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Research Article

Effect of Exogenous Sulfur on Hydrogen Peroxide, Ammonia and Proline Synthesis in White Clover (*Trifolium repens* L.)

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ABSTRACT

Sulfur is an essential element in plants, including amino acids, vitamin synthesis, and acting as an antioxidant. However, the interaction between endogenous sulfur and proline synthesis has not been yet fully documented. White clover (*Trifolium repens* L.) is known as a species highly sensitive to sulfate supply. Therefore, this study aimed to elucidate the role of sulfur in regulating proline metabolism in relation to ammonia detoxification and hydrogen peroxide (H_2O_2) accumulation in white clover. The detached leaves of white clover were immersed in solution containing different concentration of sulfate (0, 10, 100, and 1000 mM $MgSO_4$). As $MgSO_4$ concentrations were increased, the concentration of H_2O_2 increased up to 2.5-fold compared to control, accompanied with H_2O_2 detection in leaves. Amino acid concentrations significantly increased only at higher levels (100 and 1000 mM $MgSO_4$). No significant difference was observed in protein concentration. Proline and $\triangle^1$-pyrroline-5-carboxylate (P5C) concentrations slightly decreased at 10 and 100 mM $MgSO_4$ treatments, whereas it rapidly increased over 1.9-fold at 1000 mM $MgSO_4$ treatment. Ammonia concentrations gradually increased up to 8.6-fold. These results indicate that exogenous sulfur levels are closely related to H_2O_2 and ammonia synthesis but affect proline biosynthesis only at a higher level.

(Key words: Ammonia, Hydrogen peroxide, Oxidative Stress, Proline, Sulfur)

Ⅰ. INTRODUCTION

Sulfur (S) is an essential macronutrient for crop production. This nutrient is a structural component of disulfide bonds in proteins, amino acids (glutathione, methionine, cysteine), vitamins and secondary metabolites (Nakai and Maruyama-Nakashita, 2020). Plants absorb it in the form of sulfate (SO_4^{2-}) through the roots and assimilated in the leaves. Sulfate is converted to adenosine-5'-phosphosulfate (APS) by ATP sulfurylase (ATPS) and then reduced to sulfite (SO_3^{2-}) by the enzyme APS reductase (APR). Sulfite reductase (SiR) converts sulfite to sulfide and reacts with O-acetylserine lyase (OAS-TL) to form cysteine, which is then combined with glycine and glutamate to form glutathione (GSH) (Lee et al., 2011). GSH acts as an antioxidant to remove reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2).

Numerous studies have suggested regulatory interactions between S assimilation and N metabolism in higher plants (Koprivova et al., 2000; Zhang et al., 2015). S metabolism is closely related to N nutrition, and the N metabolism is strongly affected by the plant's S status. Sulfur application increases nitrogen assimilation in cadmium-stressed wheat (Khan et al., 2015). Nitrate increases sulfur uptake and assimilation-related gene expression, resulting in the increase of sulfur assimilation rate (Hesse et al., 2004). A decreased activity of sulfur assimilation-related enzymes, ATPS, APR, and OASTL, were recovered with nitrate application (Koprivova et al., 2000). Previous our works, an increase in soluble nitrogen content and a reduction in the internal S pool under S- deficiency have been observed (Lee et al., 2013a).

Proline, an amino acid, is synthesized from glutamic acid via intermediate $\triangle^1$-pyrroline-5-carboxylate (P5C). The reaction is catalyzed by P5C synthetase (P5CS) and P5C reductase (P5CR). It has been identified in the tolerance of plants to various kinds of environmental stresses. Proline accumulation contributes to maintaining osmotic balance (Lee et al., 2013b), detoxification of ammonia (Kim et al., 2004), scavenges excess free radicals (Lee et al., 2022), and regulates cellular redox

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potential (La et al., 2019). Proline synthesis is indirectly related to GSH biosynthesis because of both GSH and proline share glutamate as a common biosynthesis precursor (Moat et al., 2003). For example, proline is involved in the regulation of NADP/NADPH cycling which affects regulation in reduction of GSSG to GSH, resulting in maintenance of an increased level in reduced GSH and GSSG/GSH ratio (Hasanuzzaman et al., 2014). However, little information is known about the potential metabolic interrelationships of GSH with proline synthesis.

