Pergamon

PII: S0031-9422(97)00601-8

Phytochemistry, Vol. 47, No. 5, pp. 701–706, 1998
© 1998 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0031-9422/98 $19.00+0.00

EFFECT OF GLUTAMINE SUPPLY ON NITRATE REDUCTASE ISOFORMS IN MAIZE SEEDLINGS

NIHARIKA SHANKAR and HARI S. SRIVASTAVA*

Department of Plant Science, Rohilkhand University, Bareilly 243006, India

(*Received in revised form* 18 *June* 1997)

Key Word Index—*Zea mays*; Gramineae; Isoforms; nitrate; nitrate reductase; nitrite; protein.

Abstract—Supply of 1 to 20 mM glutamine in the presence of 5 mM nitrate to the detached roots, scutella or shoots for 24 h from 5-day-old maize seedlings inhibited NADH:NR activity in light, but increased the same in darkness. NADPH:NR in roots and scutella however, was inhibited by the amide both in light and in darkness, the inhibition in light being higher than that of NADH:NR under similar conditions. The *in vitro* provision of glutamine to the enzyme preparation from light grown roots had no effect, but Mg^{2+} or ATP inhibited NADH:NR activity, both in the absence as well as presence of glutamine. The nitrate content of the excised organs was little affected by glutamine supply but for a substantial decline in roots in light. Nitrite content on the other hand, was increased substantially in all tissues and in both light and darkness. The uptake of nitrate by intact seedlings was inhibited by glutamine. The total protein content of excised organs was unaffected by glutamine supply in dark but in light it declined in scutellum at higher concentrations of the amide and increased at lower concentrations and at each concentration in the shoots. The experiments demonstrate that NADPH:NR is more sensitive to glutamine than the NADH:NR and that the amide has a plurality of effects on nitrate uptake and assimilation process. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Ammonium, amino acids and amides as potential end products of nitrate assimilation are known to inhibit the process through the activity of nitrate reductase (NR), which is considered to be the rate-limiting enzyme in the process [1]. The inhibition by ammonium is more apparent in micro-organisms than in higher plants, and the effect of amino acids and amides on NR activity varies according to the species and the nature of the amino acid or the amide [2–4]. The inhibition of the enzyme activity by glutamine has been reported from soybean [5], tobacco [6, 7], maize [8] and in cultured spinach cells [9], although glutamine had either no effect in spinach [10] or increased *in vivo* NR activity in *Datura* [11]. In maize, NR activity was more sensitive to glutamine in roots than in shoots [8]. In a young maize seedling, there may be a differentiation between the enzyme in storage tissues, such as endosperm and scutellum, and the growing tissues, such as roots and shoots, because the enzyme in two types of organs appears to have different physiological roles [4]. These differentiations may be reflected in their response to glutamine also. Further, the enzyme is strongly influenced by the light/dark regime and exhibits hysteretic behaviour [11]. It exists in a low activity phosphorylated form in dark and in a highly active dephosphorylated form in light [12–14]. Thus, the effect of glutamine or for that matter any other critical regulator may vary according to light/dark regime also. Most of the regulatory studies have been conducted with the NADH specific NR (EC 1.6.6.1) isoform, although the presence of NAD(P)H:NR (EC 1.6.6.2) has also been demonstrated in many species including maize [4]. The current investigation was undertaken to examine whether the effect of glutamine varied according to the seedling organ, isoform and to light/dark regime. In the present study, the detached organs were used instead of intact seedlings, so that the interferences from glutamine and other amino acids released from the storage proteins in the endosperm [15] were avoided. The nitrate, nitrite and protein contents of the organs were also determined to evaluate the significance of the effect of glutamine on nitrate assimilation.

