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Table: 1, Figs : 8

Effects of coumarin on net nitrate uptake and nitrogen metabolism in roots of alfalfa (*Medicago sativa*)

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ABSTRACT

We investigated the effects of coumarin application on growth, net nitrate uptake, concentration of nitrate (NO_3^-), ammonium (NH_4^+), soluble proteins and enzymes [nitrate reductase (NR), glutamine synthase (GS) and glutamate dehydrogenase (GDH)] activities in roots of alfalfa (*Medicago sativa*). Five-d-old alfalfa seedlings were exposed to 0, 0.025, 0.05, 0.1 and 1 mM concentrations of coumarin. High concentration of coumarin (≥ 0.1 mM) decreased fresh weight (FW) production in the shoots and roots and the root to shoot (R/S) FW ratio. In addition, there were no statistically significant effects on the soluble protein in roots among all treatments. In seedlings exposed to 0.025 and 0.05 mM coumarin, the net nitrate uptake was significantly stimulated but reduced at 0.1 and 1 mM. Moreover, 0.05 mM coumarin treatments markedly increased the NO_3^- content, whereas, coumarin in the range of 0.1 to 1 mM, decreased it. Endogenous ammonium (NH_4^+) level was reduced at 0.025 to 0.1 mM, whereas, it was elevated at 1 mM. The activities of NR were repressed at the high coumarin concentration. GS levels were enhanced at 0.05 mM, whereas they were reduced by 1 mM. At the highest coumarin concentration, the activities of GDH were inhibited in roots. Therefore, low concentration of coumarin stimulated net nitrate uptake and N metabolism in roots, but higher concentration adversely affected the plant growth and the key enzymes of nitrogen metabolism in alfalfa.

Keywords: Alfalfa, glutamine synthase (GS), glutamate dehydrogenase (GDH), net nitrate uptake, nitrate reductase (NR), plant growth.

INTRODUCTION

Nitrogen is an essential macronutrient for plants growth and productivity. For higher plants, the most available form of nitrogen is nitrate. Nitrate reduction is catalyzed by nitrate reductase (NR). Ammonium is assimilated into amino acids via the combined action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (24). Glutamate dehydrogenase (GDH) may alleviate accumulation of toxic amounts of ammonium and provide the glutamate required for the biosynthesis of several protective biomolecules (9,12), including nitrogen-containing compounds such as chlorophyll (15). N metabolism influences all levels of plant function from metabolism to resource allocation, growth and development (28).

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Coumarin (1,2-benzopyrone), an allelochemical can both promote and repress the plant growth depending on the dose. Low concentrations improves the rooting in leaf explants of petunia (1) and in hypocotyls explants of soybean (18). However, higher concentrations of coumarin inhibit cell division, differentiation and germination (3,20), decrease respiration and photosynthesis by inhibiting electron transport (21) and interfere with nitrate uptake in wheat seedlings (2). A few articles have focused on nitrogen metabolism in carrot cell suspension culture (5) and leaves of alfalfa (10), but studies of this type have not yielded conclusive results. Interestingly, these enzymes responses often differ among species, organs and tissues (14,31,35). Although roots are important for nutrients and water absorption and are more sensitive to coumarin than other organs (11,33), little is known about the role of coumarin in nitrogen metabolism in roots. In addition, few studies have simultaneously studied the respective responses of net nitrate uptake and root system to coumarin. Thus the studies on the effects of coumarin on enzyme activities involved in nitrogen metabolism in roots will provide valuable information on the physiological significance of its contribution in plants to cope with coumarin toxicity.

Alfalfa (*Medicago sativa* L.) is major forage legume used for livestock feed and soil improvement. Coumarin inhibits the alfalfa seed germination and plant growth. To explain the inhibitory action, many physiological processes affected by coumarin have been investigated. For example, 1 mM coumarin stunted and swollen the root tips of alfalfa and also reduced its root hairs development (11). Since alterations in the root structure and distribution are usually accompanied by changes in nitrogen uptake (2), we hypothesized that coumarin may interferes with nitrate uptake and the key enzymes of nitrogen metabolism in alfalfa roots.

This study aimed to examine the influence of applied coumarin doses on nitrate uptake and the activities of enzymes [Glutamate dehydrogenase (GDH), Glutamine synthase (GS), Nitrate reductase (NR)] in alfalfa roots as well as plant growth. The contents of root total nitrogen nitrate, ammonium, and soluble protein were also determined. This study provides new insight into the functioning of plant nitrogen metabolism under conditions of coumarin stress.

