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Nitrogen source and the effect of growth regulators on ammonium assimilation enzymes in tissue culture of *Canavalia ensiformis* (L.) DC

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Abstract. Ammonium assimilation enzymes in *Canavalia ensiformis* (L.) DC. calli respond differentially to the N source in the culture medium when subcultured under different hormonal regimes. With 1 mM NH_4^+ the aminative and deaminative activities of GDH were not affected by the auxin, so the observed changes were due to the different nitrate concentrations in the medium. When NH_4^+ was increased to 5 mM, there was a marked increase with NAA and a NO_3^- dose effect was noticed. GS activity was not affected by the ammonium concentration but it was affected by both nitrate and the auxin type, with NAA promoting higher activities.

Key words: *Canavalia ensiformis*, growth regulators, nitrogen metabolism.

Abbreviations: BA, benzyladenine; GDH, glutamate dehydrogenase; GOGAT, glutamate synthetase; GS, glutamine synthetase; IAA, indoleacetic acid; NAA, naphthaleneacetic acid.

It is well documented that ammonium assimilation enzymes are regulated by the availability of N in the medium in whole plants (1, 2, 3, 4), excised organs (2, 5, 6, 7), or *in vitro* tissue cultures (8, 9, 10, 11). In the latter, the presence of growth regulators, generally auxins and cytokinins, are fundamental for callus induction and maintenance.

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On the other hand, the N source plays an important role in the differentiation process (12, 13, 14) and in the synthesis of secondary metabolites (15). Furthermore, the N source can influence the sensitivity of different tissues to growth regulators (14, 16).

We recently reported that the enzymatic activities of the GS/GOGAT cycle and GDH are modified differentially by the type of auxin and the auxin/cytokinin ratio in *Canavalia ensiformis* tissue culture (17). The aim of this paper is to study how N source (ammonium and/or nitrate) and growth regulators modify the activities of ammonium assimilation enzymes.

MATERIALS & METHODS

Biological material. Callus tissue, previously induced in our laboratory from anthers of *Canavalia ensiformis* (L.) DC. (18), was grown on Linsmaier & Skoog's (19) basal medium (LS) plus 0.55 mM myo-inositol, 1.18 µM thiamine-HCl and 86.2 mM sucrose, supplemented with 22.1 µM BA and 55 nM NAA or IAA. For growth kinetics and metabolic experiments, the N source of the LS medium was substituted for combinations of 1 or 5 mM NH_4Cl and 1, 5, 10, 20 and 25 mM KNO_3. One gram (fresh weight) calli were subcultured onto 10 ml LS medium supplemented with the different N source combinations (see figures). Calli were subcultured under continuous light (8 watts m^{-2}) and 25 ± 2°C for 2-3 weeks before harvesting. Growth patterns were determined using ten callus samples for each point. Each experiment was carried out at least three times.

For dry weight determinations, 300-400 mg of fresh tissue were dried in an oven at 60°C to constant weight.

Enzyme extracts. Calli were homogenized in 2.5 volumes of extraction buffer (0.05 M Tris-HCl pH 8.2, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol) and 5% (w/v) polyvinylpolypyrrolidone. The homogenates were filtered through four layers of cheesecloth and centrifuged at 18,000 x g for 30 min. The supernatants were used for the determination of the enzymatic activities. All the extraction procedures were carried out at 4°C.

Analysis. Aminative and deaminative GDH, as well as GS, activities were assayed according to Loyola-Vargas & Sánchez de Jiménez (10, 11). Blanks were used for all enzymatic determinations. For NADH-GDH, NADH disappearance was measured in the absence of other substrates. For GS activity, product formation was determined in the absence of substrates.

Protein was determined according to Peterson (20).