RESULTS AND DISCUSSION

Glutamine as one of the potential end products of nitrate assimilation is expected to inhibit NR, the first enzyme in the pathway. This expectation has been realised in several earlier investigations (see intro-

---

* Author to whom correspondence should be addressed.

duction). The inhibition of NR by glutamine was recorded in the present study also in light incubated seedling parts, although the magnitude of inhibition varied according to the isoform of NR and also to some extent according to the seedling organ. When detached roots, scutella or shoots were incubated in a medium containing different concentrations of glutamine, the NADH:NR activity in light declined, the decline usually increasing with the increase in concentration of glutamine (Table 1). At 20 mM glutamine, the maximum inhibition of 37% was observed in scutellum, followed by 28% in roots and only 18% in shoots. In dark incubated organs, however, glutamine increased NADH:NR activity. At the highest concentration of the amide, the increase was 27% in the roots, 14% in scutella and 59% in shoots. As expected, the enzyme activity was higher in light than in darkness at each concentration of glutamine and in each organ. *In vivo* regulation of nitrate reduction by glutamine has also been realised in a few studies. Inhibition of glutamine synthetase, the glutamine synthesizing enzyme, by the herbicide phoshinothrocin, abolishes the decline in NR m-RNA, which is otherwise observed due to glutamine accumulation [6]. However, in *Medicago sativa*, phosphinothrocin inhibits NR activity, which is more apparent when the enzyme is assayed *in vivo* than *in vitro* [16]. According to the authors [16], this is apparently because inhibition of GS by phosphinothrocin leads to reduced photosynthates and NADPH, required for *in vivo* nitrate reduction. In maize seedlings, the flow of glutamine and asparagine from endosperm to the embryonic axis appears to be an inherent regulatory process in the nitrate assimilation. The NR activity is suppressed in such a system and exogenous supply of glutamine causes only small inhibition [15]. However, when reserve mobilization from the endosperm is restricted, the NR expresses at higher levels and is more responsive to externally supplied glutamine.

As far as the isoformic differentiation is concerned, the experiments demonstrate greater sensitivity of NADPH:NR to glutamine than NADH:NR. The activity of this isoform declined uniformly with glutamine supply in roots and in scutella, and in both light and in darkness (Table 2). The maximum inhibition (52%) was at 20 mM glutamine in scutella and in light. In roots, the enzyme activity was higher in light than in darkness at each concentration of glutamine. However, in scutella, the difference in the activity of NADPH:NR in light or in darkness was not very distinct in glutamine supplied organs. Li *et al.* [8] also suggested that a higher sensitivity to glutamine of root than of shoot NR was due to the presence of NAD(P)H:NR exclusively in the roots.

The inhibition of NADH:NR by the glutamine is considered to be at the gene expression (repression) level [7, 9, 12]. The observed increase in enzyme activity by the amide in dark (Table 1) however, demonstrates some additional regulatory mechanism. It is known that the enzyme from dark incubated plants or plant organs is in a phosphorylated inactive from, while that from light treated ones is in a dephosphorylated form [12–14, 17–19]. Apparently, either the glutamine itself or some of its metabolic products interfered with the phosphorylation and inactivation of NADH:NR in the dark. From our *in vitro* data (Table 3), it appears that glutamine itself protects against the inhibition of enzyme by ATP and Mg^{2+} in the dark grown roots. The enzyme from the roots of dark-grown seedlings was inhibited by Mg^{2+} and/or ATP only in the absence of glutamine. However, the enzyme from light-grown seedlings was inhibited by Mg^{2+} and ATP both in the absence as well as presence of glutamine. In leaf discs from light-grown tobacco, however, glutamine has no effect on the activation state of NR [20].

As a critical regulator of nitrate reduction, glutamine is expected to affect the levels of different inor-

Table 1. Effect of glutamine concentration on NADH:NR activity in light and in darkness in detached roots, scutella and shoots

	NR activity, μmol NO_2^- hr^{-1} (g fr. wt)$^{-1}$					
Glutamine	Root		Scutellum		Shoot	
conc., mM	Light	Dark	Light	Dark	Light	Dark
0	2.07±0.24 (100)	0.83±0.04 (100)	2.73±0.20 (100)	1.48±0.14 (100)	1.90±0.19 (100)	0.71±0.05 (100)
1	1.61±0.13 (78)	1.16±0.06 (140)	2.51±0.15 (92)	1.97±0.12 (133)	1.83±0.19 (96)	0.89±0.07 (125)
5	1.69±0.11 (82)	1.02±0.07 (123)	2.52±0.24 (92)	1.40±0.13 (95)	1.70±0.14 (89)	0.94±0.09 (133)
10	1.40±0.06 (67)	1.17±0.07 (146)	2.02±0.21 (74)	1.39±0.13 (94)	1.60±0.16 (84)	1.11±0.13 (157)
20	1.49±0.14 (72)	1.06±0.09 (127)	1.72±0.17 (63)	1.69±0.13 (114)	1.56±0.12 (82)	1.13±0.09 (159)

Details of seedling growth and glutamine treatment are as in Experimental. The data are ±S.D. ($n = 6$). The values relative to control are given in brackets.