MATERIALS AND METHODS

Ⅰ. Plant material and growth conditions

Seeds of alfalfa were surface-sterilized for 20 min in 20% (v/v) sodium hypochlorite solution, washed repeatedly with distilled water and germinated at 25ºC for 36 h. Sixty germinated seeds were transferred in sterile Petri dishes (15 cm dia) containing 50 g rinsed quartz sand. We added 5 mL aerated 1/4 Hoagland's solution (containing 6 mM KNO_3) to each Petri dish. The pH was adjusted to 6.0 with 0.1 M KOH. These Petri dishes were incubated in growth chamber for 5 d (25ºC, 14/10 h light/dark photoperiod; photon flux density 300 μmol m^{-2} s^{-1}). The nutrient solution (pH 6.0) was renewed every 2 d to avoid the nutrients depletion. After 5 d of growth, the seedlings were transferred to a nutrient solution containing coumarin at concentrations of 0.025, 0.05, 0.1 and 1 mM and were incubated for 48 h. The control treatment received only complete nutrient solution. Treatments were arranged in a completely randomized block design with 5 replications.

The used concentrations of coumarin were selected based on a survey of the literature (2,3,4,5,10,11,20,30,23) and are ecologically relevant since phenolic acid concentrations are commonly found in soils at concentrations between 0.01 and 0.1 mM and influence plant growth at concentration of up to 10 mM (8,26). All reagents used were of highest analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ⅱ. Plant sampling and growth measurements

Plants shoots were cut at the quartz sand surface and roots were carefully separated from the quartz sand. After washing with distilled water, shoots and roots were dried with filter paper and then fresh weight (FW) was recorded. Part of roots were frozen in liquid N_2 and stored at -50 ℃ for plant physiological analyses, while remaining parts were dried at 70℃ in oven up to constant weight and used for N determination.

Ⅲ. Nitrate and ammonium measurements

These were measured as per method of Cruz *et al*. (12), 0.5 g frozen plant material was homogenized in a chilled mortar, dissolved in distilled water (0.5 g FW to 5 mL distilled water). Extracts were heated at 70℃ for 15 min and centrifuged at 5000 *g* for 15 min at 4℃ . The suspension was then filtered through a 0.45 μm Millipore filter membrane. The concentrations of nitrate and ammonium were analyzed using a Continuous-Flow Analyzer (Autoanalyzer 3, Bran-Luebbe, Germany). Nitrate was determined by the reduction of nitrate to nitrite and subsequent colorimetric determination of nitrite with a diazo-coupling reaction (16). Ammonium was determined by the modified Berthelot reaction (19).

Ⅳ. Protein assays

Total proteins were extracted from fresh leaves ground under liquid N with mortar and pestle. A volume of 1 mL of extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1% weight/volume (w/v) sodium dodecyl sulfate (SDS) and 1.4 μL of β-mercaptoethanol) was added to 50 mg sample. Suspensions were shaken vigorously for 10 min at 4℃ and centrifuged at 12,000 *g*, 4℃ , for 15 min. Supernatants were transferred to new tubes. Pellets were washed once again with 500 μL extraction buffer. After vortexing and centrifugation, the upper phase was added to the previously collected supernatants. Protein solutions were purified using prepacked Sephadex G-25 columns (NAPTM-25; Amersham Biosciences, Uppsala, Sweden). For column equilibration and sample elution, 25 mM Tris-HCl, pH 8.0, was used (14). Soluble protein was quantified using Coomassie Brilliant blue (8) with bovine serum albumin (BSA) as standard.

Ⅴ. Enzyme assays

NR activity was extracted and measured by the in vitro method as per Aslam *et al.* (6), with some modifications. Frozen plant material (0.5 g) was homogenized in a chilled mortar and pestle with 4 mL of 25 mM potassium phosphate buffer with (pH 8.7) containing 10 mM cysteine and 1 mM EDTA at 4℃. The homogenate was centrifuged (30,000 *g* for 15 min at 4℃) and the supernatant was assayed for NR activity. The extract (0.4 mL) was incubated in a reaction mixture, which contained 1.2 mL of 0.1 M phosphate buffer (pH 7.5, containing 0.1 M KNO_3) and 0.40 mL of 3.0 mM NADH at

30ºC for 30 min. The nitrite (NO_2^-) produced was assayed after diazotization with 1 mL of 5.8 mM sulfanilamide in 1.5 M HCl and 1 mL of 0.8 mM *N*-(1-naphthyl) ethylenediamide dihydrochloride.

