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Short Communication

Effect of Nitrate, Ammonium and Some Amino Acids on Growth and Nitrate Reductase Activity in Suspension Cultures of *Atropa belladonna*

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Growth and nitrate reductase activity (NRA) of *Atropa belladonna* cells were studied in medium supplemented with $NaNO_3$, NH_4NO_3, and amino acid precursors to tropane alkaloids. Growth and NRA were stimulated by NH_4^+; and by proline, by proline plus ornithine, but not by glutamate, in NO_3^--containing medium. Tested amino acids inhibited neither utilization of inorganic nitrogen nor growth.

Key words: Amino acid — *Atropa belladonna* — Nitrate reductase — Suspension culture — Utilization of nitrogen.

Some amino acids, such as glutamate, proline and ornithine are established precursors of the main alkaloids of *Atropa belladonna* (DL-hyoscyamine = atropine and scopolamine) (Liebisch and Schütte 1967). Glu and Pro are both utilized as sole sources of nitrogen and in combination with inorganic nitrogen by callus cultures of this plant (Salonen and Simola 1977, Salonen 1980). Orn makes a good combination for growth with Glu (Salonen 1980). Supplementation of the nutrient medium with amino acids that are good precursors of alkaloids might stimulate the generally poor production of alkaloids in tissue cultures. Orn seems to increase the level of alkaloids in shoot cultures of *A. belladonna* (Benjamin et al. 1987).

The effects of NH_4^+ and amino acids on growth and NRA vary with plant species, organs, experimental conditions and, especially, with sources of nitrogen in the nutrient media. Both growth and NR are repressed by most of the protein amino acids in suspension cultures of *Nicotiana tabacum* (Filner 1966), whereas the influence of amino acids on growth and the level of NRA are not at all correlated in cultures of *Datura innoxia* cells (Fukunaga and King 1982).

The aim of the present study was to determine whether the precursor amino acids of the tropane skeleton affect NRA in *A. belladonna* and whether the simultaneous supply of NH_4^+ ions leads to its repression. The interrelationship between NRA, growth, and utilization of NO_3^- and NH_4^+ were also studied.

Abbreviations: NAA, α-naphthylacetic acid; NR, nitrate reductase; NRA, nitrate reductase activity.

The callus line of *A. belladonna* L. was derived from the stem. In this experiment callus cultured for 30–34 months (22nd–25th passage) was used. Stock callus cultures were grown on a modified version of the medium of Wood and Braun (1961, Simola et al. 1988) that contains the micronutrients described by Nyman and Simola (1988), and the following growth factors: myo-inositol (100 mg·liter^{-1}), NAA (2 mg·liter^{-1}) and kinetin (0.1 mg·liter^{-1}). The medium was solidified with 0.9% agar.

Stock suspensions obtained from callus were grown in a medium that contained 7.5 mM NH_4NO_3 as the sole source of nitrogen (NAA 0.5 mg·liter^{-1}, kinetin 0.1 mg·liter^{-1}). The balance of ions was improved by addition of $CaCl_2 \cdot 2H_2O$ (179 mg·liter^{-1}) and Na_2SO_4 (1,340 mg·liter^{-1}). Suspensions from the 2nd–4th passages were used for experimental cultures which were grown in media (50 ml in 200-ml Erlenmeyer flasks) that contained 15 mM $NaNO_3$ or 7.5 mM NH_4NO_3 at an initial pH of 5.2. In the former medium the balance of ions was achieved with $CaCl_2 \cdot 2H_2O$ (179 mg·liter^{-1}), Na_2SO_4 (426 mg·liter^{-1}) and K_2SO_4 (523 mg·liter^{-1}). $NaNO_3$- and NH_4NO_3-containing media were supplemented with L-amino acids: 2.5 mM Pro, 2.5 mM Glu or these amino acids separately in combination with 0.25 mM Orn. These media were prepared by addition of filter-sterilized amino acids (20 × stock solutions), in the appropriate nitrogen-free nutrient solution, to the actively growing cultures after 9 days' growth. Solutions of basal nutrients were added to control media that contained $NaNO_3$ or NH_4NO_3, if not otherwise stated.

