INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2006/08–2–218–220
http://www.fspublishers.org

Response of Pea Plants (*Pisum sativum* L.) to Reduced Supply with Molybdenum and Copper

MARIETA HRISTOZKOVA, MARIA GENEVA AND IRA STANCHEVA[1]
Department of Mineral Nutrition and Water Regime, Acad M.Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Block 21, Sofia 1113, Bulgaria.
[1]Corresponding author's e-mail: ira_stancheva@abv.bg

ABSTRACT

The role of molybdenum (Mo) and copper (Cu) in regulation the activities of the enzymes involved in primary nitrogen assimilation in particular the nitrate reductase (EC 1.6.6.1) and glutamine synthetase (EC 6.3.1.2) was examined. Pea plants were grown in a phytotron chamber at 12 h photoperiod, day /night temperature 25/18 ^{o}C and photon flux density of 95 μmol m^{-1} s^{-1} until 21^{-st} day. Plants were grown at full strength Helriegel nutrient solution competed with micronutrients as in Hoagland and Arnon and reduced Mo and Cu concentrations. Although only extremely small amounts of Mo and Cu are required for normal plant growth, reduced supply with Mo and Cu to the growth medium decreased activities of the enzymes (nitrate reductase and glutamine synthetase) involved at initial steps of nitrate assimilation, fresh weight, and plastid pigment content (total chlorophyll and carotenoids). Accumulation of nitrates in plant tissues enhanced, especially in the variants with restrictive Cu concentration.

Key Words: Pea (*Pisum sativum* L.); Molybdenum; Copper, Nitrate reductase, Glutamine synthetase
Abbreviations: NR - nitrate reductase, GS- glutamine synthetase, FW-fresh weight

INTRODUCTION

Plants in balanced proportion require all the essential nutrients. Continuous shortage of a nutrient or nutrients might cause nutritional disorders and even plant death. Plant species differ regarding its Mo and Cu requirements (Gupta & Lipsett, 1981). According to Gupta (1997) pea requirement for Mo is medium, while for Cu is low (Ayala *et al.,* 1992). Molybdenum and copper are among the most recently recognized nutrient elements essential for the plant growth and development (Bottrill *et al.,* 1970; Brown & Clark, 1977). The role of Mo as a plant nutrient is related to its function as a metal component of some enzymes that catalyze nitrogen fixation, nitrate assimilation and reduction. (Kisker *et al.,* 1997; Campbell, 2001). Mo itself seems to be catalytically inactive in biological systems until is bound in complex to a unique pterin compound named Mo cofactor – MoCo. The presences of MoCo have been reported in seeds of pea (Svetsov *et al.,* 1992).

In spate of the known action of Cu on photosynthetic membranes (Quartacci *et al.,* 2001), on plasma membrane level and on its lipid composition, there is a lack of information regarding Cu effects at the function of copper in nitrogen uptake and assimilation in legumes. There are some reports about suppressed nodulation and nitrogen fixation when a copper is insufficient in the growth medium (Cartwright & Hallsworth, 1970). The study is aimed at establishing the physiological response of pea plants under conditions of reduced supply with Mo and Cu to the nutrient solution.

MATERIAL AND METHODS

Pisum sativum L. cv. Avola seeds were surface sterilized with 4% sodium hypochlorite (NaOCl) and germinated in thermostat at 25^{o}C. Five-day old seedlings were transferred to a hydroponics system - 0.8 L pots with 5 plants per pot (4 replicates per variant). Plants were grown in a phytotron chamber at 12 h photoperiod, day/night temperature 25/18^{o}C and photon flux density of 95 μmol m^{-1} s^{-1} until 21^{-th} day. Nutrient solution was prepared after Helriegel and completed with micronutrients as in Hoagland and Arnon (1950). The following variants were tested: (1) control plants – full strength nutrient solution; (2) full strength solution with twice reduced Mo concentration - 0.085 μM Mo; (3) full strength solution without Mo, (4) full strength solution with twice reduced Cu concentration – 0.15 μM Cu; (5) full strength solution without Cu.

