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Effect of inorganic nitrogen source on ammonium assimilation enzymes of *Catharanthus roseus* plants

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Abstract. The activities of the enzymes glutamate dehydrogenase (EC 1.4.1.2), glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.1.14) were measured in leaves, roots and leaf explants of *Catharanthus roseus* grown in different N sources: water (control), 20 mM KNO_3, 2 mM NH_4Cl and a mixture of the latter two. In the roots, glutamate dehydrogenase activity is fourfold higher than in leaves and it is also higher than the activities of glutamine synthetase and glutamate synthase. The aminative and deaminative GDH activities are regulated differentially depending on the nitrogen source and the tissue studied. Ferredoxin dependent glutamate synthase was detected only in photosynthetic tissues but not in the roots, where the NADH dependent enzyme was found.

The N sources substantially modified the total alkaloid content with respect to the control (water): with nitrate producing an increment of 50%, while a mixture of nitrate and ammonium produced a decrease of 45%.

Key words: Glutamate dehydrogenase, glutamate synthase, glutamine synthetase, nitrogen source.

Ammonia is assimilated into glutamine and glutamate through the combined actions of glutamine synthetase (EC 6.3.1.2, GS) and ferredoxin dependent glutamate synthase (EC 1.4.7.1, Fd-GOGAT)

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or NADH-dependent glutamate synthase (EC 1.4.1.14, NADH-GOGAT) (12). Although glutamate dehydrogenase (EC 1.4.1.2, GDH) is the primary route of nitrogen assimilation in microorganisms, several lines of evidence suggest that in higher plants functions largely in glutamate catabolism (18).

Even though the enzymes involved in this process have been thoroughly characterized in plants of agricultural importance (1, 7, 8, 9) or in particular models, such as glycophytes (17), halophytes (24), cultured cells (2, 13, 14), or detached plant organs (6, 16), very little attention has been given to the nitrogen assimilation pathways of plants that synthesize secondary metabolites. The only report that has been published on *Catharanthus roseus* is that of Stafford & Fowler (21). Furthermore, as Stewart & Rhodes (23) have emphasized, it may prove difficult to extrapolate the results obtained with cultured cells or with lower plants to plants showing a higher degree of tissue differentiation.

In a previous study we reported that nitrate reductase and nitrite reductase have completely different activities in root and leaf tissues of *C. roseus* and that only the activity of root nitrite reductase seems to be modified by the N source. The amino acid contents were also significantly affected by the different N sources, not only in the whole plant but also in *in vitro* studies using leaf explants. These results suggest that modifications in the activities of the enzymes involved in the assimilation of ammonium to organic compounds, i.e. GS, GOGAT and GDH, could partially explain the differences in the amino acid pools (8).

The present report is concerned with the effect of different nitrogen N on the activities of GDH, GS and GOGAT, and on the alkaloid content in the leaves and roots of *C. roseus.*

MATERIALS & METHODS

Plant growth conditions. *Catharanthus roseus* L. G. Don plants were grown in separate pots with soil in a growth room at 32°C with a photoperiod of 16 h light/8 h darkness. The plants were divided into four groups of 50. Each plant was watered every other day with one of the following solutions: group 1, water (control; the ammonium concentration in the soil was 1 µmol/g dry soil and the nitrate concentration was 10 µmol/g dry soil); group 2, 20 mM KNO_3; group 3, 2 mM NH_4Cl; and group 4, 20 mM KNO_3 plus 2 mM NH_4Cl.

For the experiments using explants, 10 leaves of the same physiological age were cut 3 h after the beginning of the light period and placed in Erlenmeyer flasks containing 50 ml of the above mentioned solutions. The leaves were incubated for 2, 4 and 8 h under continuous light.

Enzyme extracts. The tissues were frozen at -70°C, ground with a pestle and mortar and then homogenized with a Waring blender in 2.5 volumes (w/v) of extraction buffer containing 5% polyvynilpolypyrrolidone (w/w). The extraction buffer for GDH, GS and NADH-GOGAT contained 50 mM Tris-HCl pH 8.2, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol; for ferredoxin-GOGAT, it contained 50 mM phosphate pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol. The homogenates were filtered through four layers of cheesecloth and the filtrates centrifuged at 14,000 x g for 30 min. The supernatants were used to determine enzyme activities.

Determination of enzyme activities. Aminative and deaminative GDH activities were assayed according to Loyola-Vargas and Sánchez de Jiménez (7). GS, NADH-GOGAT and Fd-GOGAT were measured as reported previously by Loyola-Vargas and Sánchez de Jiménez (8).

