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Proline partially overcomes excess molybdenum toxicity in cabbage seedlings grown *in vitro*

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ABSTRACT. *In vitro* grown cabbage (*Brassica oleracea* var. *capitata*) seedlings exposed to excess molybdenum (Mo) ions exhibited severely reduced plant growth at the cotyledonary stage. Adding 80 mM proline (Pro) to the Mo-treated medium could help 50% seedlings to overcome the toxicity and grow true leaves. Under excess Mo stress, seedlings accumulated blue/purple anthocyanin in their cotyledons and hypocotyls. The anthocyanin content under Mo with 40 mM Pro was 4-fold higher than the control medium, MS with 40 mM Pro. The presence of Pro in the excess-Mo condition reduced chlorophyll *a*, whereas the chlorophyll *b* content was much higher than the control media of MS with and without Pro. Moreover, supplementing various concentrations of Pro into the Mo-stressed condition promoted the seedlings with higher antioxidant enzyme activities of superoxide dismutase, ascorbate peroxidate, and catalase. In addition, genes in the anthocyanin biosynthesis and accumulation pathways, phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), flavonone 3-hydroxylase (*F3H*), leucoanthocyanidin dioxygenase (*LDOX*), and glutathione-*S*-transferase (*GST*), were all upregulated. Our study indicated that, under excess Mo stress, the

antioxidant activity of cabbage seedlings was induced in an attempt to protect plants from the Mo-induced toxicity and exacerbated growth. Pro, on the other hand, functioned in producing higher antioxidant enzyme activity to partially help recover plant growth.

Key words: Anthocyanin; Chlorophyll; Growth inhibition; Antioxidant enzyme activity; Gene upregulation

INTRODUCTION

Molybdate ($Na_2MoO_4 \cdot H_2O$) is an essential element required for the growth of plants during their life cycle (Kaiser et al., 2005). Mo is distributed throughout the environment and in the soil. It has been used widely for industrial stainless steel, mining, cast iron, fertilizer manufacture, and agricultural activities (Mendel, 2005). Unlike cationic metals, the availability of Mo to higher plant species generally increases with a higher soil pH (Smith et al., 1997). Although higher plants require the balance of these essential elements by uptake from the soil, absorption of a higher Mo amount may impose toxicity symptoms, and at the same time also induce antioxidant enzyme activity and increase of anthocyanin accumulation in plant tissues to overcome the excess metal stress (Stroud et al., 2010). Excessive levels of heavy metals, such as copper, molybdenum, selenium, and zinc, in plant tissues can lead to stress conditions by inducing the formation of harmful reactive oxygen species (ROS) (Schützendübel and Polle, 2002). This consists of the exaggerated generation of free radicals (hydroxyl radical, OH^-; phenoxy radicals, RO^-; peroxy radicals, ROO^-) and other ROS (superoxide radical anion, O_2^-; singlet oxygen, $^1O_2$; hydrogen peroxide, H_2O_2) (Posmyk et al., 2009). The generation of ROS is considered to be a primary event under a variety of stress conditions (Baryla et al., 2000). In order to control the level of ROS and to protect cells under stress conditions, plant tissues are stimulated to produce several enzymes for scavenging ROS, including superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), as well as a network of low molecular weight antioxidants (ascorbate, glutathione, phenolic compounds, tocopherol, and carotenoids) (Posmyk et al., 2009). SOD is the main antioxidant enzyme, catalyzing a disproportionate amount of superoxide anion (O_2^-) to H_2O_2 (Bowler et al., 1992), whereas APX and CAT are the main enzymatic scavengers of H_2O_2 in plants and catalyze the reduction of H_2O_2 into water and molecular oxygen.

Proline (Pro), an amino acid, is essential for primary metabolism (Tan et al., 2011). Higher plants tolerant to water deficits and salinity stress usually accumulate higher levels of Pro to cope with the harsh environments (Dinakar et al., 2009). Nazarbeygi et al. (2011) reported that more Pro is produced in plant tissues encountering heavy metal stress to maintain the plant's ability to tolerate stress conditions. Rapid catabolism of Pro upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damages (Hare et al., 1998). Moreover, it can protect enzymes by scavenging ROS (Gajewska and Skłodowska, 2005).

The aim of this study was to evaluate whether L-Pro could protect cabbage seedlings from Mo stress by activating enzyme activity and antioxidant accumulation in plant tissues, as well as to understand the regulation of anthocyanin biosynthesis genes under Mo stress.

