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Molybdate Alters Sulfate Assimilation and Induces Oxidative Stress in White Clover (*Trifolium repens* L.)

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ABSTRACT

Molybdenum (Mo) in rhizosphere influences sulfate assimilation as well as a number of other physiological aspects. In this study, the activity of key enzymes in sulfate assimilatory pathways, such as ATP sulfurylase (ATPs), adenosine 5'-phosphosulphate reductase (APR), as well as the responses of reactive oxygen species (ROS), were analyzed to elucidate the metabolic and physiological effects of external Mo supply to detached leaves of *Trifolium repens* L. Mo supply with a range from 1 mM to 40 mM depressed the activity of ATPs throughout the entire time course. In the leaves exposed to 1 mM Mo, a continuous decrease in the activity of ATPs was confirmed by Native-PAGE. The APR activity was also declined by Mo treatment. The accumulation of H_2O_2 and $O_2^{\bullet-}$ were not significant up to 10 mM Mo, whereas a remarked accumulation was detected under 40 mM Mo supply. The data suggest that an external supply of Mo has an inhibitory effect on sulfate assimilation, and induces oxidative stress only at an extremely high concentration.
(**Key words :** Molybdate, Oxidative species, Sulfate assimilation, *Trifolium repens*)

Ⅰ. INTRODUCTION

In higher plants, sulfur (S) is mainly taken up in the form of inorganic sulfate, which is mediated by sulfate transporters localized in plasma membrane of roots. Once enter cell, it is activated by ATP sulfurylase (ATPs), the first enzyme of sulfate assimilation, to form adenosine 5'-phosphosulfate (APS). It has been suggested that ATPs is the rate limiting step for sulfate metabolism, overexpression of this enzyme resulted in several folds of increase in ATPs activity (Hell and Mendel, 2010). Subsequently APS is reduced to sulfite, sulfide, and finally incorporated into Cys. The process from APS to sulfite is catalyzed by APS reductase (APR). Earlier study found that the activity of APR was the lowest among enzymes of sulfate reduction pathway and it was more sensitive for the shifts of S status within plants (De Kok et al., 2005), APR thus has been considered as a key enzyme in the regulation of sulfate metabolism.

Molybdenum (Mo) is an essential micronutrient for micro-organisms, plants and animals. Plants take up Mo from soil in the form of molybdate (MoO_4^{2-}) by specific transporters (Schiavon et al., 2012). In plant or algae, molybdate-transporting proteins, MOT1 and MOT2, have been identified recently. The two carriers were characterized with ultrahigh affinity through cell membrane (Mendel, 2013). Once inside of cell, molybdate is activated by bonding to protein to form Mo-cofactor (Moco), subsequently Moco is absorbed into molybdenum enzymes such as nitrate reductase (NR) and sulfite oxidase (SO) as a part of the active center (Mendel, 2013). More than fifty molybdenum-dependent enzyme have been found, and the majority of them were identified in bacteria, while only seven in eukaryotes (Zhang and Gladyshev, 2008). However, high concentration of Mo in rhizosphere could depress sulfate uptake and assimilation (Reuveny et al., 1977; Schiavon et al., 2012), which further lead to S deficiency for plant growth. In a previous study in mulberry plants, deficient supply of S induced oxidative stress (Tewari et al., 2010). Therefore, it can be suggested that the external supply of Mo is a possible inducer of

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oxidative stress.

In plants, reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are generated as by-products of aerobic metabolism. These ROS are strong oxidizing agents which lead to oxidative damage to lipids, proteins, DNA and eventually cause cell death (Uttara et al., 2009). Low levels of ROS accumulate constantly by metabolism, but they increase when plants are exposed to abiotic stresses such as osmotic stress, drought, salinity, and high light (Atkinson and Urwin, 2012). As a strong oxidant, H_2O_2 can initiate a localized oxidative damage to leaf cells resulting in a disruption of metabolic processes and break cellular integrity triggering leaf senescence (Upadhyaya et al., 2007).

