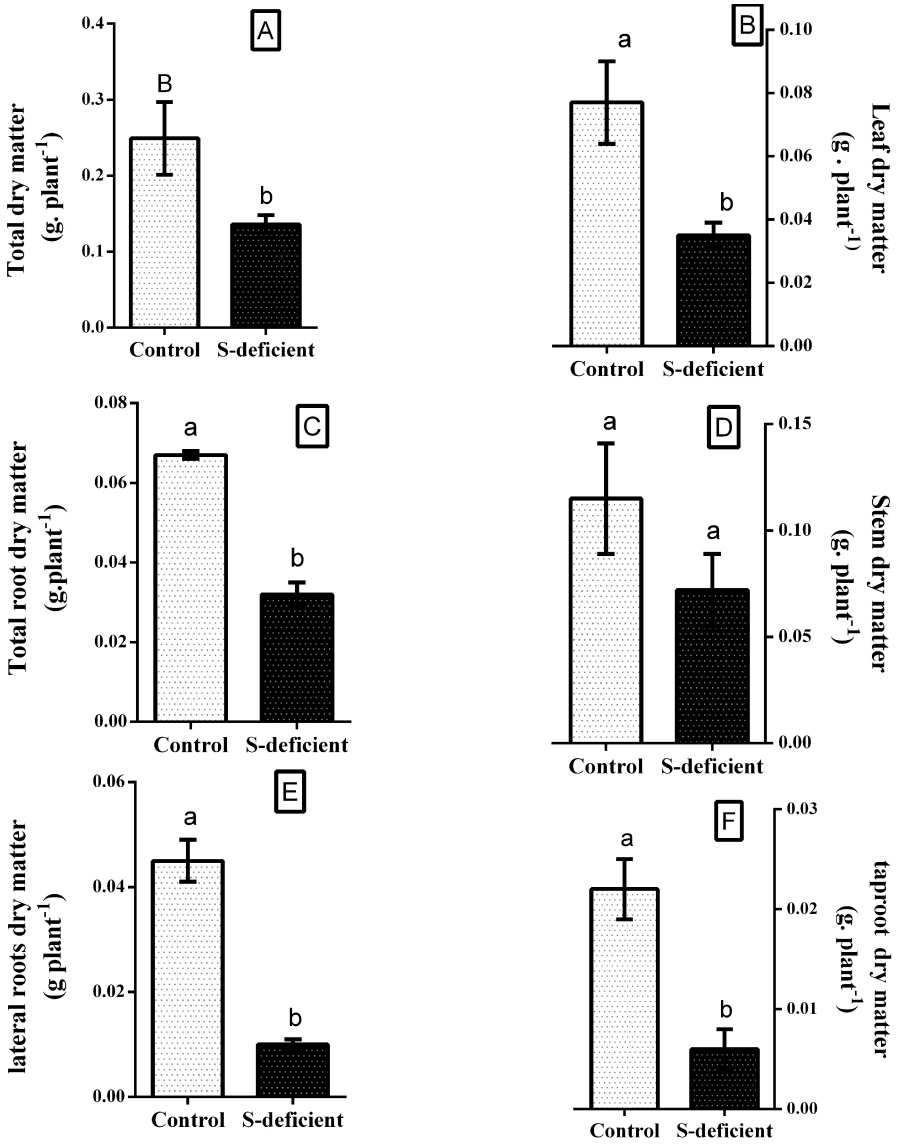

Figure 4. Effect of sulfur deficiency on biomass production of whole plant (A), leaves (B), total root (C), stem (D), lateral roots (E) and tap root (F) of *Eruca sativa*. Control: complete nutrient solution (1.005 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Vertical bars are the SE values of n=3; different letters indicate values that are significantly different ($P < 0.05$).

from vacuoles of mesophyll cells under prolonged S stress, but this release is supposed too slow to support new growth (Bell et al. 1995, Clarkson et al. 1983, Zhao et al. 1999). It was also reported in previous studies that in poor sulfur medium, the newly absorbed sulfur by plants was kept mainly in the roots (Abdallah et al. 2010). In *Brassica napus* L. grown in poor sulfur medium, less than 23% of S was moved to the leaves in S-deficient plants, whereas 55% of absorbed S was translocated to leaves when S supply was adequate (Abdallah et al. 2010, Granth and Hawkesford 2015).