Cultures were incubated at 25°C in the dark on a horizontal rotary shaker at 100 rpm. New cultures were

started by the transfer of a 21-day-old aggregated stock suspension to different media with a spatula (Simola 1973). Inocula used for NO_3^--containing media were first gently rinsed with this medium to remove NH_4^+. Cultures, 4–10 replicates, were harvested after 8, 12, 15, 20 (NH_4NO_3 media) or 30 (NO_3^- media) days' growth by vacuum filtration and washed with nitrogen-free medium. The pH of the nutrient media was measured immediately, and samples of media were stored at −20°C for analysis of levels of NO_3^-, NH_4^+, Pro and Orn. All experiments were repeated at least once.

Fresh material was used to determine NRA and intracellular concentrations of NH_4^+ and NO_3^-. The cells were extracted in a buffer that contained 0.1 M KH_2PO_4/K_2HPO_4 (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (Behrend and Mateles 1975) and 1.5% Polyclar AT (Rhodes and Stewart 1974), and 3.0 ml were used per g fresh weight. The extracts were centrifuged for 20 min at 10,000 × *g* at 4°C. The supernatants were used for the assay of NRA. This solution could be stored at least 2 days at −20°C without loss of NRA. Small aliquots were kept at −20°C for assays of NO_3^- and NH_4^+. Four different cultures were analyzed.

The NRA in vitro was assayed using NADH, as described by Filner (1966). Extract (0.1–0.2 ml) was incubated with 0.5 ml 0.1 M KH_2PO_4/K_2HPO_4 (pH 7.5), 0.1 ml 0.1 M KNO_3 and 0.1 ml 1 mM NADH in a total volume of 0.9 ml. The control assays consisted of the reaction mixture without NADH. The reaction was initiated by addition of extract at 27°C and terminated after 20 min by oxidation of excess NADH by 0.1 ml phenazine methosulfate (15 nmol/reaction mixture) (Scholl et al. 1974). After 20 min, NO_2^- was estimated according to Filner (1966).

For analysis of levels of NO_3^-, 0.5 ml of the extract for assay of NRA was diluted to 5 ml with distilled water. Proteins were precipitated by addition of 0.2 ml of 1 M zinc acetate and incubation on ice for 5 min (Høg et al. 1983). Precipitates were removed by centrifugation at 7,500 × *g* for 20 min. NO_3^- was reduced to NO_2^- as described by Young (1973, cf. Salonen 1984). Absorbance caused by the Polyclar AT-containing buffer and trace amounts of endogenous cell NO_2^- (below 0.0001% of fr wt) were subtracted from the corresponding total levels of NO_3^-. Levels of NH_4^+ in cells were determined by a modification of Weatherburn's method (1967) (cf. Salonen 1984). Similar procedures were used for determination of levels of NO_3^- and NH_4^+ in the media. Concentrations of Pro and Orn from media were analyzed as described by Chinard (1952).

The increase in fresh weight was greater during the early stages of growth (days 0–16) in NH_4NO_3-grown cultures of *A. belladonna* than in $NaNO_3$-grown cultures (Fig. 1).

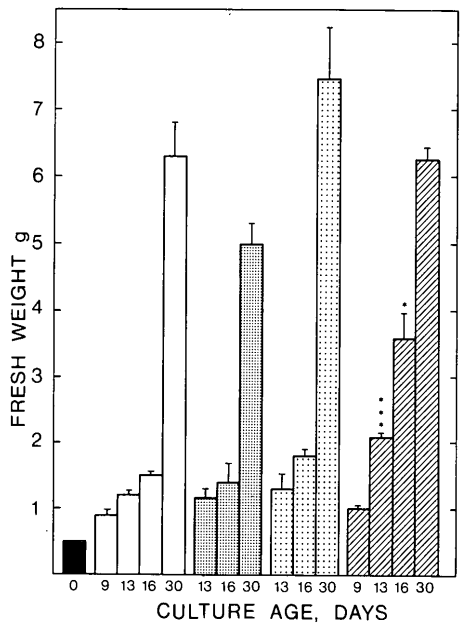

Fig. 1 Growth (fr wt) of suspension cultures of *Atropa belladonna* with 15 mM $NaNO_3$ (□), 15 mM $NaNO_3$+2.5 mM Glu (▦), 15 mM $NaNO_3$+2.5 mM Pro (▨) or 7.5 mM NH_4NO_3 (▨) as the source of nitrogen. Additions of Glu or Pro on day 10. Initial fr wt (■). The significance of differences from the corresponding controls ($NaNO_3$; days 9, 13, 16, 30) assessed by Student's t test is denoted as follows: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$. Vertical bars=S.E.