Taken together, we hypothesize that Mo involves in the regulation of sulfur metabolism and could induce oxidative stress. To test this hypothesis, the responses of sulfate assimilatory enzymes activities along with ROS accumulation were analyzed to the different supply levels of in detached leaves of *T. repens*.

Ⅱ. MATERIALS AND METHODS

1. Plant and treatment

The second or/and third full expanded leaves were collected from apex of stolon of field grown white clover (*Trifolium repens* L.), and three or four petioles of the detached leaf were immersed in a 50 mL falcon tube, containing 0.2 mM K_2SO_4, 1 mM NH_4NO_3, 0.5 mM KH_2PO_4, 0.5 mM K_2HPO_4, 0.5 mM $MgCl_2$, 14 μmol H_3BO_3, 5 μmol $MnSO_4$, 3 μmol $ZnSO_4$, 0.7 μmol $(NH_4)_6Mo_7O_{24}$, 0.7 μmol $CuSO_4$ and 0.1 μmol $CoCl_2$. For a short term of Mo treatment, the detached leaves were exposed to different concentration of Na_2MoO_4 with 1, 10, 20, 40 mM for 3, 6 and 9 h. For a longer Mo treatment, 1 mM Mo was applied for 10, 20 and 36 h. The Mo treatments were done in a greenhouse with a day/night mean temperature of 27/18°C, and a relative humidity of 65/80%. Natural light was supplemented by metal halide lamps which generated approximately 400 μM photons $m^{-2}s^{-1}$. For sampling, fresh blades were frozen immediately in liquid nitrogen and stored in deep freezer −80°C for further analysis.

2. ATP sulfurylase activity and staining

Fresh tissues were quickly ground under liquid nitrogen in pre-cooled mortars. About 100 mg fresh ground samples was homogenized in 0.6 mL extract buffer containing 10 mM Na_2EDTA, 20 mM Tris-HCl (pH 8.0), 2 mM DTT and 1% PVP with an agitator and centrifuged at 12,000 rpm for 10 minutes at 4°C. Protein concentration was determined using the method of Bradford (1976). ATP sulfurylase (ATPs) activity (EC 2.7.7.4) of crude extracts from detached leaves of white clover was determined by spectrophotometry method as described by Osslund et al. (1982). One unit of ATPs activity was defined as 1 μmol Pi liberated per minute.

For Native-PAGE analysis, electrophoresis was performed at 120 V under 4℃. Ninety microgram crude proteins were loaded on 10% gels. After running, gels were rinsed by the solution containing 200 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM Na_2MoO_4 and 0.5 U pyrophosphatase. The incubation of gels was made in the same solution, added with 5 mM ATP, at 40℃ for 30 min. The inorganic phosphorus released in the gel was sufficiently rinsed with distilled water and immersed in a minimal volume of phosphate precipitation reagent (PPR). In a few minutes, white bands of precipitated phosphate appeared, indicating the location of enzyme (Simonović et al., 2004).

3. APR activity determination

To determine the activity of APR activity (EC 1.8.4.9), about 100 mg fresh ground samples were extracted with 1 mL 100 mM Tirs-HCl, pH 8.0 containing 1 mM EDTA. After centrifugation at 12,000 rpm for 10 min, the supernatant was used for the measurement of APR activity. The activity of APR was measured with ferricyanide as the electron acceptor and followed the decrease in absorbance at 420 nm (Barrie et al., 1994). A decrease in absorbance of 1 corresponds to the reduction of 2.65 μmol of ferricyanide for an assay volume of 2.65 mL and one unit of enzyme catalyzes the reduction of 1 μmol of ferricyanide per minute.

4. H_2O_2 and $O_2^{\bullet-}$ determination

H_2O_2 was determined by the method as descripted by Lin and Kao (2001). Around 100 mg well-ground fresh tissues were extracted by 50 mM K-phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000 rpm for 10 min. Then 0.3 mL crude extraction was mixed with 0.1 mL 0.1% titanium chloride in 20% H_2SO_4, and followed by a centrifugation at 12,000 rpm for 5 min. The intensity of yellow color of the supernatant was measure at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}.