White clover (*Trifolium repens* L.) is a key species in grasslands. It is known as a species highly sensitive to sulfate supply. In the present study, we aimed to elucidate the role of sulfur in regulating proline metabolism in relation to ammonia detoxification and H_2O_2 accumulation in white clover. The responses of H_2O_2, proline, and ammonia to different $MgSO_4$ feeding levels (0. 10, 100, and 1000 mM) were examined in detached leaves of white clover.

Ⅱ. MATERIALS AND METHODS

1. Plant culture and treatments

White clover (*Trifolium repens* L.) were grown under greenhouse condition for 10 weeks. Fifty trifoliate leaves were randomly selected and cut at 2 cm above stolon base. The detached leaves were immersed in different concentration of $MgSO_4$ solution (0, 10, 100, and 1000 mM). After 24 h of external feeding, leaf tissues were collected and quickly ground with liquid nitrogen, and stored at -80℃ deep-freezer for further analysis.

2. Determination of hydrogen peroxide (H_2O_2) contents and visualization.

Hydrogen peroxide contents were determined according to Alexieva et al. (2001). Leaves tissue (approximately 200 mg) was homogenized with 0.8ml 0.1%(w/v) TCA on ice and centrifuged at 12,000 × g for 10 min at 4℃. Supernatant, 10 mM potassium phosphate buffer (pH 5.8) and 1 M potassium iodide were mixed and kept in a dark condition for 1 h. The absorbance was recorded at 390 nm and calculated using the H_2O_2 standard curve. For detection of H_2O_2, the detached leaves infiltrated under a gentle vacuum with 1 mg/ml 3,3'-diaminobenzidine containing 0.05% (v/v) tween 20, and 10 mM sodium phosphate buffer (pH 7.0) until the appearance of brown spots. Then, the leaves were boiled until the chlorophyll was completely bleached. The leaf samples were photographed (Lee et al., 2013b).

3. Measurement of $\triangle^1$-pyrroline-5-carboxylate (P5C) and proline.

For the determination of P5C and proline, fresh leaf was extracted with 3% (w/v) sulfosalicylic acid and centrifuged at 12,000 × g for 10 min. The supernatants were mixed with 10 mM 2-aminobenzaldehyde dissolved in 40% (v/v) ethanol, and then incubated at 37℃ for 2 h until yellow color appears. The absorbance was recorded at 440 nm and calculated according to the extinction coefficient 2.58 mM^{-1} cm^{-1} (Lee et al., 2022). Proline content was determined according to the method reported by Lee et al. (2009). The supernatants were mixed with ninhydrin solution containing acetic acid and 6 M H_3PO_4 (v/v, 3:2) and then mixture was boiled at 70℃ for 1 h. After cooling, toluene was added to mixture, strongly vortexed, and incubated for 24 h. The absorbance determined at 520 nm and calculated with proline as the standard.

4. Determination of amino acid and protein.

Amino acids content was determined using the ninhydrin colorimetric method (Lee et al., 2021). Fresh leaves were extracted with 100 mM potassium phosphate buffer (pH 7.0) and centrifuged at 12,000 × g for 10 min at 4℃. The supernatant was mixed with ninhydrin solution and heated at 100℃ for 10min. Then 50% ethanol was added and mixed. The absorbance was determined at 570 nm. Protein content was determined using the Bradford reagent (Sigma B6916, St. Louis, MI, USA), by applying bovine serum albumin as a standard curve.

5. Determination of ammonia.

For ammonia determination, microdiffusion of ammonia in a Conway dish was performed (Lee et al., 2013b). After microdiffusion for 18 h, the solution was collected from the center of Conway dish, reacted with Nessler's ammonium color reagent, and then quantified using the correlation equation of

the standard curve.

6. Statistical analysis

Four treatments were completely of randomized design with four replicates. Fifty detached leaves with petiole of white clover plants represented a replicate. Duncan's multiple range test was employed to compare the means of separate replicates. All statistical tests were performed using SAS 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

Ⅲ. RESULTS

1. H_2O_2 visualization and contents in detached leaves of white clover

Accumulation of H_2O_2 in leaf tissues showed as deep brown spots and large amount of spots were significantly observed as the concentration of $MgSO_4$ treatment increased (Fig. 1A). This confirmed H_2O_2 concentration in leaves. The concentration of H_2O_2 gradually increased and reached 2.3-fold higher in 1000 mM $MgSO_4$ treatment than that in control (Fig. 1B). H_2O_2 visualization of leaf discs confirmed that the amount and range of H_2O_2 spots widened as the $MgSO_4$ concentration increased.