Enzymes were extracted at 0-4^{o}C from 1.0g leaves using mortars and pestles and 5 cm^3 of optimized extraction medium 50 mM Tris-HCl (pH 8.0), 1 μM Na_2MoO_4, 10 mM $MgSO_4.7H_2O$, 1 mM EDTA, 10 mM L-cysteine, 1% PVP-40, 1g Dowex (Frechilla *et al*., 2000). The extracts were filtered through one layer of Miracloth, centrifuged at 20 000g for 20 min, and the supernatant used for the following respective assays. Nitrate reductase (NR: NADH, EC 1.6.6.2) activity was measured according to Hageman and Reed (1980). Glutamine synthetase (GS: EC 6.3.1.2) activity was determined by a biosynthetic assay based on γ-glutamil hydroxamate synthesis (O' Neal & Joy, 1973). Protein content was determined as in Bradford (1976) with BSA as a standard. The content of nitrates was determined by Nitrachek from Hawk Creek Laboratory Inc. USA and leaf tissue nitrate quick test was made.

Total chlorophyll and carotenoids in the pea leaves were exhaustively extracted in 80% acetone. The pigments

were determined spectrophotometrically after centrifugation of extract at 6 000 g for 15 min, as described by Mackinney (1941).

Data are expressed as means ± standard error where n=4. Comparison of means were performed by the Fisher LSD test (P≤ 0.05) after performing multifactor ANOVA analysis. The STASTICA (version 6.0) package was used for statistical analysis.

RESULTS AND DISCUSSION

Nitrate reductase and glutamine synthetase enzymes are consistently involved in nitrogen assimilation pathway in higher plants (Oaks, 1994), because these enzymes catalyze the first steps in nitrate assimilation a pathway of key importance for plant nutrition. In the variants with ½ Mo in the nutrient medium about 40% lower nitrate reductase enzyme activity in pea leaves was observed (figure 1). Significantly reduced enzyme activity compared with the control (80%) was measured in the Mo free variants. Molybdenum is a micronutrient required by nitrate reductase because it is involved in Mo-related cofactor of the enzyme, forming MoCo domain in the nitrate reductase enzyme structure (Mendel, 1997). The lower level of NR enzyme activity in Mo-deficient plants was observed by many authors (Hille, 1996; Gupta, 1997; Mendel, 1997; Yaneva *et al*., 2000). The activity of NR in Cu deficient plants was also found to be significantly lower than the control plants (fig. 1). Little is known about the impact of copper on the nitrate reductase biosynthesis and function. On the other hand significant differences concerning nitrate reductase activity in the variants with reduced and removed Cu ions was not found. Changes of nitrate reductase activity (fig. 1) in pea leaves with Mo and Cu shortage corresponded to the nitrate content in the studied variants (fig. 2). Distinguished high nitrate content in the variants with restrictive Cu concentrations was observed. According to the findings of Brown and Clark (1977) free amino acids and nitrates may accumulate in copper deficient plants, resulted in low NR activity in these plants which is supported by our results. Close nitrate content values in the variants with ½ Mo and Mo free variants despite of the different NR activity is probably due to lower nitrate uptake when Mo is eliminated from the growth medium.

The changes in glutamine synthetase activity in the pea leaves as a result of reduced Mo concentrations followed the same trend as in nitrate reductase (fig. 3). A decrease with 30% was observed for the variants with twice-reduced Mo and 56% for Mo free treatments. As it was reported (Oaks, 1994) a direct dependence exists between NR and GS activities, so reduced levels of NR enzyme activity will lead to the lower GS activity rates in higher plants.

Lower GS activities (45%) regarding control were measured in the variants with twice-reduced Cu as well as in Cu free variants. Some authors (Henriques, 1989; Casimiro *et al*., 1990) reported about the negative effects of copper

Table I. Plastid pigment content (mg g^{-1}) in pea plants grown on growth medium with reduced supply with Mo and Cu

Variants	Chlorophyll a	Chlorophyll b	Carotenoids	Chla/Chlb
1control	0.495±0.035^{c}	0.299±0.056^{b}	0.159±0.009^{c}	1.763±0.296
2 (1/2Mo)	0.420±0.010^{bc}	0.188±0.007^{a}	0.123±0.009^{ab}	2.239±0.101
3 (-Mo)	0.445±0.005^{b}	0.228±0.016^{a}	0.135±0.008^{b}	1.968±0.110
4 (1/2Cu)	0.453±0.005^{b}	0.210±0.010^{a}	0.126±0.010^{ab}	2.163±0.075
5 (-Cu)	0.403±0.018^{a}	0.182±0.015^{a}	0.117±0.007^{a}	2.228±0.083

Values are means ± S.E., n=4. Different letters indicate significant differences assessed by Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

Fig. 1. Nitrate reductase activity in pea leaves at different supply with Mo and Cu.