RESULTS & DISCUSSION

Growth of tissue cultures depends mainly on the concentration of growth regulators and nutrients, such as the N source. This is clearly demonstrated for *C. ensiformis* callus tissue in figures 1 & 2. In the presence of NAA, fresh weight increased as the nitrate concentration did, but this increment depended on the levels of ammonium in the medium (Fig. 1): no effect was detected with 1 mM ammonium. However, when IAA was added, changes in the N source did not influence the tissues' growth (Fig. 2).

The highest fresh weight increments obtained when using IAA were around 60% of those obtained with NAA. Nevertheless, when using dry weight increment as a growth parameter, there were no evident changes among the different growth regulator treatments, suggesting that water uptake may be affected by the N source, mainly by nitrate or by other counterions such as chloride (21).

The effect of the N source on the enzymes of N metabolism is well documented (3, 10, 22, 23) but almost nothing is known about the interaction between growth regulators and the N source. Therefore, the effects of NAA or IAA and different combinations of nitrate and ammonium as N sources were explored.

The presence of ammonium substantially modified the response of NAD(H)-GDH specific activity to auxins (Fig. 3A and 3B). With 1 mM ammonium, an increment in nitrate concentration produced a concomitant increase in NADH-GDH activity, independently

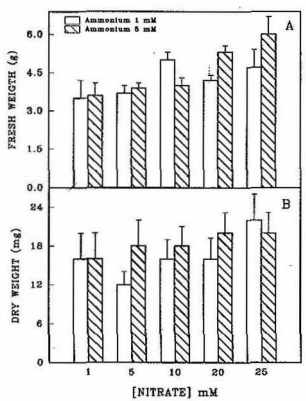

Fig. 1.— Final fresh (A) and dry (B) weights of *Canavalia ensiformis* calli cultured under different nitrogen regimes and 55 nM NAA.

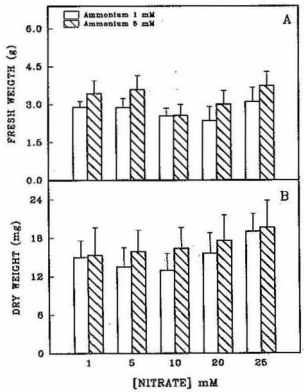

Fig. 2.— Final fresh (A) and dry (B) weights of *Canavalia ensiformis* calli cultured under different nitrogen regimes and 55 nM IAA.

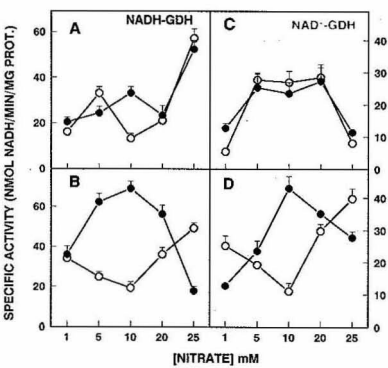

Fig. 3.— NADH-GDH (A, B) and NAD^+-GDH (C,D) activities in *Canavalia ensiformis* calli subjected to different nitrate concentrations in the presence of NH_4^+: 1 mM (A, C) and 5 mM (B, D) and of NAA (●) or IAA (○). Auxin concentration were 55 nM for both cases.

of the auxin present in the culture medium (Fig. 3A). On increasing the ammonium concentration to 5 mM, the response was a function of the auxin employed: with NAA, NADH-GDH specific activity increased up to 10 mM nitrate and then decreased; whereas, in the presence of IAA the effect of nitrate was exactly the opposite (Fig. 3B).

NAD^+-GDH specific activity in the presence of 5 mM ammonium showed a similar behavior to NADH-GDH (Fig. 3D). However with 1 mM ammonium, this enzymatic activity showed increments between 5 and 20 mM nitrate and was very low at 1 and 25 mM, independently of the auxin present in the culture medium (Fig. 3C).