For extraction of GS, 0.5 g frozen samples were homogenized in a cold mortar and pestle with 6 mL of 50 mM Tris-HCl buffer (pH 8.0, containing 2 mM $MgCl_2$, 2 mM DTT, and 0.4 M sucrose). The homogenate was centrifuged at 25,000 *g* for 20 min at 4ºC. GS activity was determined using the method of Oaks *et al.* (25). The reaction mixture contained 0.7 mL of 40 mM ATP and 1.6 mL of 0.1 M Tris-HCl buffer (pH 7.4) with 20 mM Na-glutamate, 80 mM $MgSO_4$, 20 mM cysteine, 2 mM EGTA, and 80 mM NH_2OH. The reaction was started by addition of 0.7 mL enzyme extract, incubated for 30 min at 37ºC, and stopped by adding 1 mL of ferric chloride reagent (0.37 M $FeCl_3$, 0.2 M trichloroacetic acid, and 0.6 M HCl). After centrifugation (5000 *g* for 15 min at 4 ºC) the absorbance of supernatant was read at 540 nm.

The GDH activity was measured as per the method of Loulakakis and Roubelakis-Angelakis (22). Half a g of frozen plant material was ground in a chilled mortar and pestle with 6 mL of 0.1 M Tris-HCl buffer (pH 8.2) at 4 ºC. The extract was centrifuged at 20,000 *g* for 15 min at 4 ºC and the supernatant was assayed for GDH activity. The 3.0 mL assay mixture consisted of 0.1 M Tris-HCl buffer (pH 8.2), 0.1 M $(NH_4)_2SO_4$, 0.01 M ketoglutarate, 4 mM $CaCl_2$, and 0.16 mM NADH. The reaction was initiated by adding 0.5 mL of crude enzyme extract and NADH consumption was assayed spectrophotometrically at 340 nm.

Ⅵ. Net nitrate uptake experiment

Net nitrate uptake was performed according to Abenavoli *et al.* (2) with some modification. For each coumarin treatment, sixty 5-d-old seedlings were collected after 1, 3, 6, 12, 24 and 48 h and their roots were washed carefully with nutrient solution. The seedlings were then transferred into a 20 mL of continuously aerated nutrient solution containing 0.1 mM KNO_3. Samples were taken from the solution at 5 min intervals over a 30 min total period, and the nitrate concentration was measured at 210 nm with a UV-vis spectrophotometer. The net rate of nitrate uptake was calculated from the linear phase of the nitrate depletion curve and expressed as μmoles nitrate h^{-1} g^{-1} FW. Each nitrate uptake experiment was conducted in five replicates.

Statistical analysis: Data were processed with Microsoft Excel 2003 and SPSS 11.5 software. All the values expressed are means±standard deviation (S.D.) of five replicates. The data were analyzed by one-way analysis of variance (ANOVA) to compare the means of different treatments. Differences between means were tested using Duncan tests at a 0.05 significance level.

RESULTS AND DISCUSSION

Plant growth

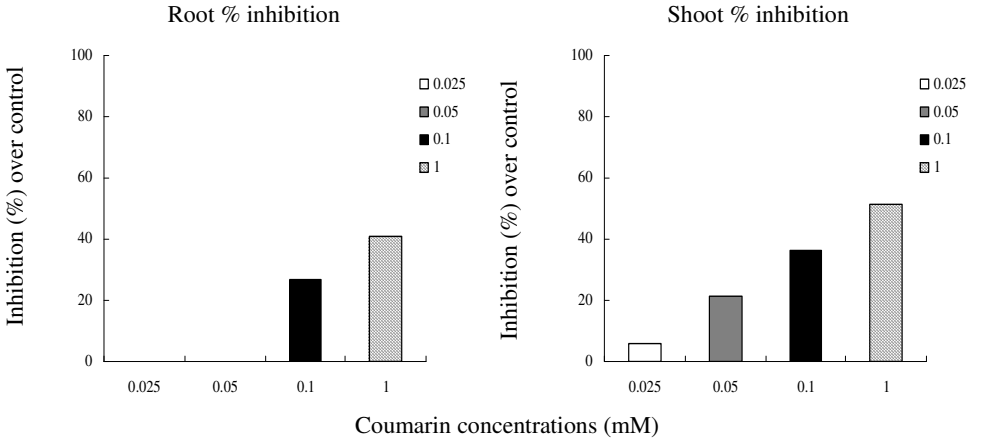
The shoot and root fresh weight decreased with increasing coumarin concentrations, but the effect was not significant except at 0.1 and 1 mM (Table 1). One mM coumarin significantly decreased the fresh weight of shoot and root by 52 and 40%,