2. Amino acid and protein concentrations in detached leaves of white clover

Amino acid concentrations were not significantly different in 10 mM $MgSO_4$ treatment compared to control, whereas it enhanced by 2- and 4-fold in 100 and 1000 mM $MgSO_4$ treatments respectively (Fig. 2A). Protein concentrations showed no significant difference in all treatments (Fig. 2B).

3. $\triangle^1$-pyrroline-5-carboxylate (P5C), proline and ammonia concentrations in detached leaves of white clover

P5C and proline concentrations were slightly decreased at 10 and 100 mM $MgSO_4$ treatments but were not significant (Fig. 3A and B). However, their concentrations were highly increased by 1.9- and 2.1-fold at 1000 mM $MgSO_4$ treatment compared to control, respectively. Ammonia concentration in the leaf tissues gradually increased by increasing the external feeding level of NH_4Cl (Fig. 3C). Ammonia concentration was 2.3-, 3.6-, and 8.6-fold higher in 10, 100, and 1000 mM $MgSO_4$ treatments, respectively, compared to control.

Ⅳ. DISCUSSION

As expected, cysteine and GSH concentrations in detached

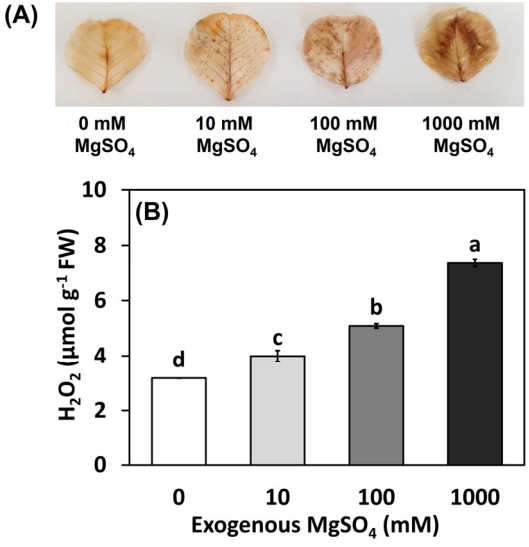

Fig. 1. Influence of exogenous $MgSO_4$ (0, 10, 100, and 1000 mM) on visualization of H_2O_2 and concentration of H_2O_2 in detached leaves of white clover. All measurements were made 24 h after treatment. The data was presented mean ± SE (n = 4). Bars labeled with the different letters are significantly different (p<0.05) according to Duncan's multiple range test.

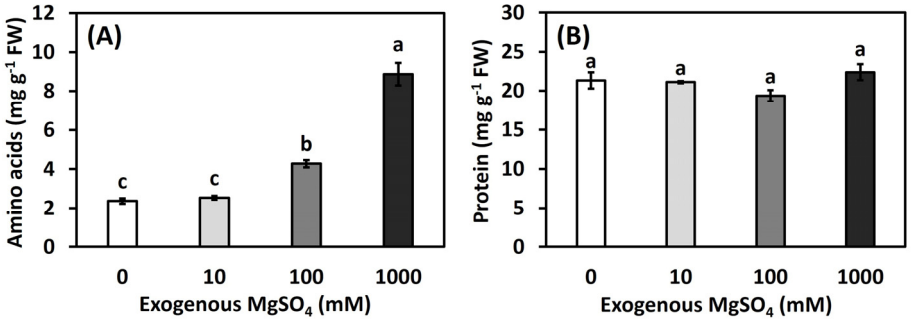

Fig. 2. Influence of exogenous $MgSO_4$ (0, 10, 100, and 1000 mM) on concentration of amino acids (A) and protein (B) in detached leaves of white clover. All measurements were made 24 h after treatment. The data was presented mean ± SE (n = 4). Bars labeled with the different letters are significantly different (p<0.05) according to Duncan's multiple range test.