Total alkaloid content was as reported by Kutney et al. (4). Protein determinations were performed as reported by Peterson, using bovine serum albumin as a standard.

RESULTS & DISCUSSION

The activity of GDH was measured in both the aminative and deaminative pathways in plants watered with the different N sources (Table I). NADH-GDH activity in the root decreased 50% only in the presence of ammonium; while in the leaves, it was not affected by any of the N sources. NAD-GDH activity showed a marked increase in roots growing in a mixture of nitrate and ammonium; whereas only minor variations could be observed in leaf tissues. These data suggest, as has been previously observed for GDH in maize (7) and the algae *Scenedesmus acutus* (20), that the aminative and deaminative activities of GDH are regulated differentially. This effect could be due to the presence of conformational isomers or isoenzymes.

GS activity in the roots increased with all added nitrogen sources: it doubled when the plants were supplemented with ammonium, increased almost six-fold with nitrate and tenfold when given the mixture (Table I). In the leaves, GS activity was always higher than that found in the roots; yet it was reduced in all nitrogen conditions, with ammonium alone producing the lowest activity. Stafford & Fowler (21) found that two different levels of nitrate produced no effect on the activity of this enzyme in *C. roseus* cells cultivated *in vitro*, and thus suggested that the regulation of GS was not dependent on nitrate availability in the medium. This finding contrasts with the marked nitrate dependence shown by GS activity in both roots and leaves (Table I).

TABLE I. Enzyme activities in *C. roseus* plants watered with different nitrogen sources, expressed as nmol of transformed substrate/min mg protein. Data shown are the mean ± 1SE of three independent experiments, each using 50 plants.

Treatment	Roots			
	NADH-GDH	NAD-GDH	GS	NADH-GOGAT
None	1503 ± 75	165 ± 16	94 ± 3	112 ± 34
Nitrate 20mM	1773 ± 124	159 ± 13	507 ± 40	55 ± 22
Ammonium 2mM	736 ± 29	117 ± 8	225 ± 18	44 ± 16
Nitrate plus Ammonium	1847 ± 92	308 ± 31	996 ± 55	229 ± 13
	Leaves			
	NADH-GDH	NAD-GDH	GS	NADH-GOGAT
None	412 ± 25	99 ± 5	1747 ± 87	460 ± 18
Nitrate 20mM	424 ± 13	78 ± 2	1310 ± 79	874 ± 43
Ammonium 2mM	407 ± 28	109 ± 13	978 ± 68	1426 ± 99
Nitrate plus Ammonium	432 ± 30	125 ± 14	1450 ± 87	2691 ± 242

GOGAT activity extracted from roots was NADH-dependent and unable to use methyl viologen as an electron donor; while the opposite occurred in leaf tissues. NADH-GOGAT activity decreased to half its initial value when the plants were grown either in the presence of nitrate or ammonium, but showed a twofold increase when the plants were exposed to the mixture. In general, biochemical studies have shown that NADH-GOGAT activity is low in mature leaves and high in roots (11) and cotyledons (3). This organ specific pattern of enzyme activity indicates that NADH-GOGAT, most likely functions with cytosolic GS to generate glutamine and glutamate for intracellular transport. The coordinated functions of NADH-GOGAT and cytosolic GS may act primarily on the initial incorporation of ammonia into root cells, the recapturing of ammonia lost by catabolism of amino acids, and the remobilization of ammonia released through processes such as the breakdown of seed storage proteins in cotyledons during germination (5).

The very low activity of GDH, compared to those of GS and GOGAT, in the leaves of plants grown without nitrogen supplement suggests a minor role for GDH in the assimilatory process of NH_4 in photosynthetic tissues. It is possible that GDH assimilates a portion of photorespiratory ammonia to generate catalytic amounts of glutamate to initiate the GS/GOGAT cycle (5). However, in the roots of the same plants GDH activity is higher than those of GS and GOGAT, and also higher than the one found in the leaves, which suggests a special role for GDH in these tissues. The importance of GDH in the roots is further stressed by the ratios of the activities of the three enzymes under the different nitrogen sources. The GDH/GS ratio has a value of 16 in roots; while in the leaves, this ratio is only 0.24. A similar behavior has been observed in maize (8) and *Bouvardia ternifolia* (19).

