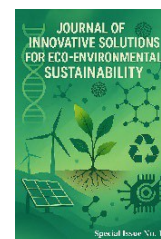




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Research Article

Effect of Foliar Application of Processed Sulfur Fertilizer on Morphological and Antioxidant Characters of *Medicago sativa* L.

Homa Mahmoodzadeh^{1*}, Mahda Mehraban², Amir Hossein Samerei¹

¹Department of Biology, Ma. C, Islamic Azad University, Mashhad, Iran.

² Management Department, Faculty of Economics and Administrative Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

Sulfur plays a crucial role in the synthesis of proteins, chlorophyll, and specific amino acids in plants. This study explores the impact of processed sulfur fertilizer on leaf and stem length, relative growth rate (RGR), quercetin content, and antioxidant levels in *Medicago sativa* L. through a greenhouse pot study conducted using a completely randomized design over 60 days. The experiment involved four levels of sulfur fertilizer (0, 7.5, 10, and 12.5 mL L⁻¹) applied across three replications. Statistical significance level $p \leq 0.05$ was determined by Duncan's test. The findings showed significant improvements in growth parameters, RGR, and quercetin antioxidant content with sulfur fertilization, particularly at 10 and 12.5 mL L⁻¹ dosages. On average, sulfur fertilizer enhanced leaf length by 86.66% and stem length by 52.38% compared to the control. Additionally, positive and significant correlations between sulfur-induced growth metrics and yield emphasize its importance in improving the productivity and quality of *M. sativa*. Sulfur application increased the SPAD number, RGR, antioxidant levels, and quercetin content by 61.90%, 89.47%, 50%, and 1450%, respectively. Therefore, processed sulfur fertilizer emerges as a promising practice for achieving higher yields and economic benefits from *M. sativa* cultivation.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a perennial herbaceous legume recognized globally for its superior nutritional value, high yield potential, and adaptability across diverse growing conditions. As an essential forage crop, alfalfa serves as a key protein source for livestock, encompassing dairy cattle, beef cattle, horses, sheep, goats, and other domestic animals. It is cultivated in over 80 countries on an area exceeding 35 million hectares, with global production approximating 436 million tons as of 2006[1]. Characterized by deep roots capable of reaching up to 4 meters and as much as 7–9 meters in well-drained soils alfalfa plants feature erect or decumbent stems reaching heights of up to 1 meter. Leaves are trifoliate with obovate leaflets measuring 10–45 mm in length and 3–10 mm in width. Its inflorescences consist of oval or rounded racemes with 5–40 yellow, blue, or purple flowers. The plants produce curly pods containing 2–8 seeds that

transition from green to brown upon maturation. Numerous cultivars exist, selected for traits such as winter hardiness, drought resistance, tolerance to heavy grazing (e.g., Spanish "Mielga" cultivars), or resistance to pests and diseases. Current breeding goals also include enhancing feeding value through better digestibility and improved fiber content [2]. Fertilizer application serves as an effective approach to optimize seedling development and overall plant quality [3]. Properly managed fertilization can rapidly adjust soil nutrient levels, increase nutrient availability to plants, promote vegetative growth, and enhance the synthesis and accumulation of plant metabolites. Fertilizers replenish essential minerals in the soil to aid terrestrial cultivation, stimulating photosynthesis rates, growth in height, root diameter expansion, and overall plant volume [4, 5]. Inorganic sulfur plays an indispensable role in facilitating optimal plant growth as well as the biosynthesis of critical compounds such as proteins and chlorophyll. Since plants absorb sulfur mostly as sulfate ions,

* Corresponding author.