MATERIAL AND METHODS

Plant growth conditions and parameter measurement

The commercial cabbage (*Brassica oleracea* var. *capitata*) hybrid variety 'KY Cross' was used for all experiments. Seeds were surface-sterilized by use of 0.6% sodium hypochloride for 15 min and washed three times with sterile water before grown in MS medium (Murashige and Skoog, 1962) supplemented with 20 g/L sucrose and 8 g/L Sigma agar. Two days after seed germination, young seedlings were removed carefully from the agar medium and transplanted into both MS medium and MS with 10 mM sodium molybdate. L-Pro at 0, 20, 40, 60, and 80 mM was supplemented into these treatments. The *in vitro* cultures were kept at 25° ± 2°C under a 16/8 h photoperiod with a light intensity of 35 to 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps (Starcoat^{TM} F28W/T5/840,170 MA; Hungary). Four days after treatments with MS and/or Mo with and without Pro, seedlings were harvested and frozen with liquid nitrogen and stored at -80°C until use. The lengths of the root, hypocotyls, and cotyledons, the cotyledon width, the percentage of seedlings with root hairs, and true leaves were recorded, respectively. The means of 15 seedlings for each treatment were collected and subjected to statistical analysis by the Fisher protected LSD test at $P \leq 0.05$ and Student *t*-test analysis at $P = 0.05$ between MS and Mo with the same Pro levels.

Cross-section of tissues

Seedling hypocotyls and cotyledons were removed from the culture plate and cross-sectioned with a surgical blade. Tissue sections were incubated in 0.5% polyethylene glycol and bubbles were expelled by a rotary vacuum pump (SV-3A No. 908) for 15 to 20 min. The sectioned samples were then examined under a light microscope (Olympus CX40) and images were recorded using an Olympus E420 digital camera.

Determination of anthyocyanins and chlorophylls

The chlorophyll (Chl) content was determined according to Hartmut and Buschmann (2001). The chlorophyll was extracted from cotyledons by using pure methanol. The chlorophyll *a* and *b* contents were determined by a spectrophotometer (Hitachi U-2900 UV-Vis Double Beam System) with adsorption values at 665.2, 652.4, and 470 nm. The concentration for Chl *a* (*Ca*), Chl *b* (*Cb*), and total Chl (*Ca*+*b*) was calculated with the following equations given for methanol as pure solvent, where the pigment concentrations are given in µg/mL extract solution:

$$Ca\ (\mu g/mL) = 16.72\ A_{665.2} - 9.16\ A_{652.4}$$
$$Cb\ (\mu g/mL) = 34.09\ A_{652.4} - 15.28\ A_{665.2}$$
$$Ca+b\ (\mu g/mL) = 24.93\ A_{652.4} - 1.44\ A_{665.2}$$

For anthocyanin determination, 0.12 to 0.2 g cotyledons was extracted in 1% HCl in methanol, and the crude extracted was left overnight at 4°C. Chloroform (500 µL) was then added and the mixture was centrifuged at 13,000 rpm (Centrifuge 5415R) at 4°C for 5 min.

The supernatant was used for anthocyanin measurement by a spectrophotometer (Hitachi U-2900 UV-Vis Double Beam System) at 510 and 700 nm after the differential pH method (Giusti and Wrolstad, 2001). The calculation followed the monomeric anthocyanin pigment concentration in the original sample using the absorbance of the diluted sample as follows: $A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$.

Total anthocyanin content (mg/L) = (A x MW x DF x 1000) / (ε x 1)

where *MW* is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), *DF* is the dilution factor, and *ε* is the molar absorptivity (26,900 $L \cdot mol^{-1} \cdot cm^{-1}$). The final anthocyanin content was converted to mg/g FW.

Antioxidant enzyme extraction and activity assay

The cotyledons were extracted with 50 mM sodium phosphate buffer, pH 7.0, and the solution was centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was analyzed for total protein and enzyme activity according to the methods adapted from Gajewska and Skłodowska (2005) and Posmyk et al. (2009). The protein content was determined according to Bradford (1976), where the mixture of 1 µL crude extraction with 299 µL Protein Assay Dye Reagent Concentrate (Bio-Rad Protein Assay) and 700 µL distilled water was incubated at room temperature for 5 min. The reaction was measured spectrophotometrically at 595 nm.

SOD activity was measured according to the methods of Masayasu and Yoshikawa (1979) and Paoletti et al. (1986). The reaction mixture consisted of 0.055 µM nitro blue tetrazolium (NBT), 50 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 16 µM pyrogallol, 1.4% Triton X-100, 0.778 mL distilled water, and 200 µL enzyme extract in a final total volume of 1 mL. The mixtures were reacted at 37°C for 10 min and then the absorption was measured spectophometrically at 540 nm. One unit of SOD activity was defined as the enzyme activity that caused the inhibition of NBT by 50% (w/v). The activity was expressed as SOD unit·mg $protein^{-1} \cdot min^{-1}$.