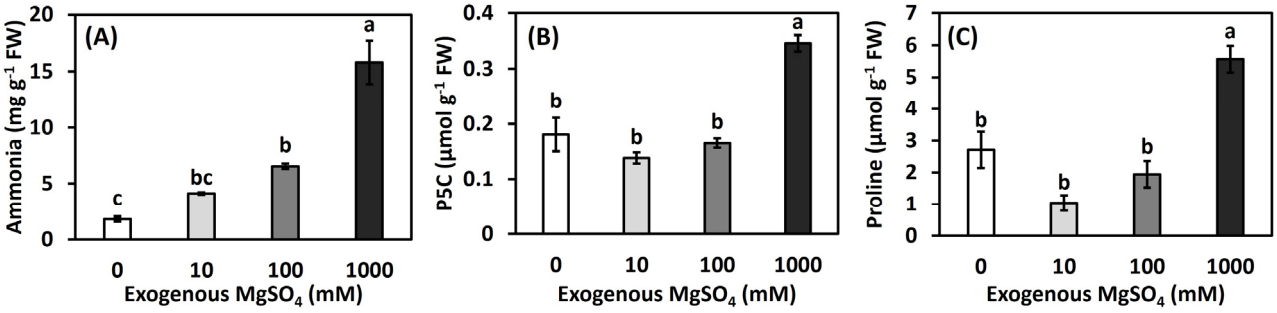

Fig. 3. Influence of exogenous $MgSO_4$ (0, 10, 100, and 1000 mM) on concentration of $\triangle^1$-pyrroline-5-carboxylate (P5C) (A), proline (B), and ammonia (C) in detached leaves of white clover. All measurements were made 24 h after treatment. The data was presented mean ± SE (n = 4). Bars labeled with the different letters are significantly different (p<0.05) according to Duncan's multiple range test.

leaves of white clover were gradually increased with increasing concentration of $MgSO_4$ feeding treatment, indicating that sulfate assimilation was enhanced by exogenous sulfate (data not shown). In addition, amino acids concentration was significantly increased by exogenous 100 and 1000 mM $MgSO_4$ treatments but did not increase protein content (Fig. 2). The enhanced amino acids may be due to the accumulation of exogenous sulfate-nduced sulfur containing amino acids (cysteine and methionine), and promotion of nitrogen assimilation.

However, detached leaves showed wilting caused by excess sulfate (1000 mM $MgSO_4$ treatment). In addition, endogenous H_2O_2 concentration and its localization with dark spots in leaves were observed in different concentrations of $MgSO_4$ treatments for 24 h (Fig. 1). H_2O_2, as a reactive oxygen species (ROS), is one of the indicators that can confirm the initial signaling of oxidative stress (Park et al., 2021). This finding is consistent with a previous report showing that a high level of S supply in onion seeding induced increase of H_2O_2 and lipid peroxidation concentrations (Chandra and Pandey, 2014). On the other hand, a low level of H_2O_2 plays as a signaling molecule in regulating redox signaling, which coincides with a low concentration of proline, as suggested by Lee et al. (2022).

It is well known that proline accumulation is generally a response to abiotic stress. Based on the data obtained during exogenous $MgSO_4$ treatments, excess sulfate (1000 mM $MgSO_4$) only highly enhanced proline and P5C concentration (Fig. 3A and B). Previous results reveal that drought or exogenous H_2O_2 increases proline accumulation (La et al., 2019; Lee et al., 2022). Additionally, it has been reported that stress-induced H_2O_2 responsive proline accumulation is related to scavenge ROS (Rejeb et al., 2014; Rehman et al., 2021). Besides, proline feeding caused a reduction in the cellular H_2O_2 under H_2O_2-induced oxidative stress (Sorkheh et al., 2012). This proline accumulation is positively related to excess ammonia production which is attributed to the decrease in *de novo* protein synthesis (Kim et al., 2004; Lee et al., 2009). In the present study, significant

increases in ammonia concentration were observed in exogenous $MgSO_4$ treatments but not in protein synthesis (Figs. 2B and 3C).

Taken together, these results indicate that exogenous sulfate supply showed positive relationships with endogenous H_2O_2 and ammonia concentration, which is associated with proline accumulation to oxidative stress and ammonia detoxification when sulfate concentration is high.

Ⅴ. ACKNOWLEDGMENTS

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