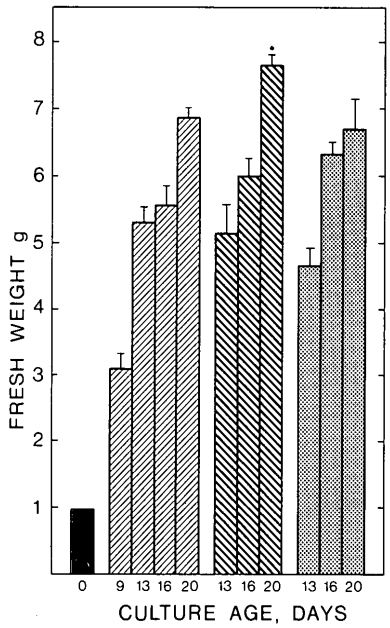

Fig. 2 Growth of suspension cultures of *A. belladonna* with 7.5 mM NH_4NO_3 (▨), 7.5 mM NH_4NO_3+2.5 mM Glu (▨) or 7.5 mM NH_4NO_3+2.5 mM Pro (▦). Additions of NH_4NO_3, Glu or Pro at 2.5 mM on day 10. Initial fr wt (■). The significance of differences from the corresponding controls (NH_4NO_3) is explained in the legend to Fig. 1.

In terms of the final fresh weights, however, no difference was observed between the two sources of inorganic nitrogen. By contrast, maximum growth of soybean and rose cells requires NH_4^+ (Bayley et al. 1972, Mohanty and Fletcher 1976).

In most cases, the growth of callus and suspension cultures is retarded by amino acids when they are the sole sources of nitrogen, but only slight effects are observed in combinations with inorganic nitrogen (cf. Salonen 1980). In this study we found that growth of suspension cultures of *A. belladonna* was scarcely affected by supplementation of medium with amino acid precursors of tropane alkaloids (Fig. 1, 2). Glu and Pro combined with high concentrations of NO_3^- had also no effect on growth of cells in a suspension culture of *Ipomoea* (Zink 1982) or *D. innoxia* (Fukunaga and King 1982), whereas both amino acids inhibited growth of tobacco cells in media that contained low concentrations of NO_3^- (Filner 1966).

By day 16, almost all the NO_3^- and about 90% of the NH_4^+ had disappeared from the medium of NH_4NO_3-grown cultures of *A. belladonna* (Fig. 3: exp. 1). The amount of NO_3^- lost from the $NaNO_3$-containing media was about 45% of the total. This amount, 377 μmol NO_3^-, was nearly equal to the NO_3^- taken up in NH_4NO_3-containing media, i.e. 394 μmol. These results show that the rates of removal of NO_3^- from $NaNO_3$- and NH_4NO_3-containing media were similar.

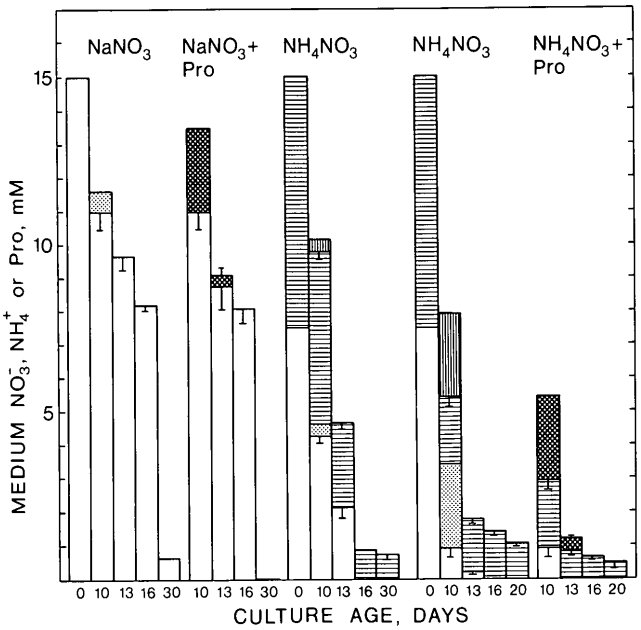

Fig. 3 Removal of NO_3^-, NH_4^+ and Pro from the medium of suspension cultures of *A. belladonna* grown with 15 mM $NaNO_3$, 15 mM $NaNO_3$+2.5 mM Pro, 7.5 mM NH_4NO_3 (exp. 1, cf. Fig. 1, 4A–B) and 7.5 mM NH_4NO_3 or 7.5 mM NH_4NO_3+2.5 mM Pro (exp. 2, cf. Fig. 2, 5A–B). Symbols: NO_3^- (□), NH_4^+ (▤), Pro (▦); additional NO_3^- (▥) or NH_4^+ (▥) on day 10.