For determination of APX activity, the reaction mixture contained 150 µM sodium phosphate buffer, pH 7.0, 1.5 µM ascorbate, 6 mM H_2O_2, 0.75 mM EDTA, and 50 µL enzyme in a total volume of 1.5 mL. The reaction was carried out for 1 min at room temperature, and the absorbance was analyzed at 290 nm. The enzyme activity was expressed as µmole ascorbate oxidized·mg $protein^{-1} \cdot min^{-1}$, according to the method of Nakano and Asada (1981).

CAT activity was measured in a mixture containing 0.5 M sodium phosphate buffer, pH 7.0, 10 mM H_2O_2, and 100 µL enzyme extract. The final volume was 1.6 mL. The reaction was carried out for 1 min and the absorbance was measured at 240 nm. The enzyme activity was expressed as µmole H_2O_2 decomposed·mg $protein^{-1} \cdot min^{-1}$, according to the method of Kato and Shimizu (1985).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The total RNA was isolated from 0.2 g cotyledons according to manufacturer protocols (Invitrogen) by use of the TRIzol reagent. RNA precipitates were resuspended in diethylpyrocarbonate (DEPC)-treated sterile water and precipitated with LiCl (at a final

concentration of 2 M). The supernatant was treated with RNase-free DNase I (Promega) at 37°C for 30 min to remove residual genomic DNA. Purified RNAs were quantified by spectrophotometry (U2000, Hitachi) and quality-checked by agarose gel electrophoresis. Total RNA (5 µg) was treated with DNase I and used for first-strand cDNA synthesis by priming with oligo-d$(T)_{15}$ and catalyzed with Superscript II Reverse Transcriptase (Invitrogen) at 42°C for 1.5 h. Real-time PCR and data analysis were performed in the Applied Biosystems 7300 Real-Time PCR System with SYBR Green PCR Master Mix (Applied Biosystems) and the endogenous control for quantification was the *Actin* gene. The primers used were according to Yuan et al. (2009). Before running the real-time PCR, the primer efficiency of the target genes and housekeeping genes was evaluated by testing at 100, 200, and 300 nM. The primers are shown in Table 1. The real-time PCR condition was 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was amplified in triplicate and all PCRs were performed on the Applied Biosystems 7300 Real-Time PCR System. With the housekeeping gene *Actin*, the relative amount of the gene transcript was presented as $2^{-\Delta CT}$, according to the ΔC_T method described in the real-time PCR Applications Guide (Applied Biosystems).

Table 1. Oligo nucleotide primers used for real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis in this study.

Genes	Primer sequence (5'→3')	Primer concentrations (nM)	Accession No.
ACTIN	F: CTGTGACAATGGTACCGGAATG	100	AF044573
	R: ACAGCCCTGGGAGCATCA	100	
PAL	F: CAGAGCAACACAACCAAGACGTGAA	300	BH716217
	R: TCTCCTCCAAGTGTCGTAGATCGATG	100	
CHS	F: GCGCATGTGCGACAAGTCGAC	200	EF408921
	R: CCTGTCGAGCGTCGAGAGAAGGA	200	
F3H	F: GTCATCTTCAGGGAGAGTCTGTTCA	200	DQ288239
	R: TCGCTGTACTCCTCCGTCACTT	200	
LDOX	F: GTGGACAGCTTGAGTGGGAAGATTAC	300	AY228485
	R: GTACTCACTCGTAGCTTCAATGTAATCAG	100	
GST	F: CTTGTAGCCATTTGGTCAA	300	BH738469
	R: GAGACTTGCCCAAAAGGTTCGT	100	

RESULTS

Influence of proline on plant growth under excess Mo

Excess Mo in the MS medium affected seedling growth significantly at 4-day intervals by decreasing the growth rate. Moreover, 10 mM Mo supplemented with Pro also decreased the seedling growth, root and hypocotyl lengths, cotyledon length, and cotyledon width. By adding different concentrations of Pro to excess-Mo media, seedlings could grow out true leaves, where the higher the concentration of Pro, the higher the percentage of leaf growth. Specifically, the treatment of Mo plus 80 mM Pro reached 53.33% of seedlings with true leaves, whereas in the control MS with or without Pro, all grew normally (Table 2, Figure 1A). Seedlings produced no root hair in the Mo-alone and Mo plus Pro treatments as compared with the control MS with Pro (Figure 1A).

Table 2. Effect of proline (Pro) on the growth of molybdenum (Mo)-treated cabbage seedlings.