The detection of $O_2^{\bullet -}$ was determined by hydroxylamine oxidation (Wang and Luo, 1990). Around 200 mL crude extraction was mixed with 0.36 mL 50 mM K-phosphate buffer (pH 7.8) and 0.04 mL NH_2OH-HCl, the mixture was incubated at 25℃ for 20 min. Then it reacts with 0.2 mL 17 mM sulfnilamind and 0.2 mL 7 mM α-naphthylamine under 25℃ for 20 min. The absorbance in the aqueous solution was record at 530 nm. A standard curve with $NaNO_2$ was used to calculate the production rate of $O_2^{\bullet -}$ from the chemical reaction of $O_2^{\bullet -}$ and hydroxylamine.

5. Statistical Analysis

Tukey's studentized range test was used to compare the means of three replicates. Unless stated otherwise, conclusions are based on differences between the means, with a significant level at $p< 0.05$ by using SAS 9.1.3 software.

Ⅲ. RESULTS

1. Effects of molybdate on sulfate assimilatory enzyme

The addition of 1mM Mo led to a 49% decrease in ATPs activity after 3 h treatment (Fig. 1A). And 10, 20 and 40 mM Mo as well significantly depressed ATPs activity with a decrease of 53%, 55% and 60% when compared with control. However, it was notable that the various level of Mo ranging from 1 mM to 40 mM showed very much similar depression of ATPs activity (Fig. 1A). A similar pattern was exhibited at 6 and 9 h after treatment (Fig. 1B and C).

The activity of APR was quickly depressed by 87% to 90% within 3 h of Mo external supply (Fig. 2A). After 6 or 9 h of Mo exposure, a similar down-regulate tendency was observed (Fig. 2B and C).

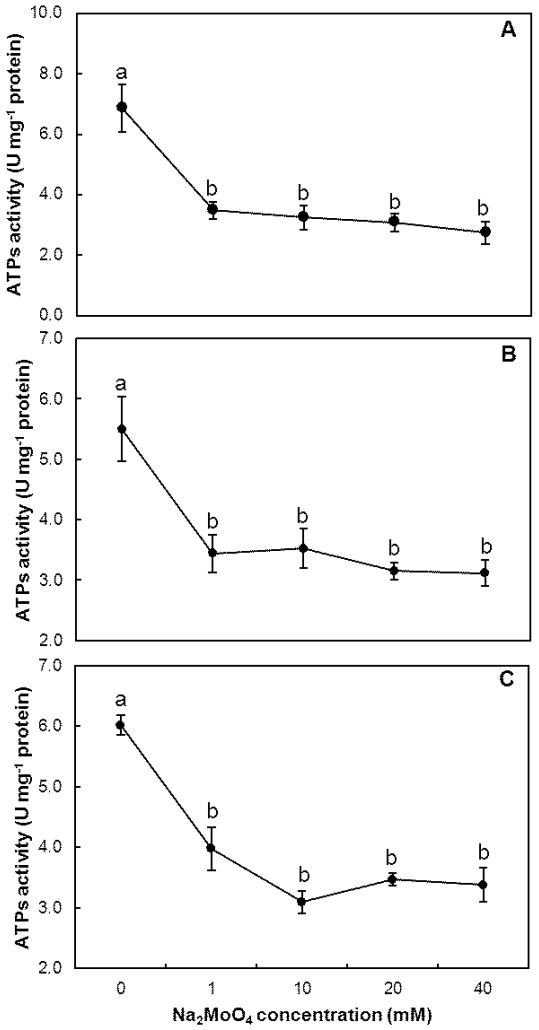

Fig. 1. Changes of ATP sulfurylase activity in the detached leaves of *T. repens*. Detached leaves were exposed to different concentration of Mo for 3 h (A), 6 h (B) and 9 h (C). All values are means ± SE of triplicates. Different letters indicate significant different at $p<0.05$ according to the Tukey's studentized range test.

To visibly assess ATPs activity *in vitro*, electrophoresis was performed by using fresh crude extraction. For 1 mM Mo treatment, ATPs protein was depressed gradually with a progress of exposing time up to 36 h (Fig. 3).