All the N sources produced an increase in Fd-GOGAT activity in the leaves. The high GOGAT activity in leaves could be attributable to the major role that the enzyme plays in the recycling of

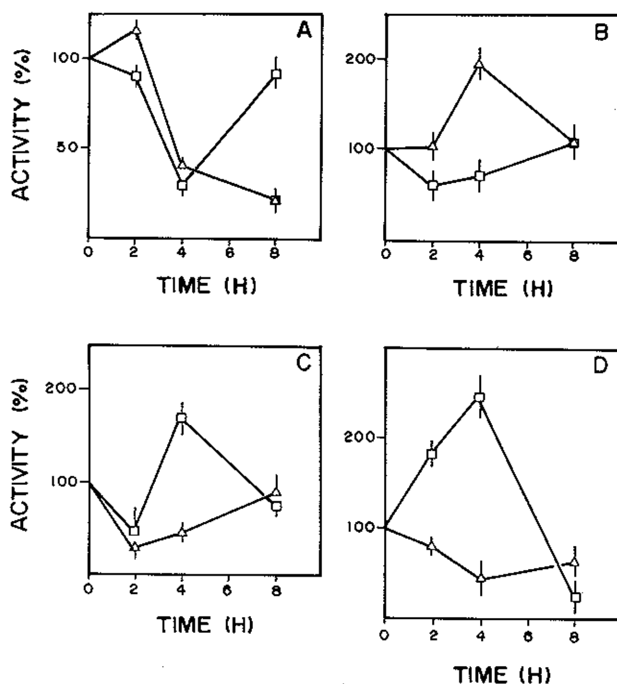

Fig. 1. NADH-GDH (Δ) and NAD^+-GDH (□) activities in leaf explants incubated in 50 ml of water (A), 20 mM KNO_3 (B), 2 mM NH_4Cl (C) and 20 mM KNO_3 + 2 mM NH_4 Cl (D). NADH-GDH activity at time zero were 437 (A), 405 (B), 460 (C) and 426 (D) nmoles NADH min^{-1} mg $protein^{-1}$ and for NAD^+-GDH were 76 (A), 85 (B), 98 (C) and 105 (D) nmoles NADH min^{-1} mg $protein^{-1}$. Time zero was taken as 100%. Each value is the mean ± SE of three independent experiments.

ammonia derived from photorespiration (12), or that it helps in the export of reducing equivalents from the chloroplast. It is possible that the energy charge of the cell is responsible for this status, since it is known that ATP inhibits glutamate dehydrogenase and stimulates glutamine synthetase (23, Loyola-Vargas & Sánchez de Jiménez, unpublished data).

Our results support the idea that GDH could play an important role in the ammonia detoxication process in the roots and also suggest that it might have a catabolic role, which in turn regulates the supply of carbon for different reactions of intermediary metabolism.

In general, in roots the enzyme activities increased in the following order: GS>GDH>GOGAT except in the case of the mixture of nitrate plus ammonium. In the leaves the order was always GOGAT>GS>GDH suggesting that glutamine could be synthesized

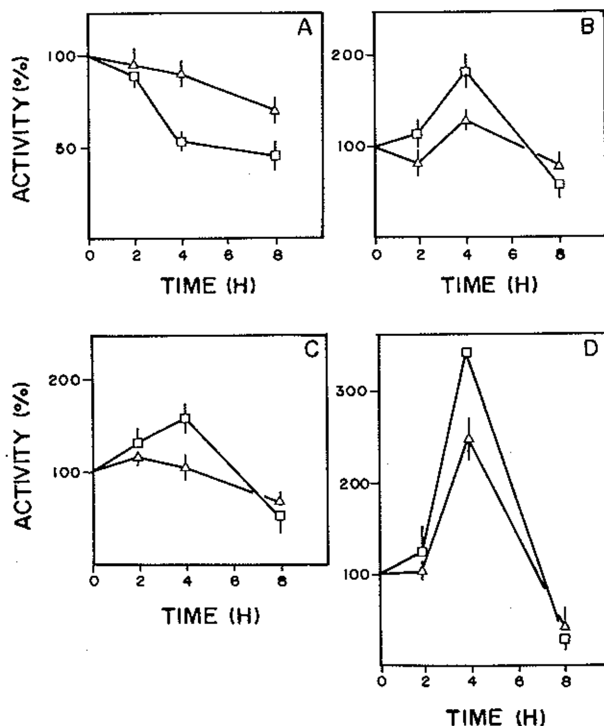

Fig. 2. GS (□) and Fd-GOGAT (Δ) activities in leaf explants incubated in 50 ml of water (A), 20mM KNO_3 (B), 2 mM NH_4Cl (C) and 20 mM KNO_3 + 2 mM NH_4Cl (D). GS activity at time zero were 802 (A), 764 (B), 900 (C) and 917 (D) nmoles glutamylhydroxamate min^{-1} mg $protein^{-1}$ and for Fd-GOGAT were 420 (A), 390 (B), 435 (C) and 403 (D) nmoles glutamate min^{-1} mg $protein^{-1}$. Time zero was taken as 100%. Each value is the mean ± SE of three independent experiments.

in the roots and transported to the leaves where the elevated activity of Fd-GOGAT converts it to glutamate which accumulates in this tissue (data not shown).