Treatments	Without Pro	Pro 20 mM	Pro 40 mM	Pro 60 mM	Pro 80 mM
Root length (mm)					
MS	47.52 ± 7.86^{b*}	31.43 ± 8.66^{c*}	54.97 ± 11.56^{a*}	50.79 ± 12.03^{ab*}	45.54 ± 14.55^{b*}
Mo10 mM	15.23 ± 2.66^{d}	13.45 ± 3.58^{d}	12.45 ± 3.12^{d}	12.44 ± 2.16^{d}	13.12 ± 2.81^{d}
Hypocotyl length (mm)					
MS	23.22 ± 7.11^{c*}	21.21 ± 8.23^{c*}	38.26 ± 4.47^{a*}	34.29 ± 6.28^{b*}	35.42 ± 6.28^{*ab}
Mo10 mM	9.28 ± 2.14^{d}	7.86 ± 1.16^{d}	8.29 ± 1.59^{d}	7.12 ± 0.8^{d}	7.83 ± 1.90^{d}
Cotyledon length (mm)					
MS	11.76 ± 1.32^{a}	9.64 ± 1.50^{b*}	12.48 ± 1.61^{a*}	12.52 ± 1.32^{a*}	12.04 ± 1.05^{*a}
Mo10 mM	9.17 ± 1.00^{bc}	8.39 ± 0.68^{c}	9.49 ± 1.17^{b}	8.83 ± 1.11^{bc}	9.09 ± 0.72^{bc}
Cotyledon width (mm)					
MS	6.30 ± 0.74^{b*}	4.97 ± 0.78^{cde}	7.10 ± 0.66^{a*}	7.15 ± 1.45^{a*}	5.99 ± 0.35^{b*}
Mo10 mM	4.72 ± 0.56^{cd}	4.49 ± 0.48^{cde}	4.52 ± 0.47^{cde}	4.25 ± 0.45^{de}	4.16 ± 0.40^{e}

Values are reported as means ± SD. Means followed by the same superscript letter in a row of MS with Pro and Mo with Pro are not significantly different by the LSD test at $P \leq 0.05$ (N = 15). *Values between MS and Mo (column) are significantly different by the Student *t*-test analysis at P = 0.05 and N = 15.

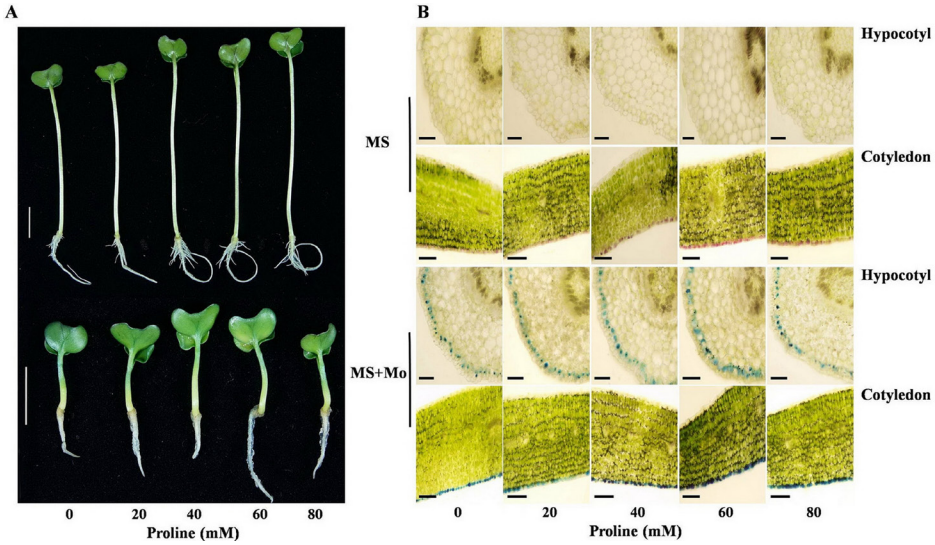

Figure 1. Four-day-old cabbage seedlings grown *in vitro* on MS with and without molybdenum (Mo). Both were supplemented with proline (Pro) at different concentrations of 0, 20, 40, 60, and 80 mM (row from left to right). **A.** MS (top) and Mo with Pro treatments (bottom) (bar = 5 mm). **B.** Anthocyanin pigment evident in the epidermal cell layer of hypocotyls and cotyledons (bar = 0.1 mm).

Anthocyanin pigment accumulated in the epidermal cells of Mo-treated seedlings

The hypocotyl sections showed accumulation of the blue pigment in the epidermal cell layer under both the Mo-only and Mo with Pro treatments, whereas no pigment was observed in the hypocotyl epidermis in both the MS-only and MS with Pro treatments during the 4-day experiment (Figure 1B). Blue and purple pigments were also present in the cotyledon cross-sections of Mo-treated seedling no matter if Pro was supplemented or not, whereas control without Mo showed regular pink coloration in cotyledons (Figure 1B). These results suggest that the presence of excess Mo was correlated with the blue pigment formation in cabbage tissues.