Email: homa.mahmoudzadeh915@gmail.com

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most sulfur-containing fertilizers consist of sulfate salts. In recent decades, sulfur deficiencies have garnered increased attention due to their negative impacts on soil fertility, crop quality, and yield potential. A scarcity of sulfur results in slower growth rates, reduced leaf sizes, and chlorosis symptoms in plants. For healthy plant development, sulfur requirements comprise approximately 0.1–0.5% of dry biomass weight. Furthermore, sulfur shortages can disrupt chloroplast functionality due to insufficient sulfur levels within these organelles [6]. Such deficiencies markedly reduce photosynthetic efficiency but can be mitigated gradually through supplemental sulfur applications [7]. Research has demonstrated significant benefits from sulfur fertilization in crops such as date palm [8]. Kumar et al. (2022) reported that 20 kg/ha S application gave the highest growth and yield attributes in cluster bean. The significantly higher growth attributes, such as plant height, number of branches per plant, leaf area index, etc are increased with increasing sulphur levels [3]. Although sulfur is considered a macronutrient in most ecosystems under natural conditions, its availability can occasionally be limited. Studies estimate that roughly 100 million hectares globally suffer from sulfur deficiency in soil. Reductions in atmospheric sulfur emissions, combined with shifts toward concentrated mineral fertilizers, have compounded the scarcity of sulfur within agricultural systems, ultimately leading to widespread symptoms of deficiency among crops. Consequently, adequate sulfur supplementation via fertilizers becomes increasingly vital for optimal yields and improved crop quality. Elemental sulfur fertilizer stands out in agricultural applications due to its high sulfur concentration. Unlike other mineral fertilizers that contain sulfur, it offers the most concentrated form, which helps reduce application costs. This type of fertilizer is particularly advantageous in regions where rainfall exceeds evaporation since it relies on water to aid its gradual distribution in the soil. Once applied, elemental sulfur releases sulfur slowly, ensuring a steady supply for plants throughout their entire growing period while minimizing losses caused by sulfates (VI) leaching into the soil. Consequently, under specific conditions, elemental sulfur can effectively function as a slow-release fertilizer [9, 10]. The effects of foliar-applied sulfur fertilizer on alfalfa growth factors are currently not clearly understood and need further investigation. Considering these factors, this study focuses on assessing the impact of varying levels of sulfur fertilizer on the growth, leaf yield, relative growth rate, and antioxidant and quercetin content of *Medicago sativa*.

2. Materials and Methods

This study investigated the effects of processed sulfur fertilizer on the morphological and antioxidant properties of *Medicago sativa* L. The experiment was conducted using a completely randomized design with three replications and four fertilizer concentrations (0, 7.5, 10, and 12.5 mL L⁻¹ of sulfur fertilizer). Each pot contained 10 *Medicago* seeds, planted at a depth of 1 cm, and was maintained in a greenhouse environment with regular irrigation. The temperature ranged between 25 and 30 °C under a daily photoperiod of 16 hours of light and 8 hours of darkness. The seeds used for the study were sourced from the Pakan Isfahan Seed Company. The chemical composition of the “processed sulfur fertilizer” was sulfur powder, KOH and Ferula extract. The fertilizer solutions were prepared in wash bottles, and foliar sprays were applied weekly. Treatment processes were recorded during each application, and fresh fertilizer solutions were prepared prior to every spray. Distilled water was used as a control in the experiment. The study lasted for 60 days, during which five foliar spray treatments were administered. Growth measurements, including the total length of leaves and shoots, were taken every 20 days for 5 plants per replication and documented in centimeters.

2.1. Chlorophyll Measurement

Two months into the study, prior to initiating any harvesting procedures, the chlorophyll content was assessed using the SPAD-502 meter (manufactured by MINOLTA Co., Ltd., Japan) for each experimental replica.

2.2. Harvesting

Following two months of treatment, plants were carefully harvested. Shoots and roots were separated for further analysis and parameter recording.

2.3. Relative Growth Rate

The total shoot length was recorded during the first and fourth weeks, and the RGR was computed using a specified formula [3].

$$RGR = \frac{2.303 \times \log_{10}(L_2/L_1)}{t_2 - t_1}$$

where L_1 = shoot length at first week;
 L_2 = shoot length at fourth week;
 t_1 = first week;
 t_2 = fourth week.

2.4. Preparation of Plant Extract

Air-dried and finely ground plant material (80 mesh, 20 g per sample) was subjected to extraction using an aqueous ethanol solution (ethanol: water, 80:20 v/v) for six hours at room temperature in an orbital shaker (Gallenkamp, UK). The extracts were separated from the residual material by filtration through Whatman No. 1 filter paper. The residual material underwent two additional rounds of extraction using fresh solvent, and the resulting extracts were pooled together. These combined extracts were then concentrated, and the solvent was removed under reduced pressure at 45 °C using a rotary evaporator (EYELA, SB-651, Rikakikai Co. Ltd., Tokyo, Japan). The final dried crude extracts were weighed to determine yield and stored at -4 °C in a refrigerator until further analyses.