Table 2. Effect of glutamine concentration on NADPH:NR activity in darkness and in light in detached roots and scutella

	NR activity μmol NO_2^- hr^{-1} (gf r. wt)$^{-1}$			
Glutamine	Root		Scutellum	
conc., mM	Light	Dark	Light	Dark
0	1.58±0.14 (100)	1.12±0.11 (100)	1.88±0.17 (100)	1.52±0.18 (100)
1	1.19±0.14 (75)	0.71±0.06 (63)	1.45±0.07 (77)	1.55±0.14 (102)
5	1.20±0.08 (76)	0.65±0.04 (58)	1.20±0.06 (64)	1.05±0.09 (69)
10	1.09±0.09 (68)	0.48±0.03 (42)	1.10±0.12 (58)	1.03±0.08 (68)
20	0.97±0.06 (61)	0.55±0.04 (50)	0.91±0.06 (48)	0.86±0.08 (56)

Details as in Table 1.

Table 3. *In vitro* effects of ATP and Mg on NADH:NR activity from roots of light or dark grown seedlings

	NR activity, μmol NO_2^- hr^{-1} (g fr. wt)$^{-1}$			
	Light		Dark	
Treatment	−Glutamine	+Glutamine	−Glutamine	+Glutamine
None	2.19±0.11 (100)	2.07±0.09 (100)	0.91±0.03 (100)	0.94±0.07 (100)
ATP	1.47±0.09 (67)	1.43±0.08 (69)	0.78±0.04 (86)	0.91±0.09 (97)
$MgCl_2$	1.62±0.14 (74)	1.51±0.13 (76)	0.74±0.05 (81)	0.86±0.09 (91)
ATP+$MgCl_2$	1.33±0.10 (61)	1.44±0.15 (69)	0.67±0.09 (74)	0.85±0.09 (90)

Enzyme was extracted from the roots of the seedlings raised for 5 days with 1/2 strength Hoagland's soln containing 5 mM KNO_3 as sole nitrogen source, in light or in darkness. The preparation was incubated at 10° for 30 min before assay with ATP, $MgCl_2$ (2 mM each) and 0 (−) or 10 mM (+) glutamine. Data are average ±S.D. (n = 4). Values relative to control are given in brackets.

ganic and organic nitrogenous metabolites. In the present study, the amide had no effect on nitrate content of the isolated organs, except for a decline in roots. In light incubated roots, accumulation of nitrate at 20 mM glutamine was 49% of the control (minus glutamine) (Table 4). However, in dark incubated roots, and in scutella or shoots incubated in light or in darkness, glutamine supply had little effect on nitrate content. The accumulation of nitrate in roots and scutella was higher in light than in darkness at each concentration of glutamine. However, shoots accumulated more nitrate in darkness than in light. The inhibition of nitrate accumulation in the excised roots, could have been due to reduced nitrate uptake by the roots. In the present study, we encountered difficulties in measuring nitrate content of the media (to measure nitrate uptake) due to interference from the leachates from the excised organs. But in an experiment with intact seedlings incubated for 3 hr in light in different concentrations of glutamine, an inhibition of the uptake of nitrate was observed, which ranged from 42 to 58% (data not given). Apparently, the nitrate uptake mechanism/system of isolated scutella or shoots, which was most likely through diffusion process, was insensitive to glutamine. In other studies, nitrate uptake by the yeast *Candia nitriphila* is unaffected by glutamine [21], but is apparently inhibited in soybean [22]. Quillere *et al.* [23] have also suggested that lower nitrate accumulation in genetically transformed tobacco plants, which have 74 to 133% higher endogenous glutamine content than the wild-type, is due to reduced nitrate uptake. Thus besides the inhibition of nitrate reduction, the nitrate uptake process also appears to be targeted in the regulation of nitrate assimilation by glutamine.