The present study showed that the levels of chlorophyll in the leaves were influenced by the S-starvation, a decrease in the order of 68%, 53% and 73% was recorded in total chlorophyll, chlorophyll *a* and *b*, respectively. This reduction could be associated to the decrease in numerous S compounds in S-deficient plants, such as cystein, methionine and S-adenosylmethionine (SAM) (Hoefgen and Nikiforova 2008, Nikiforova et al. 2003, Prosser et al. 2001). Cysteine and Methionine are two rich amino acids which act as structural and functional elements of chloroplast targeted proteins (Droux

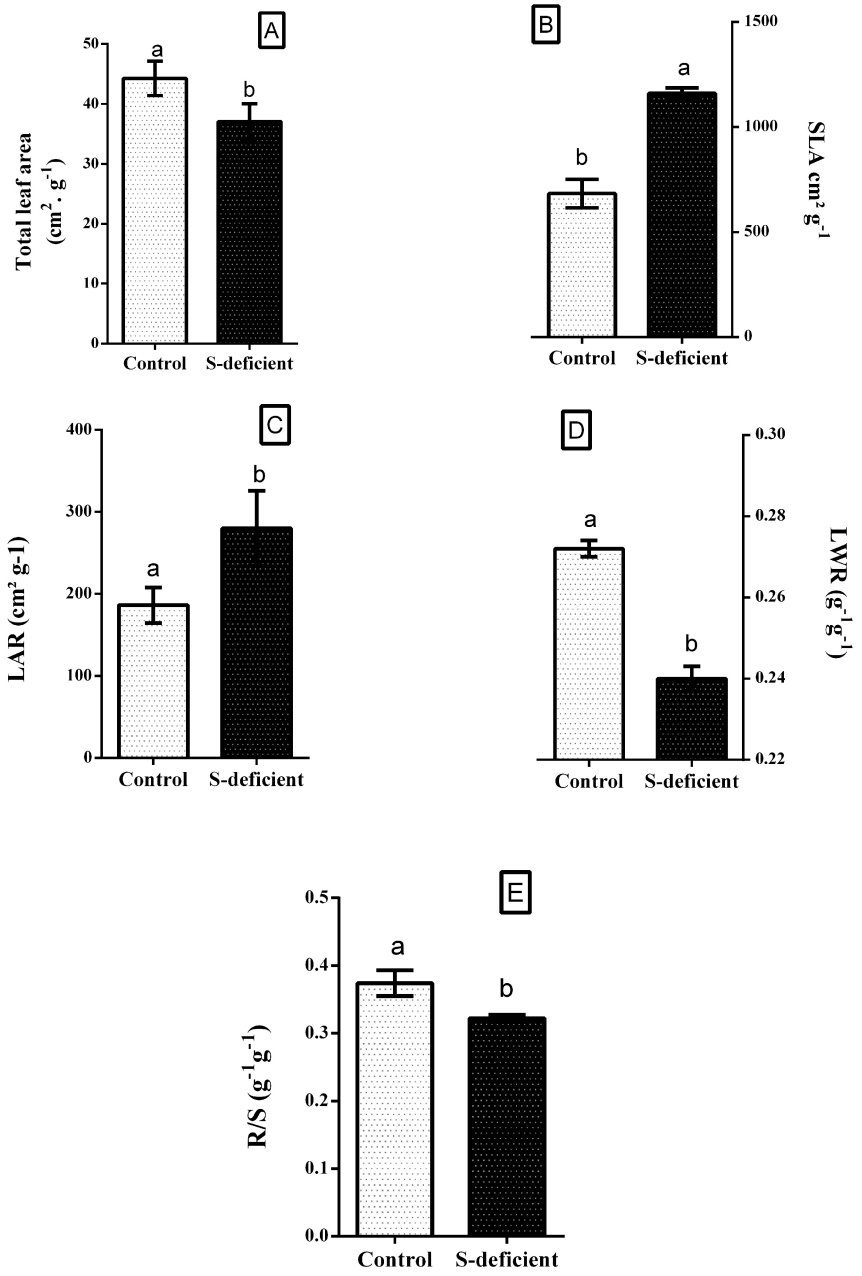

Figure 5. Effect of sulfur deficiency on Tolal Leaf Area (A), Specific Leaf Area (B), Leaf Weight Ratio (C), Leaf Area Ratio (D) and R/S ratio (E) of *Eruca sativa* submitted to sulfur deficiency. Control: complete nutrient solution (1.005 mol.m^{-3} of S); S-deficient: S-deficient nutrient solution (0.05 mol.m^{-3} of S). Vertical bars are the SE values of n=3; different letters indicate values that are significantly different ($P < 0.05$).

2004, Juszczuk and Ostaszewska 2011) and the reduction in sulfur supply may affect the levels of those amino acids which can affect the chlorophyll apparatus efficacy. The SAM is methionine derivate that is involved in the chlorophyll synthesis pathway as a methyl group donor (Hoefgen and Nikiforova 2008, Varin et al. 2010). The same result was recorded in Rice plants submitted to sulfur deficiency, the chlorophyll content was reduced by 48% following a serious decrease in *Rubisco* level, and a reduction of 61% in the