2.5. Estimation of Total Flavonoid Content

The total flavonoid content (TFC) was quantified using a standard method with minor modifications. For the assay, 1 mL of sample solution (1 mg mL⁻¹) was combined with 4 mL of distilled water and 0.3 mL of 5% NaNO₂ solution. After allowing the mixture to react for 5 minutes, 0.3 mL of 10% AlCl₃ solution was introduced and incubated for an additional 5 minutes. Subsequently, 2 mL of 1M NaOH was added, and the solution was thoroughly mixed. After a total incubation time of 30 minutes, the absorbance was measured at 510 nm against a blank solution. The flavonoid content determination was performed in triplicates and expressed as milligrams of quercetin per 10 grams of plant extract. A calibration curve based on quercetin was utilized for this calculation.

2.6. DPPH Assay for Antioxidant Activity in *Medicago* Extracts

The free radical scavenging activity (RSA) of diluted *Medicago* extracts was assessed via the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. To prepare the stock solution, 24 mg of DPPH were dissolved in 100 mL of methanol. Filtration of the stock solution through methanol produced a workable mixture with an approximate absorbance of 0.973 at 517 nm. For testing, a sample of 3 mL DPPH solution was mixed with 100 µL of the leaf extract in a test tube, while 3 mL of DPPH with 100 µL of methanol served as the standard control. The tubes were then incubated in complete darkness for 30 minutes. Absorbance measurements were taken at 517 nm, and the percentage of

antioxidant activity or RSA was calculated using the corresponding formula:

$$\% \text{ of antioxidant activity} = \frac{Ac - As}{Ac} \times 100$$

where: Ac—Control reaction absorbance; As—testing specimen absorbance.

2.7. Statistical Analysis

The statistical evaluation was performed with analysis of variance (ANOVA) and Duncan's mean difference comparison test, utilizing the SPSS statistical software package version 18. Visual representations were created using Microsoft Excel 2023.

3. Results and Discussion

The application of varying sulfur (S) rates and the progression of the growth period had a significant impact on the leaf length of *Medicago* plants, as detailed in Table 1. A significant increase $P < 0.05$ in leaf length was observed, particularly at 60 days after treatment (DAT). When compared to the control group, the addition of different S levels resulted in an increase in leaf length by 11.2 cm. Among the treatments, the tallest plant was noted under the 12.5 mL L⁻¹ application, while the shortest plant occurred in the control group.

Table 1. Effects of different levels of sulphur on the Leaf length of *Medicago* at different days after transplanting (DAT)

S fertilizer concentration (mL L ⁻¹)	20 DAT	40 DAT	60 DAT
0	3.2± 0.5 ^c	5.1±0.9 ^c	5.9±1.3 ^c
7.5	7.2±0.8 ^b	8±1.8 ^b	9.8±2.1 ^b
10	8.3±1.2 ^a	9.3±0.8 ^a	10.4±1.9 ^a
12.5	6.1±0.4 ^b	8.8±1.5 ^b	11.2±2.3 ^a

Means in each column followed by similar letters are not significantly different at the 5% probability level using Duncan's multiple range test.

S fertilizer significantly $P < 0.05$ enhanced the stem length of *Medicago*, as shown in Table 2, with the growth steadily increasing alongside higher S concentrations up to 12.5 mL L⁻¹. The greatest stem length was observed in plants treated with 12.5 mL L⁻¹ of S, which was significantly greater compared to all other tested levels, while the shortest stem length was recorded in the control group.

Table 2. Effects of different levels of sulphur fertilizer on the stem length of *Medicago* at different days after transplanting (DAT).

S fertilizer concentration (mL L ⁻¹)	20 DAT	40 DAT	60 DAT
0	17 ± 3.5 ^c	18 ± 2.9 ^c	21 ± 1.9 ^c
7.5	20 ± 4.8 ^b	23 ± 4.8 ^b	26 ± 4.1 ^b
10	24 ± 3.2 ^a	26 ± 2.8 ^a	32 ± 2.9 ^a
12.5	25 ± 4.4 ^a	27 ± 2.5 ^a	33 ± 4.3 ^a

Means in each column followed by similar letters are not significantly different at the 5% probability level using Duncan's multiple range test.