The detached organs accumulated significant

Table 4. Effect of glutamine concentration on nitrate content in light and in darkness in detached roots, scutella and shoots

	Nitrate, μ mol (g fr. wt)$^{-1}$					
Glutamine	Root		Scutellum		Shoot	
conc., mM	Light	Dark	Light	Dark	Light	Dark
0	62.5±5.8 (100)	20.6±0.8 (100)	60.4±4.0 (100)	30.1±1.9 (100)	53.4±4.6 (100)	101.4±9.8 (100)
1	52.1±4.3 (83)	19.4±0.7 (94)	58.4±4.6 (97)	31.4±1.6 (104)	54.6±5.1 (102)	98.6±8.8 (97)
5	39.4±2.8 (63)	18.7±1.3 (91)	57.6±5.7 (95)	27.9±2.6 (93)	56.7±2.6 (106)	97.6±6.7 (96)
10	38.6±4.1 (62)	19.4±1.2 (94)	60.0±4.2 (99)	28.6±3.0 (95)	51.6±5.1 (97)	103.2±5.2 (102)
20	30.5±1.9 (49)	18.9±0.7 (92)	56.4±2.8 (93)	27.4±1.8 (91)	50.8±6.1 (95)	97.0±6.4 (96)

Details as in Table 1.

amount of nitrite under incubation conditions. The nitrite accumulation increased substantially with increasing concentrations of glutamine in all three organs, in both the light and in darkness (Table 5). The increase in nitrite content (over control) at 20 mM glutamine was the highest (about 8-fold) in roots incubated in light and was the lowest (about 1.3-fold) in scutella incubated in darkness. The accumulation of nitrite was usually higher in darkness than in light in each organ and at each concentration of glutamine, except for the roots at higher concentrations of glutamine. The accumulation of nitrite in glutamine supplied seedling parts is apparently due to the inhibition of nitrite reductase. In another study, Faure *et al.* [24] have reported a correlated inhibition of NR and nitrite reductase by the products of nitrate assimilation. The inhibition of nitrite reductase activity by glutamine has been reported in sunflower cotyledons [25]. Further in soybean leaves, the formation of NR transcript is abolished completely in the presence of 10 mM glutamine in the nutrient solution [26]. Thus, glutamine seems to be exerting a broader feed back inhibitory effect on the nitrate reduction process. A perusal of our data on NR activity and on nitrate and nitrite contents reveals that the reduction of nitrite was more sensitive to inhibition by glutamine than that of nitrate to nitrite. One would also expect a general inhibition of protein accumulation by glutamine as the amide inhibited nitrate uptake and nitrate and nitrite reduction by the isolated organs. However, in roots 1 or 5 mM glutamine caused a substantial increase in protein content in light, while higher concentrations had little effect (Table 6). It had however, no effect on protein content at any concentration in darkness. In scutella there was inhibition of protein content in light at 10 or 20 mM glutamine, and almost no effect at lower concentrations in light or at any concentration in darkness. In shoots, glutamine caused a gradual increase in protein content with increase in its concentration in light, although it had little effect in darkness, as was the case in roots or scutella. The increase in protein content is apparently

Table 5. Effect of glutamine concentration on nitrite content in light and in darkness in detached roots, scutella and shoots

	Nitrite, μ mols (g fr. wt)$^{-1}$					
Glutamine	Root		Scutellum		Shoot	
conc., mM	Light	Dark	Light	Dark	Light	Dark
0	2.8±0.1 (100)	5.5±0.4 (100)	11.4±0.3 (100)	15.4±1.3 (100)	0.9±0.1 (100)	1.8±0.0 (100)
1	8.5±0.6 (304)	11.6±0.7 (210)	13.0±0.7 (114)	15.0±1.2 (97)	1.4±0.8 (149)	2.9±0.1 (166)
5	17.2±0.9 (613)	11.6±1.0 (210)	12.7±0.6 (111)	17.9±1.6 (116)	2.9±0.7 (303)	3.4±0.2 (193)
10	17.6±1.2 (625)	12.9±1.3 (233)	14.6±0.5 (128)	20.4±1.9 (132)	3.0±0.2 (318)	4.2±0.2 (236)
20	22.3±1.4 (794)	14.4±1.4 (261)	18.1±1.6 (159)	20.6±2.0 (134)	4.6±0.4 (478)	4.9±0.2 (273)

Details as in Table 1.