The total anthocyanin content of the cotyledons, ranging from 0.007 to 0.03 mg/g FW for treatments of Mo with and without Pro, was significantly different from the control MS with and without Pro. The anthocyanin content under excess Mo with 40 mM Pro increased almost 4-folds as compared with the MS with 40 mM Pro treatment (Figure 2A). The pigment solution appeared pink to purple in the Mo with and without Pro treatments, whereas the control was light pink (Figure 2B).

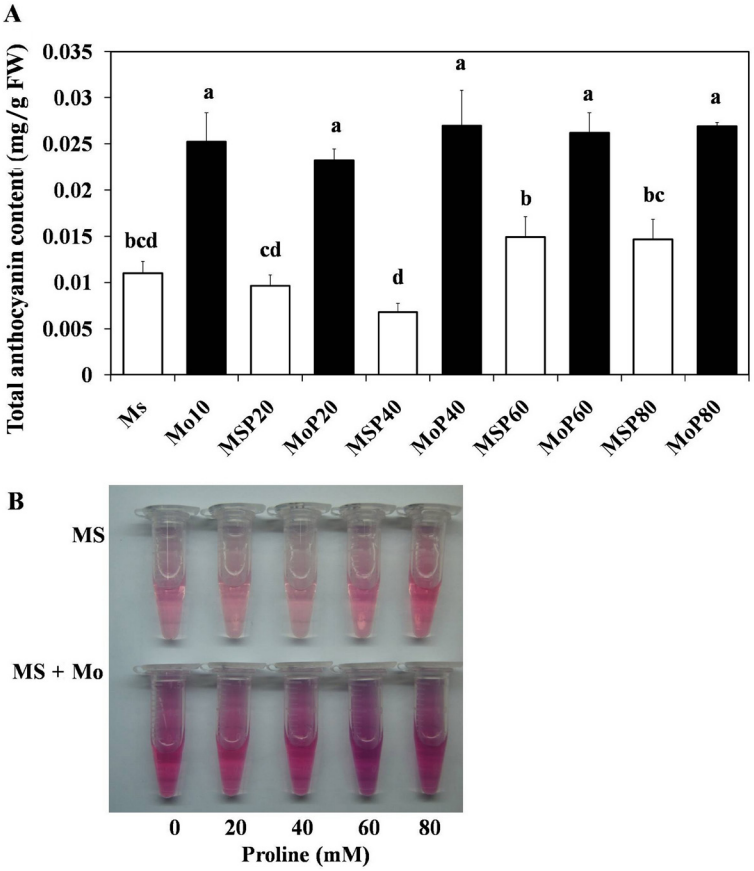

Figure 2. Anthocyanin changes in cabbage seedlings under MS (control) and molybdenum treatments, both supplemented with proline (P) at 0, 20, 40, 60 and 80 mM for 4 days. **A.** Total anthocyanin content. **B.** Appearance of anthocyanin extracts from cabbage seedling shoots using 1% HCl in methanol. Values are reported as means ± SD of three replications. Means followed by the same letter are not significantly different by LSD test, $P \leq 0.05$ and $N = 3$.

The total Chl and Chl *b* contents were increased (Figure 3A). The Chl *a* content was higher in MS with 40 mM Pro, and significantly different from Mo treatment at the same Pro level, and also higher than the control MS. The Chl *a* content in medium with Mo only was not significantly different from Mo plus Pro treatments. Seedlings treated with both Mo and Pro had much higher Chl *b* than seedlings in MS with Pro (Figure 3A). The seedlings

and their extracted pigments showed dark green coloration under Mo plus Pro levels (Figure 1A and B).