ability of PSI to produce the $NADP^+$ (Lunde et al. 2008). Furthermore, it is well known that the sulfur is a constituent of thioredoxin system and ferredoxin proteins and plays a crucial role in the coordination of Calvin cycle with light reaction in chloroplast (Leustek and Saito 1999, Saito 2004). Similar results of low chlorophyll content under sulfur starvation have been reported in *Morus* alba (Tewari et al. 2010), *Phaseolus vulgaris* (Juszczuk and Ostaszewska 2011), *Zea mays* (Pagani and Echeverría 2012) and *Lycopersicum esculentum* (Kassem et al. 2015). The S induced disruptions of CO_2 fixation system would explain clearly changes occurred in biomass production and morphological parameters recorded in S-deficient plants when compared to control. The total dry matter of *Eruca sativa* was negatively affected by poor sulfur supply, a reduction in dry weight was recorded in both aerial and underground parts. The inhibitory effect of sulfur deficiency on growth of *Eruca sativa* is due to a decrease in the synthesis of sulfur-containing metabolites mostly in *Eruca sativa* as a plant of Brassicaceae family. Several other works have shown this negative effect of S-deficiency on the overall development of the plant in *Brassica napus* (Blake-Kalff et al. 1998), *Arabidopsis thaliana* (Akiko et al. 2005, Kutz et al. 2002), *Trifolium repens* L. (Varin et al. 2010) and *Medicago truncatula* (Casieri et al. 2012 , Gao et al. 2015). This slowdown in the development of the plant suggests the importance of sulfur in the biochemical progress. In plants submitted to S-deficiency a decrease of biomass, proteins level, chlorophyll and cellular S/C/N ratio imbalance were observed in several works **(**Juszczuk and Ostaszewska 2011, Gao et al. 2015 Thomas et al. 2000, Zhao et al. 1999**)**. The reduction of dry weight was relatively more pronounced in leaves than in root, this is in agreement with the suggestion that more metabolites are allocated from shoot to roots under S deficiency conditions and it's could be associated to maintain the ability of roots to acquire mineral nutrient (Gao et al. 2015, Scherer et al. 2008,). The same modification in roots/shoot ratio was recorded in plants submitted to Mg, P and N starvation (Dan et al. 2007, Hermans et al. 2006, Kutz et al. 2002, Lopez-Bucio et al. 2003, Nikiforova et al. 2003).

The environmental conditions and nutrient status of plants affect directly the morphology of leaves, which play a crucial role in the promotion of whole plant growth. The relationship between leaf area growth and total plant growth in terms of mass depend not only on photosynthetic rate but also on how carbon is partitioned among new leaf area, leaf and root biomass, production and respiration (Weraduwage et al. 2015). In the present work, S-deficiency affected significantly the leaf morphology of *Eruca sativa.* The reduction of leaf area is probably due to the reduction in division of mesophile cells. In wheat, the sulfur deficiency was accompanied by a 10-fold decrease in the number and size of mesophile cells (Burke et al. 1986). The effect of sulfur deficiency on Leaf area ratio (LAR) was relatively small but significant. LAR wish indicate the assimilative capacity of the plant was increased by sulfur deficiency. This result means that the decrease in total leaf area (-16%) was less important than the decrease in biomass of the whole plant (-83%).

Leaf thickness is related to the efficiency of resource acquisition, this is supposed to impact seriously the function of leaf. Light absorbed by the leaf, diffusion pathway of CO2 through leaf tissues, photosynthetic and growth rate are all depending on leaf thickness (Syvertsen et al. 1995, Nielsen et al. 1996, Garnier et al. 1999). The leaf weight expressed per unit of plant biomass (LWR) decreased which increased SLA and lower thickness of leaves. These results suggested that *Eruca sativa* submitted to S deficiency changed its morphology by increasing the LWR and reducing leaf thickness. These observations were probably associated to a low availability of metabolites required for biomass leaf production and/or changes in their partitioning between area growth and leaf thickness. This statement could be supported by numerous disruptions observed in leaves of S-deficient plants such as content of chlorophyll, disturbance in PSI and PSII, decreased photosynthetic activity, and reduced activity of *Rubisco* (Kassem et al. 2015, Lunde et al. 2008, Juszczuk and Ostaszewska 2011). These changes of morphological features in total biomass partitioning have been shown to occur when plants are submitted to environmental constraints (Erice et al. 2010, Guendouz et al. 2016). They reflect the plasticity of plants and might constitute an adaptive strategy of plants submitted to S deficiency.

CONCLUSION

We showed in this study that, at application of sulfur stress, the plants act negatively in terms of growth and biomass production. Significant reduction has been recorded in the biomass of all the plant parts, but the aerial part (leaves and stem) remains the most affected by sulfur restriction. The chlorophyll content was also influenced by S-deficiency; the leaves of *Eruca sativa*

grown in S-deficient environment have the lowest chlorophyll content. The leaf area and the specific leaf ratio were also reduced under S -starvation. While the specific leaf area increased markedly. These results will present a basis for other investigations more prolonged that will enable us to better understand the response of *Eruca sativa* towards sulfur deficiency by studying other biochemical parameter in the context of their impact on the rate of production and the pharmacological quality *Eruca sativa*.

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