In *A. belladonna* NH_4^+ was not as effectively taken up as NO_3^- when present at an equal concentration (Fig. 3). NH_4^+ is often preferentially removed from nutrient solutions that contain both NO_3^- and NH_4^+ when the concentration of NH_4^+ is lower than that of NO_3^- (e.g. soybean, Bayley et al. 1972). Contradictory results have, however, been obtained with rose cells (Mohanty and Fletcher 1976).

Pro was exhausted from the medium by *A. belladonna* cells within 6 days up to day 16, and it was removed more rapidly than NH_4^+ in NH_4NO_3-grown cultures (Fig. 3: exp. 2). Orn was also completely utilized from the media between days 10 and 16 (data not shown).

In suspension cultures of *A. belladonna*, NH_4^+ significantly increased the NRA during days 9–16 whereas, in $NaNO_3$-grown cultures, NRA developed more slowly (Fig. 4A). Recently, NRA and NR protein were found to increase in corn plants treated with NH_4NO_3 (Remmler and Campbell 1986). Negative effects of NH_4^+ have, however,

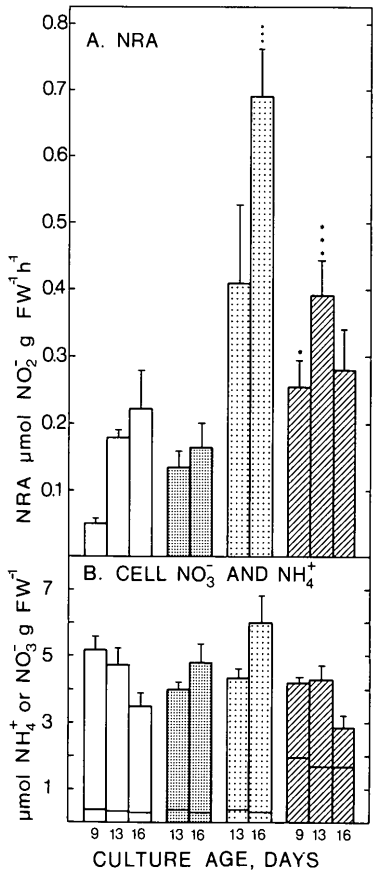

Fig. 4A–B NRA (A) and cell levels of NH_4^+ and NO_3^- (B) in suspension cultures of *A. belladonna* grown with 15 mM $NaNO_3$ (□), 15 mM $NaNO_3$+2.5 mM Glu (▥), 15 mM $NaNO_3$+2.5 mM Pro (▣) or 7.5 mM NH_4NO_3 (▨). In Fig. 4B, the upper part of columns shows the amount of NO_3^- and the lower part that of NH_4^+. The significance of differences from the corresponding controls ($NaNO_3$) is as explained in the legend to Fig. 1.

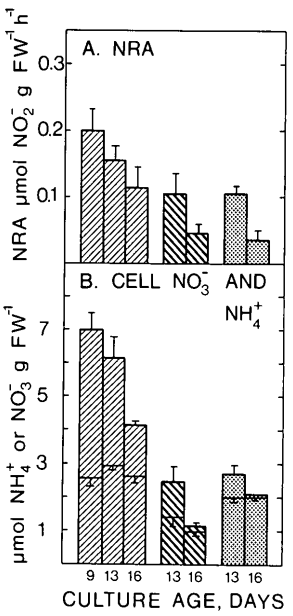

Fig. 5A–B NRA (A) and cell levels of NH_4^+ and NO_3^- (B) in suspension cultures of *A. belladonna* grown with 7.5 mM NH_4NO_3 (▨), 7.5 mM NH_4NO_3+2.5 mM Glu (▧) or 7.5 mM NH_4NO_3 +2.5 mM Pro (▦). The amount of NO_3^- and NH_4^+ is indicated as in Fig. 4B. NR activities did not differ significantly from the corresponding controls (NH_4NO_3).

been reported in rose cells (Jordan and Fletcher 1980) and *Ipomoea* cultures (Zink 1982).