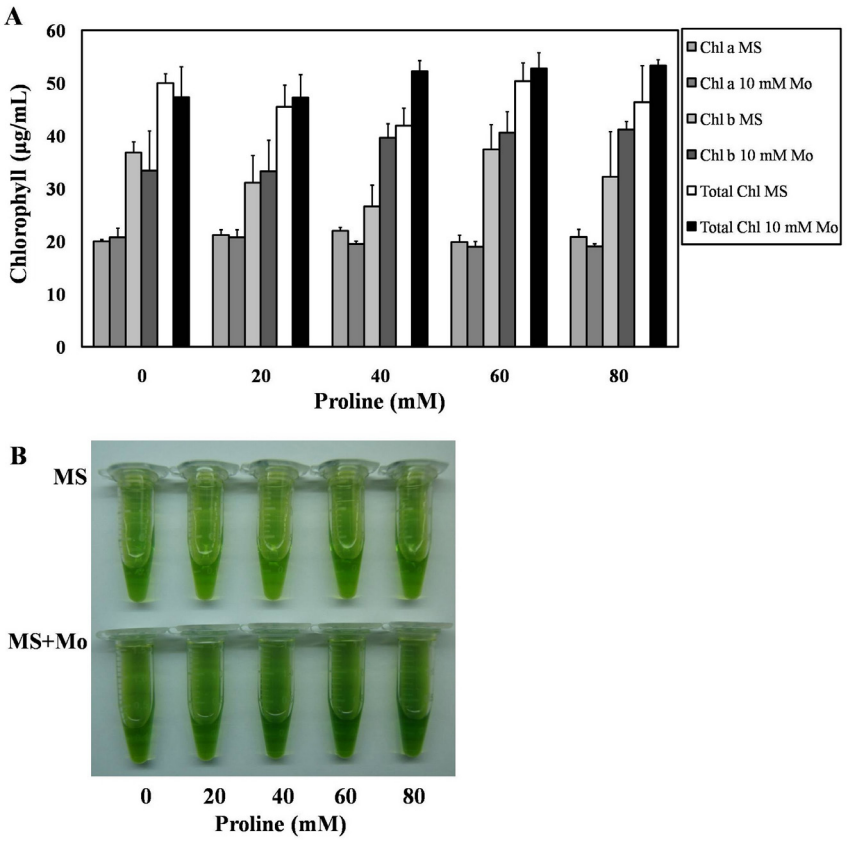

Figure 3. Chlorophyll changes in cabbage seedlings under MS (control) and molybdenum (Mo) treatments, both supplemented with proline at 0, 20, 40, 60, and 80 mM for 4 days. **A.** Chlorophyll a and b and total chlorophyll content. **B.** Appearance of chlorophyll extracts from cabbage seedling shoots using 100% methanol. Values are reported as means of three replications ± SD.

Influence of proline on anthocyanin biosynthetic genes of Mo-stressed seedlings

Since the anthocyanin contents under excess Mo with and without Pro all increased, we examined the expression level of genes in the flavonoid pathway (Figure 4), including *PAL*, *CHS*, *F3H*, *LDOX*, and *GST*. These were all upregulated under Mo stress either with or without Pro treatment (Figure 5). High expression levels of *PAL* (Figure 5A), *CHS* (Figure 5B), and *F3H* (Figure 5C) were detected in the cotyledon of Mo-stressed seedlings. *LDOX* was dramatically increased from low to high concentrations of Pro up to 60 mM, and then decreased (Figure 5D). *GST*, the gene responsible for accumulation of anthocyanin in the vacuoles, had a higher transcript level in Mo with 40 mM Pro, followed by that in control medium free of Mo but with Pro (Figure 5E).

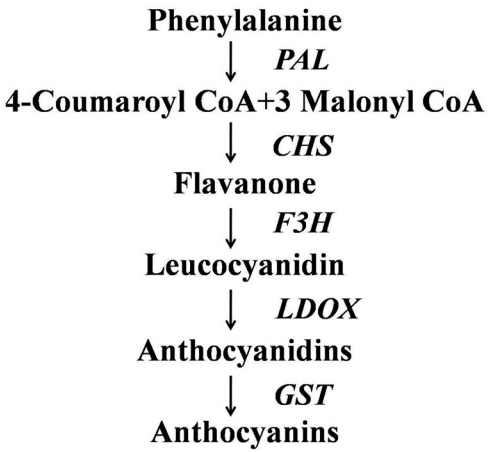

Figure 4. Simplified schematic flow chart of the flavonoid pathway leading to anthocyanins synthesis. The enzymes in this pathway are PAL, CHS, F3H, LDOX, and GST. The pathway was adapted from Petroni and Tonelli (2011).