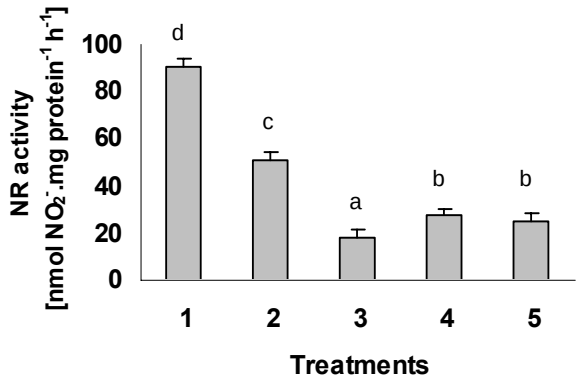
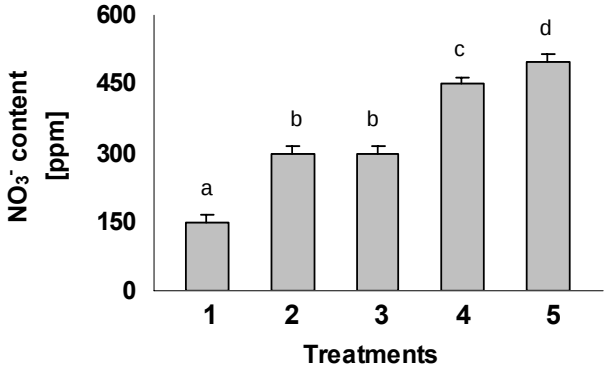


Fig. 2. Nitrate content in pea leaves in response to different supply with Mo and Cu.



deficiency on ultrastucture of chloroplasts. Because chloroplastic GS is a predominant enzyme in the leaves, lower Cu supplied to the plants in our experiment could decrease GS activity in the pea leaves.

The plastid pigments content (chlorophyll *a*, b and carotenoids) also decreased (by comparison with the control) in the variants with reduced Mo and Cu in the growth medium as well as in the variants free of these elements (Table I). Significant differences regarding the chlorophyll *b* content among the variants with reduced Mo and Cu supply were not found. The chlorophyll *a / b* ratio was increased in the experimental variants compared with the control. Our results are in agreement with the study of Bottrill *et al.* (1970) that the molybdenum deficiencies

Fig. 3. Glutamine synthetase activity at different supply with Mo and Cu.

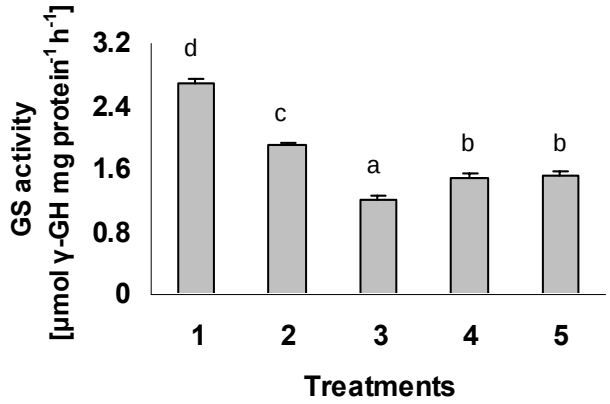
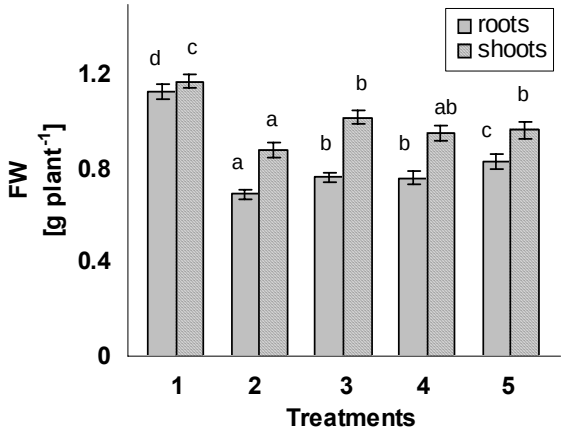


Fig. 4. Root and shoot fresh weight of pea grown at restrictive Mo and Cu concentrations.



decreased the chlorophyll content of spinach leaves. Gupta (1997) reported about plant chlorosis, due to the inability of the plant to form chlorophyll caused by Mo deficiency.

Noticeably reduced chlorophyll *a* content and carotenoids compared with the control were observed in the Cu free variants (Table I). These results are consistent with the findings of Hille (1996) for the Cu deficient sugar beet leaves. Ayala *et al.* (1992) reported that PSII preparations from Cu depleted pea plants showed a decreased carotenoid content in light harvesting chlorophyll *a/b* complex of photosystem II.