2. Oxidative species were induced by exogenous Mo supply

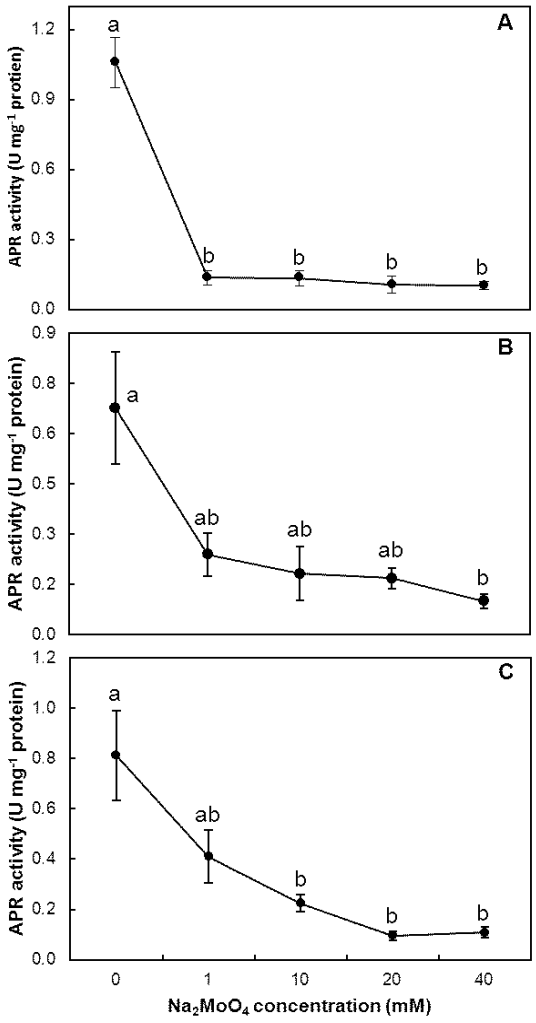

Fig. 2. Changes of APR activity in the detached leaves of *T. repens*. Detached leaves were exposed to relative different concentration of Mo for 3 h (A), 6 h (B) and 9 h (C). All values are means ± SE of triplicates. Different letters indicate significant different at $p<0.05$ according to the Tukey's studentized range test.

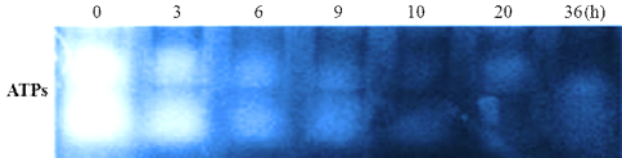

Fig. 3. Native-PAGE analysis of ATP sulfurylase. Detached leaves of *T. repens* were exposed to 1 mM Mo for 36 h.

To assess whether exogenous Mo induces oxidative stress or not, the concentration of H_2O_2 and $O_2^{\bullet-}$ were assayed. The concentration of H_2O_2 and $O_2^{\bullet-}$ was not significantly changed when Mo supply was below 1 mM regardless of exposing time (Table 1). However, a significant accumulation of ROS generally were observed when Mo supply was over 10 mM with a progress of exposing time. For the leaves exposed to 40 mM Mo for 9 h, the concentration of H_2O_2 and $O_2^{\bullet-}$ was increased by 22% and 471%, respectively, compared with control (Table 1).