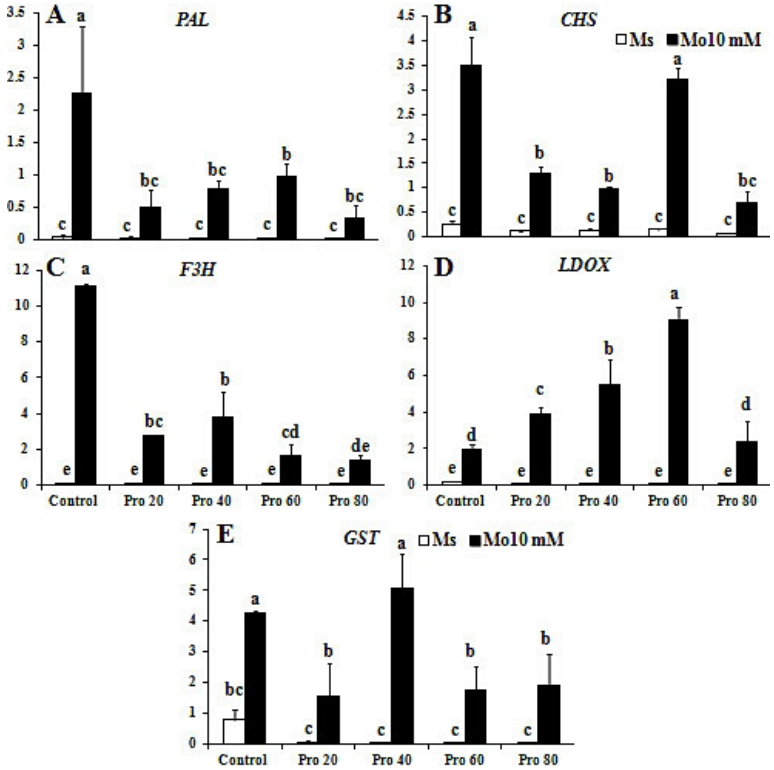

Figure 5. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of the expression of anthocyanin structural gene expression: *PAL* (**A**), *CHS* (**B**), *F3H* (**C**), *LDOX* (**D**) and *GST* (**E**) from cabbage seedling shoots exposed to MS and molybdenum with and without proline at different concentrations, 0, 20, 40, 60, and 80 mM for 4 days. Data are reported as means ± SD of three replications. Means followed by the same letter are not significantly different by LSD test at $P \leq 0.05$ (N = 3).

Modulation of antioxidative enzyme activities by proline under excess-Mo condition

In general, the enzyme activities of SOD, APX, and CAT were all increased in seedlings under Mo stress, either with or without Pro (Figure 6). Protein contents were not significantly different between the treatments (Figure 6A). SOD activity was the highest at Mo with both 20 and 40 mM Pro (Figure 6B). APX activity also increased under Mo excess, compared with the control, either with and without Pro, especially at 80 mM Pro. APX activity increased under excess Mo plus 20 to 80 mM Pro (Figure 6C). CAT activity also increase under Mo with 80 mM Pro (Figure 6D).

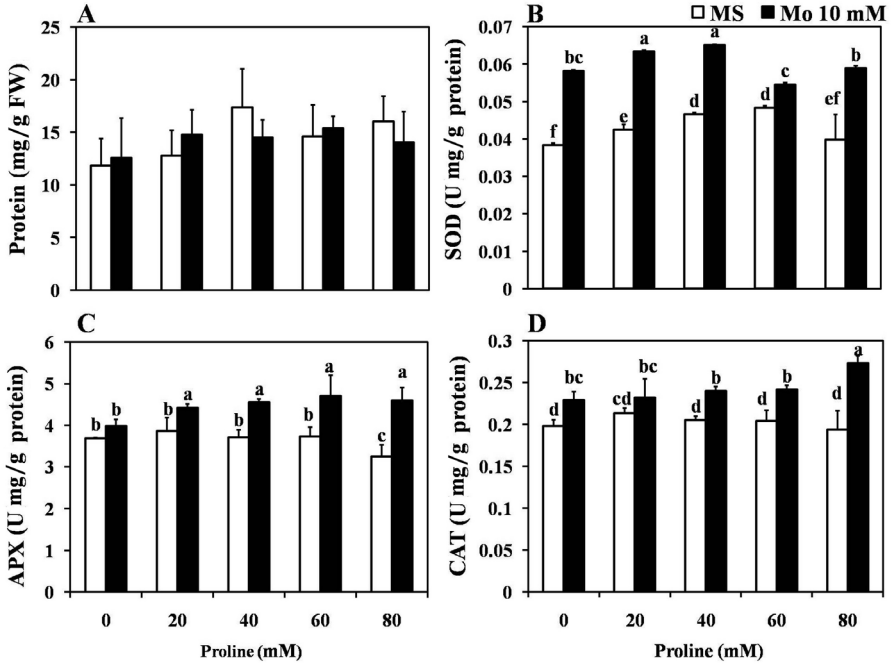

Figure 6. Protein content (**A**) and the kinetics of the antioxidant enzymes, superoxide dismutase (SOD) (**B**), ascorbate peroxidase (APX) (**C**), and catalase (CAT) (**D**). The activity is presented for cabbage seedlings shoots exposed to MS with proline and 10 mM molybdenum (Mo) with proline at 0, 20, 40, 60 and 80 mM for 4 days. Data are reported as means ± SD of three replications. Means followed by the same letter are not significantly different by LSD test $P \leq 0.05$ (N = 3).