respectively (Fig. 1). These results indicated that effects of coumarin on alfalfa seedlings growth are concentration-dependent. In contrast, 0.1 and 1 mM coumarin increased the root to shoot fresh weight ratio (R/S ratio) by 16 and 24%, respectively (Table 1, Fig. 2), confirming that alfalfa allocated more fresh weight to roots to maximize the capacities for nutrients and water absorption. Coumarin inhibits the root length, and alters root morphology and histology of different plant species, such as wheat (2), maize (3) and alfalfa (11) at high concentrations.

Table 1. Effects of coumarin on seedling fresh weight and root to shoot ratio (R/S)

Coumarin mM)	Root Fresh Weight (mg)	Shoot Fresh Weight (mg)	Root to Shoot Ratio (R/S)
0	15±2a	33±2a	0.45±0.03c
0.025	15±2a	31±4a	0.48±0.04bc
0.05	14±3a	28±3a	0.50±0.03bc
0.1	11±1b	21±2b	0.52±0.01b
1	9±0c	16±3c	0.56±0.01a

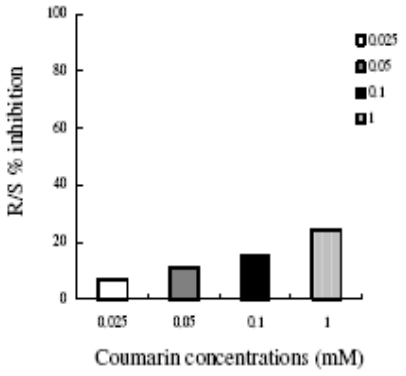
Data are means of five replicates ± SE. Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's test at the 5% probability level.



Figures 1. Inhibitory effects of applied coumarin on root and shoot growth of alfalfa seedlings.

Net nitrate uptake rates

In our studies, net nitrate uptake in alfalfa seedlings was induced after 24 h (Fig. 3). This is similar with the results obtained in the previous studies (2). Moreover, all coumarin treatments influenced the net nitrate uptake rates and the effects were concentration-dependent (Fig. 3). At 1 h, net nitrate uptake was not much different in any treatment. Compared with control, 0.05 mM coumarin significantly increased the net nitrate uptake rates by 38, 39, 35, 22 and 40% at 3 h, 6, 12,24 and 48 h, respectively, whereas 0.025 mM coumarin caused increases of 16 and 25% at 6 and 12 h (Fig. 3). On the other hand, net nitrate uptake rates were reduced at higher concentration (0.1 and 1 mM) from 3 to 48 h. The inhibitory effect of 1 mM coumarin on net nitrate uptake rate



Figures 2. Stimulatory effects of applied coumarin on root to shoot Ratio (R/S) of alfalfa seedlings

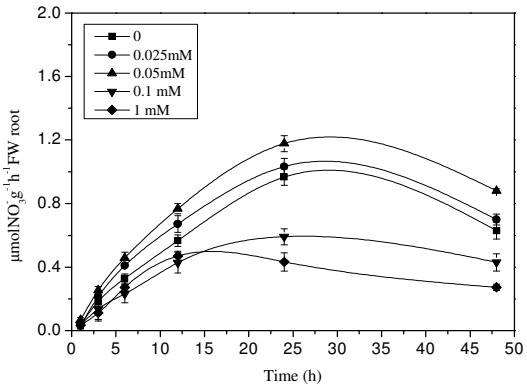


Figure 3. Net rates of nitrate uptake in roots of alfalfa under coumarin treatments