We did not observe distinguish Mo and Cu deficiency symptoms on the experimental pea plants but fresh weight of roots and shoots (fig. 4) was reduced with 40% and 25% respectively by the lessening Mo concentrations. Similar effects were observed in the plants with the reduced Cu supply where root and shoot biomass respectively decreased with 30% and 20%. Although extremely small amounts of Mo and Cu are required for normal plant growth insufficient Mo and Cu concentrations applied resulted in stunting of the plant growth and biomass accumulation.

In conclusion, restrictive supply with Mo and Cu to the growth medium resulted in lack of the deficiency symptoms in pea plants, but in contrast activities of the enzymes (nitrate reductase & glutamine synthetase) involved at initial steps of nitrate assimilation was strongly affected. Reduced Mo and Cu concentrations resulted in decrease in plastid pigment content in the pea leaves, shoot and root biomass and nitrate accumulation.

REFERENCES

Ayala, M.B., J.L. Gorge, M. Lachica and G. Sandmann, 1992. Changes in carotenoids and fatty acids in photosystem II of Cu–deficient pea plants. *Physiol. Pl*., 84: 1–5

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem*., 72: 248–54

Bottrill, D.E., J.V. Possingham and P.E. Kriedemann, 1970. The effect of nutritient deficiency of photosynthesis and respiration in spinach. *Pl. Soil*, 32: 424–38

Brown, J.C. and R.B. Clark, 1977. Copper as essential to wheat reproduction. *Pl. Soil*, 48: 509–23

Campbell, W.H., 2001. Structure and function of eucaryotic NAD(P)H: nitrate reductase. *Cell. Mol. Life Sci*., 58: 194–204

Casimiro, A., J. Barroso and M.S. Pas, 1990. Effect of copper deficiency on photosynthetic electron transport in wheat plants. *Physiol. Pl.*, 79: 459–64

Cartwright, B. and E.G. Hallsworth, 1970. Effects of copper deficiency on root nodules of subterranean clover. *Pl. Soil*, 33: 685–98

Frechilla, S., E.M. Gonzalez, M. Royuela, F.R Minchin, P.M. Aparicio–Tejo and C. Arrese–Igor, 2000. Source of nitrogen nutrition (nitrogen fixation or nitrate assimilation) is a major factor involved in pea response to moderate water stress. *J. Pl. Physiol*., 157: 609–17

Gupta, U.C., 1997. *Molybdenum in Agriculture*. Cambridge University Press, UK.

Gupta, U.C. and J. Lipsett, 1981. Molybdenum in soil, plants, and animals. *Advances in Agronomy*, 34: 73–115

Hageman, R.H. and A.J. Reed, 1980. Nitrate reductase from higher plants. *Methods Enzymol*., 69: 270–0

Henriques, F.S., 1989. Effects of copper deficiency on the photosynthetic apparatus of Sugar beet (Beta vulgaris L). *J. Pl. Physiol*., 135: 453–8

Hille, R., 1996. The mononuclear molybdenum enzymes. *Chem. Rev*., 96: 2757–816

Hoagland, D.R. and D.I. Arnon, 1950. The water–culture method for growing plants without soil. *Calif. Agr. Exp. Sta. Circ*., 347: 1–39

Kisker, C., H. Schindelin and D.C. Rees, 1997. Molybdenum–cofactor–containing enzymes: structure and mechanism. *Ann. Rev. Biochem*., 66: 233–67

Mackinney, G., 1941. Absorption of light by chlorophyll solution. *J. Biol. Chem.,* 140: 315–22

Mendel, R.R., 1997. Molybdenum cofactor of higher plants: biosynthesis and molecular biology. *Planta*, 203: 399–405

Oaks, A., 1994. Primary nitrogen assimilation in higher plants and its regulation. *Canadian J. Bot.* 72: 739–50

O'Neal, D. and K.W. Joy, 1973. Glutamine synthetase of pea leaves, I Purification, stabilization and pH optima. *Arch. Biochem. Biophis*., 159: 113–22

Quartacci, M.F., E. Cosi and F. Navari–Izzo, 2001. Lipids and NADH–dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. *J. Exp. Botany*, 52: 77–84

Svetsov, A.A, A.V Amelin, N.V. Novikova and N.P. Lvov, 1992. The molybdenum cofactor of pea seeds. *Sov. Plant Physiol*., 39: 104–9

Yaneva, I.A., V.D. Baydanova and R.V. Vankova–Radeva, 2000. Nitrate reductase activation state in leaves of molybdenum–deficient winter wheat. *J. Pl. Physiol*. 157: 495–501

(Received 20 August 2005; Accepted 18 February 2006)