The differential regulation of the aminative and the deaminative activities of GDH may be a function of both the auxin and the N source. In the presence of 1 mM ammonium and NAA, a change in the nitrate concentration did not modify the NADH-GDH/NAD^+-GDH ratio (Table I). When the auxin employed was IAA, there was significant change in the ratio. An increment in the ammonium concentration to 5 mM produced a change in the ratio with both auxins. From these results, it is clear that there is a differential effect of growth regulators on both senses of the reaction catalyzed by GDH. Similar results were obtained when specific activities were expressed in terms of dry weight since no significant changes in the total soluble protein pool were observed in the callus cultured on different media (data not shown).

Since GDH is an oligomeric protein possessing both activities (24), it may be possible for auxins to induce conformational changes through covalent modifications, like phosphorylation (25, 26), which would favor one activity over the other, or by modifying the Km for ammonium or glutamate, as has been shown to occur during osmotic stress in maize (27).

An alternative explanation concerns a release of Ca^{2+} by plant membranes in response to auxin (28), an ion from which NADH-GDH is dependent. Since the deaminative activity requires at most one magnitude order less (26) of the calcium concentration (29) needed by the aminative reaction, this increment in the Ca^{2+} pool could produce the observed auxin differential effects on GDH activity.

Table I. NADH-GDH/NAD^+-GDH ratio as affected by ammonium concentration and auxins

Nitrate (mM)	NADH-GDH/NAD^+-GDH ratio			
	Ammonium 1 mM		Ammonium 5 mM	
	IAA	NAA	IAA	NAA
1	3	1.35	1.67	2.85
5	1.19	1.3	1.13	2.65
10	1.84	1.71	10.81	1.77
20	0.74	1.05	0.86	1.62
25	7.1	1.24	4.59	0.65

The possibility that the ammonium pool is causing the observed increment in GDH activity can be ruled out by the fact that in the presence of 5 mM ammonium, 10 mM nitrate and IAA, this enzymatic activity decreases, suggesting again that the effect is due to the auxin in the medium.

The observed GS activity in the calli subcultured in IAA is much lower (near zero) than that obtained in NAA (Fig. 4). The response pattern was similar with 1 or 5 mM ammonium and a range of nitrate concentrations. As the nitrate concentration increases, there is also an increase in GS activity when the calli are subcultured in NAA, with a peak between 5 and 10 mM and remaining fairly constant to up to 25 mM. In contrast, in the presence of IAA this enzymatic activity did not show any changes when the nitrate concentration was modified.

The data may suggest that NAA induces the *de novo* synthesis of the enzyme or alternatively, that there is a change in the composition of the subunits of the enzyme (as has been demonstrated during the development of *Phaseolus vulgaris* (30)) that yield an enzyme with higher activity level.

In summary, our data show that the enzymes involved in the assimilation of ammonium in tissue cultures of *Canavalia ensiformis* respond differentially to the N source in the culture

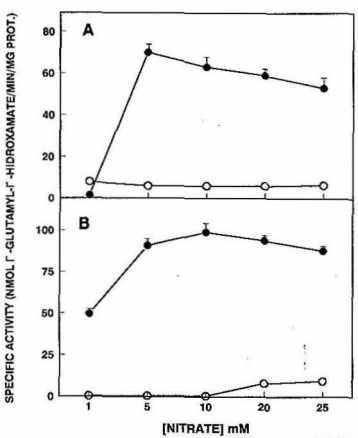

Fig. 4.— GS activity in *Canavalia ensiformis* calli subjected to different nitrate concentrations in the presence of NH_4: 1 mM (A) and 5 mM (B) and of NAA (●) or IAA (○). Auxin concentration were 55 nM for both cases.

medium, when subcultured under different hormonal regimes. The differences in the responses could be due, in the first place, to a higher pool of one of the auxins in the tissues, caused by their different metabolic rates, rather than to a difference in the affinity for either auxin, since it has been shown that the affinity constants for IAA and NAA are similar in soybean suspension cultures (31). Furthermore, the NO_3^-/NH_4^+ ratio might regulate the uptake and/or the metabolism of growth regulators as has been reported for potato (32) and sunflower (33), and also the cell sensitivity to auxins (16, 34).

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