was stronger than 0.1 mM after 24 h (Fig. 3). Roots exhibit at least two mechanisms for nitrate uptake (2,29). Uninduced plant have a low, constitutive level of nitrate uptake rate. Upon exposure to nitrate, "induced plant", the uptake rates increase; therefore, nitrate stimulates its own uptake (2,29). Subsequent accumulation in plant tissues of nitrate can result in nitrate uptake to decline through feedback regulation (2). However, Abenavoli *et al*.(2001) found that coumarins at 0.025 mM to 1 mM stimulated the net nitrate uptake in wheat seedlings (2). The different responses may depend on plant species studied. The effect of coumarin on nitrate uptake is related to parallel influences on root anatomy and morphology (2), and is driven by energy coupled with the transmembrane H^+ gradient (13). Coumarin at 0.025 and 0.05 mM might result in subapical swelling of tip, thereby increasing the root surface available for nitrate absorption (2). This, in turn, may increase nitrate availability for the induction process. In addition, lower concentration of coumarin might also increase the ctivity of ATPase: H^+ pumps (20), which increases the nitrate uptake. On the other hand, alfalfa treated with the highest concentration (1 mM) of coumarin was stunted and root tips were swollen (11), thereby reducing nitrate uptake.

N forms and key enzymes for N metabolism in roots

The coumarin had no effect on the soluble protein content in roots (Fig. 4). Similarly, Abenavoli *et al.* (2003) did not observe effects of coumarin on the soluble protein content during the culture cycle of carrot cells (5). Previous studies have found that coumarin markedly reduces the incorporation of labelled Phe into protein in some crop seeds (32) and protein concentrations in leaves of alfalfa (10), thus suggesting that coumarin inhibits the growth by interfering in protein biosynthesis (10). Although the mechanisms require further clarification, the maintenances of soluble protein levels under coumarin stress may be beneficial for root growth.

Compared with control, 0.05 mM coumarin increased the NO_3^- concentration in roots by 12%, while high concentrations of 0.1 and 1 mM coumarin decreased it (P <0.05) (Fig.5). The different responses of NO_3^- concentrations in roots to coumarin are closely

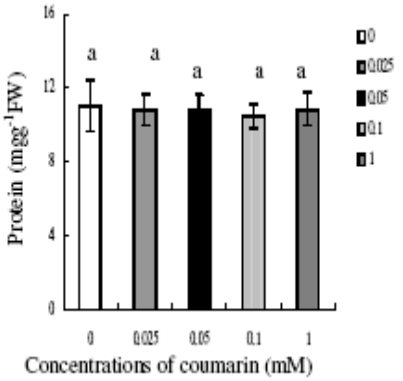


Figure 4. Effects of coumarin treatments on protein contents in roots. Bars represent SD of means. Different letters indicate values that differ significantly from the control at $P<0.05$.

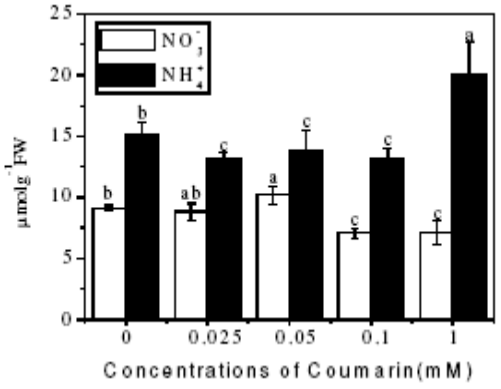


Figure 5. Effects of coumarin treatments on $NO3^-$ and $NH4^+$ contents in roots. Bars represent SD of means. Different letters indicate values that differ significantly from the control at $P<0.05$.

linked to the nitrate influx. The activity of NR decreased ($P < 0.05$) in the presence of 0.1 and 1 mM coumarin, respectively. One mM coumarin reduced the activity of NR by 50% (Fig. 6). The nitrate content directly influences the NR protein synthesis and activation, as the NR is a substrate inducible enzyme (34). Thus, coumarin-induced stimulation or inhibition of NR activity in the alfalfa roots may be due to high or low nitrate availability. Also, the direct effects of coumarin on NR activity are not excluded.