Table 3 illustrates that Sulfur (S) fertilizer had a significant impact on SPAD values. Different concentrations of S fertilizer

resulted in notable variations in SPAD measurements. The average SPAD values recorded were 21, 27, 33, and 26 for the control group and the S-treated plants, respectively. The application of varying levels of S fertilizer also had a marked effect on the relative growth rate (RGR) of *Medicago* plants. A steady increase in RGR was observed with rising S fertilizer concentrations up to 10 mL L⁻¹, after which higher doses (12.5 mL L⁻¹) caused a decline. The highest RGR of 1.8 was noted at 10 mL L⁻¹, while the lowest RGR of 0.9 was found in the untreated control plants. Moreover, *Medicago* plants subjected to different levels of S fertilizer were analyzed for their quercetin content, measured in milligrams per 10 grams of plant extract. As detailed in Table 3, the control group contained 2 mg 10 g⁻¹ of quercetin, whereas treated plants with 7.5, 10, and 12.5 mL L⁻¹ of S fertilizer exhibited quercetin levels of 12, 22 and 32 mg 10 g⁻¹, respectively.

Table 3. Effects of different levels of sulphur fertilizer on the SPAD, RGR and Quercetin content of *Medicago*.

S fertilizer concentration (mL L ⁻¹)	SPAD number	RGR	Quercetin content (mg 10 g ⁻¹ extract)
0	21± 2.5 ^c	0.9± 0.1 ^c	2± 0.1 ^d
7.5	27±3.9 ^b	1.4±0.2 ^b	12±2.2 ^c
10	33±4.1 ^a	1.8±0.25 ^a	22±4.25 ^b
12.5	26±1.4 ^b	1.6±0.3 ^b	32±3.3 ^a

Means in each column followed by similar letters are not significantly different at the 5% probability level using Duncan's multiple range test.

When the free-radical compound DPPH interacts with an unpaired electron, its strongest absorption occurs at 517 nm, producing a distinct purple color. When a free-radical scavenger antioxidant reacts with DPPH, it reduces the radical to form DPPHH, which exhibits lower absorbance at the same wavelength due to reduced hydrogen presence. This transition from the radical DPPH to DPPHH results in noticeable decolorization, shifting toward a yellow hue as more electrons are captured. Table 4 highlights that *Medicago* plants treated with 12.5 mL L⁻¹ of sulfur fertilizer exhibited an absorbance of 0.458 at 517 nm, with antioxidant activity measured at 53.69%—higher than the control group, which demonstrated an absorbance of 0.986 at the same wavelength. Notably, antioxidant activity significantly improved with S fertilizer up to a concentration of 10 mL L⁻¹ but showed a decline at the 12.5 mL L⁻¹ application level.

Table 4. Antioxidant activity of *Medicago* plants treated with S fertilizer (DPPH test).

S fertilizer concentration (mL L ⁻¹)	Absorbance at 517 nm		% of Antioxidant
	Control	Sample	
0	0.986	0.541	45.131
7.5	0.923	0.467	49.404
10	0.990	0.402	59.393
12.5	0.989	0.458	53.690

Calculation of Antioxidant activity of *Medicago* with DPPH; % of antioxidant activity (Control plants) = $[(Ac - As) \div Ac] \times 100$; % of antioxidant activity = $[(0.986 - 0.541) \div 0.986] \times 100$; % of antioxidant activity = 45.131

Calculation of Antioxidant activity of *Medicago* with DPPH; % of antioxidant activity (7.5mL L⁻¹ of S fertilizer) = $[(Ac - As) \div Ac] \times 100$; % of antioxidant activity = $[(0.923 - 0.467) \div 0.923] \times 100$; % of antioxidant activity = 49.404

Calculation of Antioxidant activity of *Medicago* with DPPH; % of antioxidant activity (10 mL L⁻¹ of S fertilizer) = $[(Ac - As) \div Ac] \times 100$;

% of antioxidant activity = $[(0.990 - 0.402) \div 0.990] \times 100$; % of antioxidant activity = 59.393

Calculation of Antioxidant activity of *Medicago* with DPPH; % of antioxidant activity (12.5mL⁻¹ of S fertilizer) = $[(Ac - As) \div Ac] \times 100$; % of antioxidant activity = $[(0.989 - 0.458) \div 0.989] \times 100$; % of antioxidant activity = 53.690