Table 6. Effect of glutamine concentration on protein content in light and in darkness in detached roots, scutella and shoots

Glutamine conc., mM	Protein, mg (g fr. wt)$^{-1}$					
	Root		Scutellum		Shoot	
	Light	Dark	Light	Dark	Light	Dark
0	13.2±1.8 (100)	15.6±2.6 (100)	19.2±2.6 (100)	27.6±3.6 (100)	21.0±2.0 (100)	26.4±2.6 (100)
1	18.7±2.6 (142)	15.8±2.4 (101)	18.7±2.1 (97)	30.9±2.6 (112)	23.3±1.6 (111)	24.6±1.3 (93)
5	17.8±3.4 (135)	15.4±1.6 (99)	18.4±2.6 (96)	30.8±2.9 (111)	25.5±1.7 (121)	24.1±1.4 (91)
10	13.2±1.3 (100)	16.7±1.4 (107)	14.5±1.9 (75)	30.6±1.8 (131)	27.5±1.6 (131)	23.8±2.6 (90)
20	12.9±1.9 (98)	17.0±2.3 (109)	10.7±1.0 (56)	30.1±3.1 (109)	29.9±2.7 (142)	29.1±3.1 (110)

Details as in Table 1.

due to the incorporation of glutamine nitrogen into other amino acids and eventually into proteins. In roots, apparently higher concentrations of the amide caused some toxic effects on a process linked to protein metabolism.

EXPERIMENTAL

Seed and seedling material. Seeds of *Zea mays* L. Ganga safed-2 were purchased from National Seed Corporation, New Delhi. They were washed thoroughly with H_2O and planted on 3 layers of filter paper in Petri plates (14 cm) with the embryo side touching the paper. The filter papers were moistened with 1/2 strength Hoagland's soln. containing no nitrogen. The seedlings were raised for 5 days in a controlled environment room at 26±2° temp. and 67 Wm^{-2} light (16 hr photoperiod). The uniformly grown seedlings were selected, and roots, scutella and shoots were taken for further treatments.

Treatment with glutamine. Scutella, roots or shoots were incubated in 1/2 strength Hoagland's soln containing 5 mM KNO_3 as sole nitrogen source (1 g in 50 ml) and 0, 1, 5, 10 or 20 mM l-glutamine in 14 cm Petri plates. Chloramphenicol (10 $\mu g\ ml^{-1}$) was added to the incubation medium to prevent bacterial contamination. The incubation was carried on for 24 hr either in the light (67 Wm^{-2}) or in darkness at 26°.

Enzyme extraction and assay. The NADH and NADPH:NR were obtained as a single prepn by extracting the freshly harvested samples with a pestle and mortar at 2°, in an extraction medium containing 1 mM EDTA, 5 mM cysteine and 0.5% casein in 0.1 M K-Pi buffer (pH 7.8) (1:4). The homogenate was centrifuged for 20 min in cold at 12000 rpm. The supernatant was used for both NADH and NADPH:NR assays by the methods of ref. [27] and ref. [28], respectively. In a few experiments, the enzyme was partially purified to confirm that the two isoforms were actually different in their existence. This was done using blue Sepharose (Fluka CL 6-B) chromatography of the crude enzyme prepn and subsequent elution of the enzyme with either 100 μM NADH or NADPH. Although the blue Sepharose treatment increased the activities of both isoforms 4- to 6-fold, the observed effect of glutamine on either of the isoforms was similar to that observed when crude enzyme prepn was used for assay.

Protein measurement. The samples of scutella, roots or shoots were extracted with 10% TCA and the protein content in the pellets was determined by the method of ref. [29].

Nitrate and nitrite contents. 2 g of freshly harvested samples were extracted with 40 ml of hot (60–70°) H_2O. 2 × 1 ml aliquots of the extracts were taken out for colorimetric measurement of nitrite [30]. The rest of the samples were used for nitrate measurement by using a Orion model 290 A (Orion Research Inc. Boston, MA) nitrate electrode and ion meter.