The reduction of NO_3^- produces NH_4^+. High levels of endogenous NH_4^+ are harmful to higher plants (27). Ammonia is rapidly assimilated into organic N via the GS/GOGAT cycle or via the GDH alternative pathway (24). Consequently, ammonium tissue concentrations are the outcome of a complex balance among absorption, formation and assimilation of ammonia. In this study, coumarin caused significant decreases in endogenous NH_4^+ concentration at lower concentration (0.025, 0.05 and 0.1 mM) and an increase at the highest concentration (1 mM) (Fig. 5). The modified NH_4^+ levels presents an additional evidence of altered nitrogen metabolism during coumarin stress. Coumarin enhanced the GS activity in roots at lower concentration (Fig. 7). Similar to our observations, Abenavoli *et al*. (2003) reported that in suspension culture, 0.05 mM coumarin stimulated the GS activities in carrot cells (5). The increase in GS activity was probably linked to the induction of the cytosolic GS isoform (GS1) protein and transcripts by coumarin. Besides, the stimulation of GDH activity was observed in carrot cells in suspension culture (5) and in leaves of alfalfa (10). In contrast, 1 mM coumarin decreased the activities of GS and GDH in alfalfa roots (Figs. 7 and 8). This discrepancy might be caused by the different plant species and tissues used. Coumarin can induce accumulation of reactive oxygen species (ROS) in plant species, which causes oxidative stress (4). Thus, reductions in these enzymes activities may be attributed to oxidative modifications of these enzyme proteins. Reduction of ammonium at lower concentration might be responsible for the induction of GS activity observed in alfalfa roots. Enhancement of this ion observed at 1 mM coumarin treatment could be explained by the

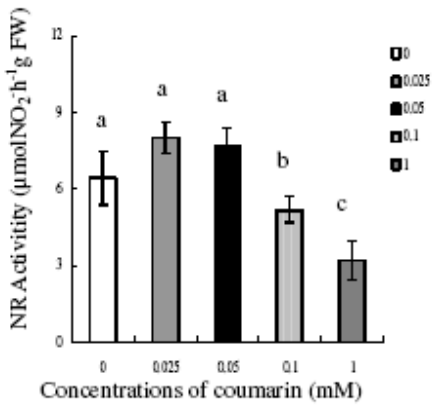


Figure 6. Effects of coumarin treatments on the activities of NR in roots. Bars represent SD of means. Different letters indicate values that differ significantly from the control at $P<0.05$.

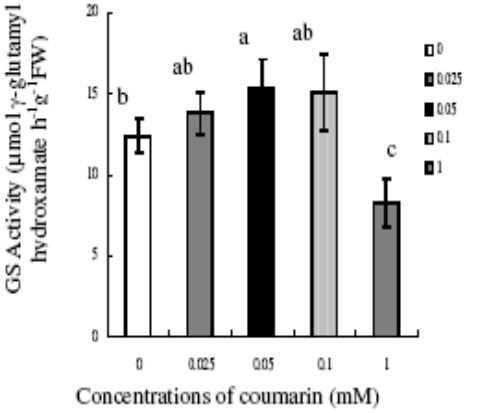


Figure 7. Effects of coumarin treatments on the activities of GS in roots. Bars represent SD of means. Different letters indicate values that differ significantly from the control at $P<0.05$.

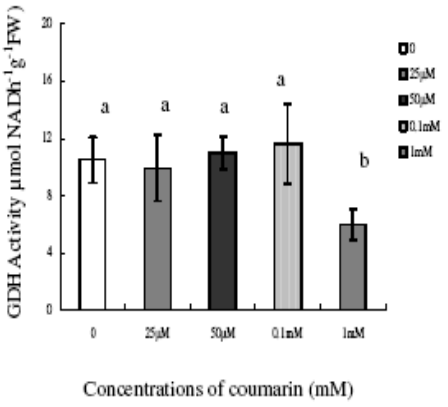


Figure 8. Effects of coumarin treatments on the activities of GDH in roots. Bars represent SD of means. Different letters indicate values that differ significantly from the control at $P<0.05$.

significant reduction in GS and GDH activity. Therefore, both GS and GDH played key roles in scavenging excessive endogenous ammonium in roots of alfalfa, and GS was more important at low coumarin (≤0.1 mM)

CONCLUSIONS

High coumarin concentrations decrease the growth but increased the inhibitory activities of key enzymes (NR, GS and GDH) involved in nitrogen assimilation and catabolism. The adverse effects of high coumarin concentration declined the net nitrate

uptake and nitrate along with an increase of NH_4^+ contents in alfalfa roots. Thus, the inhibitory effects of higher coumarin concentration (0.1 and 1mM) on seedlings took place partly due to the influence on nitrogen metabolism. The low concentrations of coumarin (0.025 and 0.5mM) stimulated the net nitrate uptake and N metabolism in roots. Thus, the positive or negative effects of coumarin on N metabolism in roots of alfalfa seedlings depended on its concentration.