DISCUSSION

Mo is an essential micronutrient that is required at a minimum level by plants for metabolism (Kaiser et al., 2005; Mendel, 2005, 2007). As a heavy metal, high concentrations will damage plant cells. McGrath et al. (2010) reported that high Mo concentrations decreased the shoots and yields in tomato, oilseed rape, red clover, and ryegrass. It was shown in our results that Mo reduced/inhibited the hypocotyl length, and shoot and root growth. By the

addition of Pro to the excess-Mo medium, cabbage seedlings could partially recover their growth, as indicated by the emergence of true leaves, whereas few were observed in the excess-Mo condition. This result indicates that Pro offered protection together with anthocyanin pigmentation against plant stress conditions such as heavy metals and salinity, as also reported previously (Sharma and Dietz, 2006; Chutipaijit et al., 2011). According to Szabados and Savoure (2009), Pro acts as a signaling molecule and can aid plants in recovering from stress conditions. In the treatment of excess Mo plus Pro, dark green pigment appeared in the seedling extracts, whereas under MS with Pro, the seedlings appeared light green (Figure 3B). The trend of Chl *b* accumulation (Figure 3A) also correlated with the excess-Mo condition of dark green color, whereas the control with more Chl *a* appeared light green, as also reported previously (Willstätter and Stoll, 1928). High total Chl and the accumulation of Chl *b* content might be an effect of Mo (Pirzad et al., 2011). Moreover, Datta et al. (2011) also observed increased levels of Chl *a* and *b*, total Chl, and sugar under 7.5 ppm Mo in *Cicer arietinum.*

Under stress conditions such as heavy metals, drought, temperature, etc., plants could be protected by a mechanism that increases its antioxidative properties, such as producing flavonoids, including anthocyanin, and antioxidative enzymes to overcome metal toxicity (Hung et al., 2008; Keilig and Ludwig-Müller, 2009; Sperdouli and Moustakas, 2012). Anthocyanins have been implied to confer tolerance to diverse environmental stressors (Close and Beadle, 2003; Gould, 2004). In addition, plants under salinity stress responded by producing protection agents through an increase of Pro, glutathione *S*-transferase, antioxidant enzymes, and anthocyanin contents (Hoque et al., 2008; Chutipaijit et al., 2011). When Pro was added to MS medium along with a high concentration of Mo, cabbage seedlings produced more anthocyanin than controls in MS with Pro treatments. Enzyme activities of SOD, APX, and CAT were all increased under Mo with Pro. This indicates that exogenous proline could induce antioxidant protection against stress conditions. According to Hoque et al. (2008), exogenous proline mitigates the detrimental effects of salt stress, in its superior ability to increase the activities of antioxidant enzymes, catalase and peroxidase, of tobacco Bright Yellow-2 (BY-2) culture cells in suspension under salt stress. Moreover, proline provides protective action against NaCl-induced oxidative damage by reducing protein carbonylation, enhancing antioxidant defense and methylglyoxal detoxification systems, and decreasing levels of ROS accumulation and lipid peroxidation, as well as improving membrane integrity (Banu et al., 2009). Khedr et al. (2003) reported that exogenous proline improves salt tolerance by up-regulating stress-protective proteins.

Under Mo excess with and without Pro conditions, there was significant difference in the expressions of the *PAL*, *CHS*, *F3H*, *LDOX*, and *GST* genes involved in anthocyanin pigmentation. Mo upregulated early genes of the pathway (*PAL*, *CHS*, and *F3H*), whereas addition of 40 mM Pro to Mo upregulated genes of the last steps, such as *LDOX* and *GST* (Figure 5). This upregulation is similar to the results of Qi et al. (2011) in that jasmonate (JA) induced trichome initiation and anthocyanin biosynthesis. The anthocyanin accumulation was increased in the *Coi1-2 pap1-D* double mutant of *Arabidopsis* in response to JA treatment. It also upregulated high transcript levels of the anthocyanin biosynthetic genes *DFR*, *LDOX*, and *UF3GT.* In addition, the genes responsible for flavonoid and anthocyanin syntheses pathways, *PAP1*, *CHS*, *CHI*, *F3H*, *F3'H*, *FLS*, *DFR*, *LDOX*, and *UF3GT*, in *Arabidopsis* were upregulated by treatment with sucrose, which is the most efficient trigger for mRNA accumulation of the selected genes coding for enzymes involved in the flavonoid biosynthesis pathway (Solfanelli et al., 2006).

The results of this investigation clearly explained the effect of Pro in alleviation of the growth inhibition imposed in Mo-stressed cabbage seedlings. High Pro concentrations helped recover plant growth under stressed conditions by inducing true leaf growth. In addition, Pro supplemented to the excess-Mo condition increased antioxidant enzyme activity, probably to scavenge ROS, and upregulated anthocyanin genes expression to protect plant cells from the Mo toxicity.

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