Since some of the N metabolism enzymes respond very quickly to external N sources, we also studied the N metabolism in leaf explants over a period of 8 h. When the explants were incubated in water, the activities of both NADH-GDH and NAD-GDH slowly decreased over the 8 h period, with the aminative activity disappearing more rapidly (Figure 1A). This further supports the fact that these two activities could be differentially regulated as it occurs in the whole plant. The extracts from explants that were given a source of N showed an increased activity over the first 4 h followed by a rapid decrease (Figure 1B-D).

In the presence of ammonium, the activity of GS increased rapidly over the first 4h and then decreased again to levels below

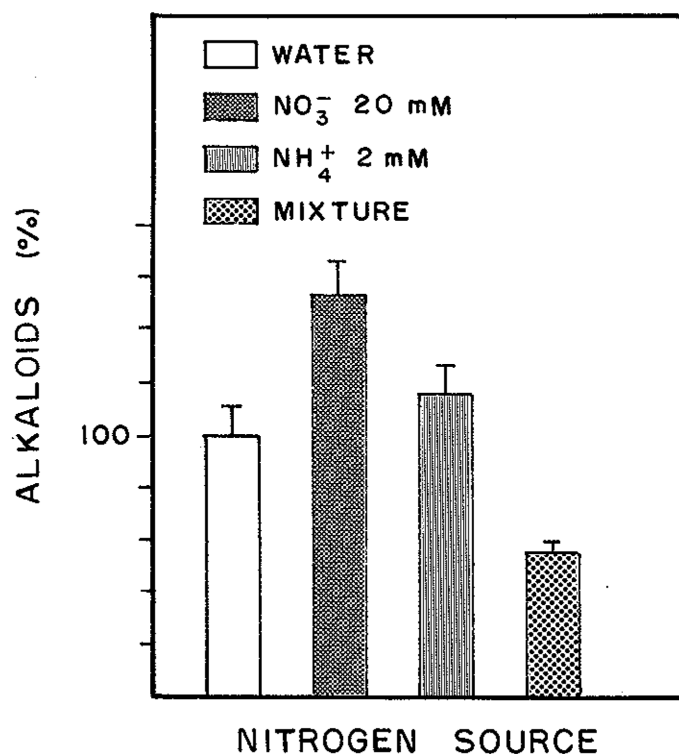

Fig. 3. Alkaloid content in the leaves of plants incubated with water (▭), 20 mM KNO_3 (▦), 2 mM NH_4Cl (▥) and 20 mM KNO_3 + 2 mM NH_4Cl (▩). The alkaloid content in the control plants was taken as 100%. Each value is the mean ± Se of three independent experiments.

the original activity (Figs 2C & D); whereas in the control explants, it showed the opposite effect (Fig. 2A). The activity of GOGAT decreased in extracts of tissues incubated in water and in ammonium. However, in the latter case, the enzyme returned to its basal levels after 8 h. Only with nitrate alone did GOGAT show some increase over the first 4h before falling to its original level.

The results presented here show that *Catharanthus roseus* possesses all the enzymes involved in ammonia assimilation and that they are differentially modified by exogenous sources of N. The response in the leaves differs from that in the roots, determining which amino acids accumulate. In the roots, the GS/GOGAT cycle works mainly in the synthesis of glutamine for export. The substrate for this enzyme could be provided by GDH, as suggested by the fourfold higher GDH activity in roots than in leaves (Table I).

Since the indole alkaloids, which are of great economic interest, are derived from the primary pathways of amino acid metabolism, we consider it necessary to understand how these are regulated and how this regulation could affect the secondary pathways leading to these compounds.

Fig. 3 shows that nitrate produced an increase of 50% in the alkaloid content while the mixture of nitrate and ammonium reduced the alkaloid content by 45%. These data, along with the measured enzymatic activities might suggest a link between N assimilation and the pathways for the synthesis of secondary metabolites if we consider that both of the N molecules in these alkaloids come from tryptophan and serine, which in turn come from glutamine and glutamate.

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