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REFERENCES

1. Abenavoli, M.R. and Muscolo, A. (1996). Physiological changes induced by coumarin in leaf explants of *Petunia hybrida*. *Plant Physiology and Biochemistry* Special Issue, 10th FESPP Congress pp. 310.
2. Abenavoli, M.R., Santis, D.E.C., Sidari, M., Sorgon, A.A., Badiani, M. and Cacco, G. (2001). Influence of coumarin on the net nitrate uptake in durum wheat (*Triticum durum* Desf. cv. Simeto). *New Phytologist* **150**: 619-627.
3. Abenavoli, M.R., Sorgona, A., Albano, S. and Cacco, G. (2004). Coumarin differentially affects the morphology of different root types of maize seedlings. *Journal of Chemical Ecology* **30**:1871-1883.
4. Abenavoli, M.R., Cacco, G., Sorgona, A.,Marabottini, R., Paolacci, A.R., Ciaffi, M. and Badian, M. (2006). The inhibitory effects of coumarin on the germination of durum wheat (*Triticum turgidum* ssp. durum, CV. SIMETO) seeds. *Journal of Chemical Ecology* **32**: 489-506.
5. Abenavoli, M.R., Sorgona, A., Sidari, M., Badinai, M. and Fuggi, A. (2003). Coumarin inhibits the growth of carrot (*Daucus carota* L. cv. Saint Valery) cells in suspension culture. *Journal of Plant Physiology* **160**: 227-237.
6. Aslam, M., Hukffaker, R.C. and Rains, D.W. (1984). Early effects of salinity on nitrate assimilation in barley seedlings. *Plant Physiology* **76**: 321-325.
7. Baleroni, C.R.S., Ferrarese, M.L.L., Souza, N.E. and Ferrarese-Filho, O. (2000). Lipid accumulation during canola seed germination in response to cinnamic acid derivatives. *Biologia Plantarum* **43**: 313-316.
8. Bradford, M.M. (1976). A rapid and sensitive method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.
9. Chaffei, C., Gouia, H. and Ghorbel, M.H. (2003). Nitrogen metabolism in tomato plants under cadmium stress. *Journal of Plant Nutrition* **26**: 1617-1634.
10. Chen, Y.P., Zhang, L. and Wang, J.C. (2011). Effects of coumarin application on plant growth and nitrogen metabolism in leaves of *Medicago sativa*. *Allelopathy Journal* **28**: 105-114.
11. Chon, S.U., Choi, K.S., Jung, S., Jang, H.G., Pyo, B.S. and Kim, S.M. (2002). Effects of alfalfa leaf extracts and phenolic allelochemicals on early seedling growth and root morphology of alfalfa and barnyardgrass. *Crop Protection* **21**: 1077-1082.
12. Cruz, C., Dominguez-Valdivia, M.D., Aparicaic-Tejo, P.M., Lamsfus, C. and Martins-Loucao, M.A. (2006). How does glutamine synthetase activity determine plant tolerance to ammonium? *Planta* **223**: 1068-1080.
13. Daniel-Vedele, F., Filleur, S. and Caboche, M. (1998). Nitrate transport: a key step in nitrate assimilation. *Current in Opinion Plant Biology* **1**: 235-239.
14. Dluzniewska, P., Gessler, A., Dietrich, H., Schnitzler, J.P. and Teuber, M.H. (2007). Nitrogen uptake and metabolism in *Populus*×canescens as affected by salinity. *New Phytologist* **173**: 279 -293.
15. Forde, B.G. and Lea, P.J. (2007). Glutamate in plants: Metabolism, regulation, and signaling. *Journal of Experimental Botany* **58**:2339-2358.
16. Gine, M.F., Bergamin, F.H., Zagatto, E.A.G. and Reis, B.F. (1980). Simultaneous determination of nitrate and nitrite by flow injection analysis. *Analytica Chimica Acta* **114**:191-197.