Acknowledgements—This research work was financially supported by a grant (SP/SO/A10/93) from the Department of Science and Technology, Govt. of India, New Delhi.

REFERENCES

1. Srivastava, H. S., *Phytochemistry*, 1980, **19**, 725.
2. Filner, P., *Biochimica et Biophysica Acta*, 1966, **118**, 299.
3. Oaks, A., Aslam, M. and Boesel, I., *Plant Physiology*, 1977, **59**, 391.
4. Srivastava, H. S., *Phytochemistry*, 1992, **31**, 2941.
5. Callaci, J. J. and Smarelli, J. Jr., *Biochimica et Biophysica Acta*, 1991, **1088**, 127.
6. Deng, M. D., Moureaux, T., Cherel, I., Boustin, J. P. and Caboche, M., *Plant Physiology and Biochemistry*, 1991, **29**, 239.
7. Vincentz, M., Moureaux, T., Leydecker, M.-T.,

Vaucheret, H. and Caboche, M., *Plant Journal*, 1993, **3**, 315.

8. Li, X.-J., Larson, D. E., Glibetic, M. and Oaks, A., *Physiologia Plantarum*, 1995, **93**, 740.
9. Shiraishi, N., Sato, T., Ogura, N. and Nakagawa, H., *Plant Cell Physiology*, 1992, **33**, 727.
10. Sanchez, J. and Heldt, H. W., *Plant Physiology*, 1990, **92**, 684.
11. Lillo, C., *Physiologia Plantarum*, 1994, **90**, 616.
12. Huber, J. L., Huber, S. C., Campbell, W. H. and Redinbaugh, M. G., *Archives of Biochemistry and Biophysics*, 1992, **296**, 58.
13. MacKintosh, C., *Biochimica et Biophysica Acta*, 1992, **1137**, 121.
14. McMichael, R. W. Jr., Bachmann, M. and Huber, S. C., *Plant Physiology*, 1995, **108**, 1077.
15. Sivasankar, S. and Oaks, A., *Plant Physiology*, 1995, **107**, 1225.
16. Lacuesta, M., Gonzalez-Moro, B., Gonzalez-Murua, C. and Munoz-Rueda, A., *Physiologia Plantarum*, 1993, **89**, 847.
17. Kojima, M., Wu, S.-J., Fukui, H., Sugimoto, T., Namori, T. and Oji, Y., *Physiologia Plantarum*, 1995, **93**, 139.
18. Merlo, L., Ferreti, M., Passera, C. and Ghisi, R., *Physiologia Plantarum*, 1995, **94**, 305.
19. Srivastava, H. S. and Shankar, N., *Current Science*, 1996, **71**, 702.
20. Ferrario, S., Valadier, M.-H. and Foyer, C. H., *Planta*, 1996, **199**, 371.
21. Hipkins, C. R., Kau, D. A. and Cannons, A. C., *Biochemical Journal*, 1993, **295**, 611.
22. Muller, B. and Touraine, B., *Journal of Experimental Botany*, 1992, **43**, 617.
23. Quillere, I., Duffose, C., Roux, Y., Foyer, C. H., Caboche, M. and Morot-Gaudry, J.-F., *Journal of Experimental Botany*, 1994, **45**, 1205.
24. Faure, J. D., Vincentz, M., Kronenberger, J. and Caboche, M., *Plant Journal*, 1991, **1**, 107.
25. La Habba, P., Ageura, E. and Maldonado, J. M., *Planta*, 1988, **173**, 52.
26. Wu, S., Lu, Q., Kriz, A. L. and Harper, J. E., *Plant Molecular Biology*, 1995, **29**, 491.
27. Srivastava, H. S. and Ormrod, D. P., *Plant Physiology*, 1984, **76**, 418.
28. Dailey, F. A., Warner, R. L., Somers, D. A. and Klein, A., *Plant Physiology*, 1982, **69**, 1200.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *Journal of Biological Chemistry*, 1952, **193**, 265.
30. Srivastava, H. S., Ormrod, D. P. and Hale, B. A., *Environmental Pollution*, 1994, **86**, 37.