Sulfur is a vital macronutrient for plant growth and metabolism. Its deficiency during cultivation can profoundly impact plant morphology, resulting in smaller and fewer leaves, reduced biomass, and impaired development. Sulfur is integral to the synthesis of amino acids like cysteine, as well as enzymes, chlorophyll, proteins, and vitamins. These processes support vital metabolic activities in plants, including enzyme regulation, photosynthesis, respiration, and antioxidant metabolism, such as glutathione-mediated removal of reactive oxygen species. Research has shown that sulfur deficiency hampers chlorophyll production and photosynthetic efficiency across various plant species. This study corroborates earlier findings by Abdalla et al. (2021), where lettuce growth was stimulated through sulfur supplementation in hydroponic systems [11]. Similarly, Chung et al. (2022) demonstrated enhanced fresh weights in hydroponically grown lettuce ('Cheongchima') treated with high sulfur levels (2 mM or 64 mg L⁻¹) compared to controls [12]. Nawirska-Olszańska (2021) found that the antioxidant properties, determined both as ABTS and DPPH radical-scavenging activities, were highest in cabbage harvested from the plot treated with potassium sulfate [13]. Slightly lower activities were found in cabbage from the control plot, which also exhibited the lowest bioactive component content determined. Higher levels of vitamin C, phenolics, and chlorophyll a and b were detected in cabbage treated with potassium sulfate, while a higher concentration of carotenoids was seen when elementary sulfur and ammonium sulfate were used. Muscolo et al. (2020) observed similar improvements in red onion quality when sulfur-enriched fertilizers derived from agricultural waste were applied. Antioxidant activity indicators such as DPPH, ORAC, and ABTS were elevated in treated plants. Notably, polyphenol concentrations increased across all treatments, while sulfur compounds dominated the volatile profiles due to higher levels of S-alkenyl cysteine sulfoxides—the precursors of the aroma compound thiosulfinate. The study concluded that SB-OR-enriched fertilizers were most effective in enhancing red onion quality and antioxidant properties [14]. Research by Akhgar et al. (2024) explored the interaction between nitrogen and sulfur fertilizers alongside a growth regulator on rapeseed ('Hyola 401') traits. Treatments included pure nitrogen fertilizer at varying levels (0, 90, 180, or 270 kg ha⁻¹), sulfur fertilizer (0, 35, or 70 kg ha⁻¹), and the growth regulator (Shootex) at rates of 0 and 200 mL L⁻¹. Results indicated that combining 270 kg ha⁻¹ nitrogen with 70 kg ha⁻¹ sulfur yielded the highest seed and oil outputs (4081 and 1135 kg ha⁻¹ respectively), whereas the lowest yields (1086 and 368 kg ha⁻¹) were observed with sulfur application alone under no nitrogen fertilizer [15]. This highlights the importance of synergistic application of nitrogen and sulfur fertilizers alongside appropriate growth regulators for optimal rapeseed cultivation. Quercetin, a well-studied flavonoid present in fruits and vegetables, is renowned for its health benefits, including potent antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer properties. Its antioxidant action mitigates oxidative stress by scavenging free radicals and preserving cellular integrity. Quercetin also suppresses inflammatory cytokine production, providing therapeutic potential for inflammation-related conditions. Importantly, it has demonstrated anticancer efficacy by inhibiting cancer cell growth and inducing apoptosis. Its cardiovascular benefits include lowering blood pressure, improving endothelial function, and reducing cholesterol levels, making it a promising candidate for disease prevention [16-20]. In this research project, the application of sulfur fertilizers significantly boosted quercetin content by an astounding 1450% compared to control samples. This remarkable enhancement illustrates the effectiveness of sulfur fertilizers in increasing valuable secondary metabolites like

quercetin in plants a finding with considerable implications for both agricultural production and human health applications.

4. Conclusion

Applying different concentrations of sulfur (S) fertilizer significantly impacted the growth parameters, including leaf and stem length, SPAD number, relative growth rate (RGR), and the antioxidant as well as quercetin content of *M. sativa*. The highest leaf and stem lengths were recorded in plants fertilized with 12.5 mL L⁻¹ of S fertilizer. On average, sulfur application increased leaf length by 86% and stem length by 52% compared to control plants. Furthermore, noticeable improvements were observed in SPAD values, RGR, and the antioxidant and quercetin content when compared to untreated plants. These findings suggest that farmers could achieve economically advantageous yields of *M. sativa* by applying 12.5 mL L⁻¹ of S fertilizer although RGR and antioxidant activity peaked at 10 mL L⁻¹. However, further research is warranted to confirm these results across diverse soil types and climatic conditions.

Author Contributions

Mahmoodzadeh designed the study and wrote the first draft. Samerei and Mehraban performed the lab tests. All authors approved the final version of the manuscript.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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