17. Hoagland, D.R. and Arnon, D.I. (1950). The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular* **347**: 32-50.
18. Jansson, E. and Svensson, S. (1980). Coumarin effects on *Glycine max* hypocotyl explants. *Physiology Plantarum* **48**: 486 - 490.
19. Krom, M. (1980). Spectrophotometric determination of ammonia: Study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. *Analyst* 105: 305-316.
20. Kupidlowska, E., Kowalec, M., Sulkowski, G. and Zobel, A.M. (1994). The effect of coumarins on root elongation and ultrastructure of meristematic cell protoplast. *Annals of Botany* **73**: 525-530.
21. Li, Z.X., Qin, S.J., Gao, H., Lu, D.G. and Ma, H.Y. (2009). Effects of phenolic compounds on root respiration and root activity of *Cerasus sachalinensis* Kom. *Allelopathy Journal* **24**:113-122.
22. Loulakakis, K.A. and Roubelakis-Angelakis, K.A. (1990). Intracellular localization and protein of NADH-glutamate dehydrogenase from *Vitis vinifera* L.: Purification and characterization of the major leaf isoenzyme. *Journal of Experimental Botany* **41**:1223-1230.
23. Lupini, A., Sorgonà, A., Miller, A.J. and Abenavoli, M.R. (2010) Short-term effects of coumarin along the maize primary root axis. *Plant Signaling and Behavior* **5**:1395-1400.
24. Miflin, B.J. and Habash, D.Z. (2002). The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *Journal of Experimental Botany* **53**: 979-987.
25. Oaks, J., Stulen, K., Jones, Winspear, M.J. and Booesel, I.L. (1980). Enzymes of nitrogen assimilation in maize roots. *Planta* **148**: 477-484.
26. Rice, E.L. (1984). *Allelopathy*. Academic Press, Orlando, FL: Pp. 424.
27. Sánchez, E., Rivero, R.M., Ruiz, J.M. and Romero, L. (2004). Changes in biomass, enzymatic activity and protein concentration in roots and leaves of green bean plants (*Phaseolus vulgaris* L. cv. Strike) under high NH_4NO_3 application rates. *Scientia Horticulturae* **99**: 237-248.
28. Scheible, W.R., Morcuende, Czechowski, R., Fritz, T., Osuna, C., Palacios-Rojas, D., Schindelasch, N., Thimm, D., Udvardi, O. and Stitt, M.K.M. (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. *Plant Physiology* **136**: 2483-2499.
29. Sidari, M., Pusino, A., Gessa, C. and Cacco, G. (1998). Effect of imazamethabenz-methyl on nitrate uptake in wheat (*Triticum durum* L.). *Journal of Agricultural and Food Chemistry* **46**: 2800-2803.
30. Tartoura, K., Rocha, A. and Youssef, S. (2004). Synergistic interaction between coumarin 1,2-benzopyrone and indole-3-butyric acid in stimulating adventitious root formation in *Vigna radiata* (L.) Wilczek cuttings: I. Endogenous free and conjugated IAA and basic isoperoxidases. *Plant Growth Regulation* **42:** 253-262.
31. Teixeira, J. and Pereira, S. (2007) High salinity and drought act on an organ-dependent manner on potato glutamine synthetase expression and accumulation. *Environmental and Experimental Botany* **60**: 121-126.
32. Van Sumere, C.F., Cottenie, J., Degreef, J. and Kint, J. (1971). Biochemical studies in relation to the possible germination regulatory role of naturally occurring coumarin and phenolics. *Recent Advances in Phytochemistry* **4**: 165-221.
33. Wang, J.C., Wu, Y., Wang, Q., Peng, Y.L., Pan, K.W., Luo, P. and Wu, N. (2009). Allelopathic effects of *Jatropha curcas* on marigold (*Tagetes erecta* L.). *Allelopathy Journal* **24**:123-130.
34. Wang, R., Guegler, K., LaBrie, S.T. and Crawford, N.M. (2000). Genomic analysis of nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *The Plant Cell* **12**: 1491-1510.
35. Zhou, W., Sun, Q.J., Zhang, C.F., Yuan, Y.Z., Zhang, J. and Lu, B.B. (2004). Effect of salt stress on ammonium assimilation enzymes of the roots of rice (*Oryza sativa*) cultivars differing in salinity resistance. *Acta Botanica Sinica* **46**: 921-927.