No close relationship between NRA and cell levels of NO_3^- was apparent in our experiments when NO_3^- was used as the sole source of inorganic nitrogen (Fig. 4B). By contrast, intracellular levels of NO_3^- were positively correlated with NRA in NO_3^-- and NH_4NO_3-grown *Spirodela* (Ferguson 1969), whereas the inverse relationship was found in cultures of rose cells (Jordan and Fletcher 1980) and in tobacco (Wakhloo and Staudt 1988). Cell levels of NH_4^+ remained relatively constant in *A. belladonna*. Higher levels of NH_4^+ were detected in NH_4NO_3-grown cultures than in NO_3^--grown cultures (Fig. 4B, 5B), but these levels of NH_4^+ did not inhibit growth (Salonen 1984).

NRA was enhanced by Pro and by a combination of Pro and Orn in $NaNO_3$-grown cultures of *A. belladonna* (Fig. 4A, Table 1). Similar enhancement by Pro (1 mM) has been found in *Ipomoea* cells in combination with NO_3^- (20 mM) (Zink 1982), but not in suspension cultures of *D. innoxia* (Fukunaga and King 1982). In tobacco cells, both Pro and Glu (0.1 mM) repressed induction of NR in the presence of 2.5 mM NO_3^- (Filner 1966). At a high initial level of NO_3^- (17.8 mM) in the medium, Glu decreased NRA in tobacco whereas Pro had no effect (Behrend and Mateles 1975). NRA in NO_3^--grown cultures of *A. belladonna* was not affected by Glu or by a combination of Glu and Orn (Fig. 4A, Table 1).

No clear changes in NRA in response to inclusion of amino acids in the medium were found in *A. belladonna* cultures grown in NH_4NO_3-containing media (Fig. 5A, Table 1). During days 13 and 16, these cultures were near the stationary phase of growth unlike in the case shown in Figure 4A, in which cells were actively growing. The concentration of NO_3^- in cells, estimated on the basis of the total water content of *A. belladonna* cells on day 16, was between about 0.05 mM (NH_4NO_3+Pro) and about 1.6 mM (NH_4NO_3). NRA was very low in all these cultures (Fig. 5A). Corresponding levels of NO_3^- in cells were 3–6 mM in $NaNO_3$-containing media. Adequate levels of NO_3^- were available as a substrate for NR in most cultures.

The suspension cultures of *A. belladonna* were able to reduce effectively the NO_3^- taken up from the medium, and the accumulation of NO_3^- in the cells was rather small.

Table 1 Effect of combinations of glutamate and proline with ornithine on NRA in suspension cultures of *A. belladonna* growing in $NaNO_3$ or NH_4NO_3 on day 13

Nitrogen source	NRA (μmol $NO_2^- \cdot g^{-1} \cdot h^{-1}$)[a]	(%)[b]
$NaNO_3$ (15 mM, control)[c]	0.40±0.15	100
+Glu (2.5 mM)+Orn (0.25 mM)	0.53±0.12	132
+Pro (2.5 mM)+Orn (0.25 mM)	0.89±0.12 *	223
NH_4NO_3 (7.5 mM, control)[c]	0.16±0.02	100
+Glu (2.5 mM)+Orn (0.25 mM)	0.13±0.02	81
+Pro (2.5 mM)+Orn (0.25 mM)	0.14±0.02	88

[a] Values are means ± S.E. The significance of differences from the corresponding controls is as explained in the legend to Fig. 1. On day 16, NRA was lower than on day 13 except in $NaNO_3$-grown control cultures.

[b] The percentages presented relate to the control values.

[c] The values are not consistent with those presented in Fig. 4. There were some differences in NR activities between the cultures obtained from inocula of different ages, but the same trends were seen in all experiments.

NRA was enhanced by NH_4^+ or Pro. An increase in NRA due to reduced nitrogen is not generally found in higher plants. Inhibitory effects of amino acids were not observed. Thus, the addition of some precursor amino acids of tropane alkaloids to the nutrient medium does not repress the utilization of inexpensive inorganic nitrogen.

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