Ⅳ. DISCUSSION

Molybdate is a structural analog of sulfate, thus exogenous supply of this metalloid to plants intends to alter sulfate uptake and subsequent metabolism. In present study, both ATPs and APR activities were decreased by Mo supply. Similar results were observed in the tobacco cells (Reuveny, 1977). In addition, Schiavonet et al. (2012) found in *Brassica juncea* that the rate of sulfate uptake was significantly decreased after 24 h when Mo was supplied with a concentration equaled to that of sulfate, while the internal sulfate distributed in roots and leaves seemed more susceptible to excess of Mo, representing a decrease from 1 h onwards. Additionally, Mo feeding sharply decreased cysteine content only 10 min after Mo treatment; in contrast, glutathione was not repressed until 24 h of Mo treatment in leaves. Reuveny (1977) observed that Mo repressed ATPs in cells grown with sulfate, but not the cells grown with Cys. Moreover, it was evidence that sulfur flux through sulfate assimilation was controlled by APR of 72% (Scheerer et al., 2010), accompanied with a massive decrease in APR activity as like in our study. Therefore, it can be suggested that sulfate flux into the detached leaves of *T. repens* might be restricted by exogenous Mo. Overall, it clearly indicates that excess of Mo could involve in down regulation of sulfate assimilation via the restriction of sulfate influx as well as the decrease in sulfate assimilatory enzymes activity.

Mo was transported by Sultr5;2 (also named MOT1) belonging to a member of sulfate transporter superfamily, and it was mainly expressed in endodermic and stele cells of root (Schiavon et al., 2012). After absorption, Mo served as a substrate of the reaction catalyzed by ATPs, but there was no molybdenum-containing product from this reaction due to adenosine 5'-phosphomolybdate was either not formed

Table 1. The concentration of H_2O_2 and $O_2^{\bullet -}$ in detached leaves of *T. repens* exposed to different Mo concentrations

Na_2MoO_4 concentration (mM)	0	1	10	20	40
H_2O_2 (μmol g^{-1} FW)					
3 hours	31.4 ± 1.5^{b}	34.0 ± 1.5^{ab}	33.6 ± 2.1^{ab}	34.2 ± 1.5^{ab}	36.8 ± 2.1^{a}
6 hours	30.0 ± 0.5^{c}	31.0 ± 1.6^{bc}	34.5 ± 3.4^{ab}	34.2 ± 1.1^{ab}	35.5 ± 1.2^{a}
9 hours	30.5 ± 0.1^{b}	31.0 ± 0.5^{b}	33.9 ± 1.1^{ab}	36.5 ± 4.4^{a}	37.3 ± 1.6^{a}
$O_2^{\bullet -}$ (nmol g^{-1} FW)					
3 hours	1.1 ± 0.3^{b}	1.2 ± 0.2^{b}	1.2 ± 0.6^{b}	2.0 ± 0.9^{b}	4.9 ± 0.8^{a}
6 hours	0.6 ± 0.0^{b}	0.7 ± 0.2^{b}	1.1 ± 0.3^{b}	3.6 ± 0.4^{a}	4.0 ± 0.4^{a}
9 hours	0.7 ± 0.2^{b}	0.6 ± 0.2^{b}	1.3 ± 0.3^{b}	3.4 ± 0.7^{a}	4.0 ± 0.5^{a}

Data represent the means ± SE of three replicates. Values in each row followed by different letters are significantly different at $p<0.05$ according to the Tukey's studentized range test.

or not stable (Reuveny, 1977). It has been speculated that Mo could compete with sulfate ion for sulfate transporter and ATPs enzyme resulting in a lower sulfate metabolism and wasteful use of ATP (Hale et al., 2001; Shinmachi et al., 2010). *MOT1* was up-regulated in *B. juncea* plants supplied with Mo, and the increase of Mo transporter accompanied with an accumulation of Mo in both roots and leaves (Schiavon et al., 2012). Therefore, it can be assumed that there was a raise in content of Mo in the molybdate-treated detached leaves in this study. Previous study revealed that excess Mo induced physiological disorders and changes metabolic pathways, and consequently was reduced plant growth and thus productivity (Rout and Das, 2002; Schiavon et al., 2012). In this study, either 1 or 10 mM Mo failed to increase ROS concentration in detached leaves of *T. repens*. The probable reason is that anthocyanin, which is produced in response to stress (Chalker-Scott, 1999), could sequestrate vaculolar Mo, thus avoiding toxic from high Mo to vital biochemical processes in cells (Hale et al., 2001).

In summary, excess of Mo involves in depression of ATPs and APR activity, and it could be used as an inhibitor of sulfate assimilation.

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