EFFECT OF SOURCE OF NITROGEN ON GROWTH, ALKALOIDAL CONTENT AND ENZYMES ACTIVITIES IN *ATROPA ACUMINATA*

M.BASHIR KHAN, Bio-chemist, Pakistan Forest Institute, Peshawar.

ABSTRACT

Total biomass, percentage of tropane alkaloids, total alkaloid per plant, ornithine decarboxylase (ODC) activity, arginine decarboxylase (ADC) activity and putrescine levels were determined separatly in roots, stems and leaves of *Atropa acuminata* fed with nutrient solution having different source of nitrogen fertilizer. All sources of nitrogen fertilizer increased the total biomass and the highest increase (27.2% of control) was in the plants fed with both ammonium and nitrate. The highest increase (34.7% of control) in the alkaloid concentration was in the plants fed with only ammonium. The increase in the Putrescine level, ODC and ADC activites was higher in the plants fed with only ammonium or both ammonium and nitrate as compared to those plants fed with only nitrate.

INTRODUCTION

Nitrogen is the most important among the mineral nutrients, because it is an integral part of protein and nucleic acid structure as well as of apoenzymes. The two major sources of inorganic nitrogen taken up by roots of higher plants are nitrate and ammonium. The subject of whether plants should be supplied with nitrate- or ammonium- based fertilizer is a matter of great practical importance and has been reviewed by Haynes and Goh (1978) and Kirkby (1981). In general, calcifuges-or plants adapted to acid soils -and plants adapted to low soil redox potential have a preference for ammonium ion (Ismunadji and Dijkshoorn, 1971). In some cases the highest growth rate is obtained with a combination of both ammonium ion and nitrate (Gashaw and Mugwira, 1981) or with ammonium ion alone (Sommer and Six, 1982a).

Nitrogen fertilization stimulates growth, delays senescence and also changes plant morphology. With an added supply of nitrogen shoot elongation is enhanced and the root elongation is inhibited (Klemm, 1966). The effects of ammonium ion and nitrate on root morphogensis differ markedly. With ammonium ion there is a greater reduction in elongation, the roots are short and thick (Bhat, 1983) and lateral root formation is stimulated (Klemm, 1966). The reasons for the different effects of nitrogen forms on the root morphogenesis are unknown; differences in both pathways of assimilation in the roots and in plant hormonal balance are probably involved.

Solanaceous species have been extensively studied for the effect of nitrogen as fertilizer. All the earlier work about fertilizer effect on solanaceous species has been reviewed by James (1947) who concludes that," Nitrogen fertilizers, in particular have been found to stimulate growth and increase the alkaloid content of *Atropa belladonna*". According to Brewer and Hiner (1950) ammonium nitrate used alone is the best application for increasing dry weight production of belladonna leaves. According to Waller and Nowacki (1978) it is possible to produce two-to ten fold increase of alkaloid by treating the plant with high levels of nitrogen. Sakson (1979) reported that NPK fertilization had no significant effect on alkaloid content of *A.belladonna*. Ceylan and Vomel (1980) reported for *A.belladonna* that N fertilizer at 50-250 kg/ha resulted in a general

increase in yield, with the greatest response occurring in the 3rd year of cultivation. Gershenzon (1984) writes in his review, "studies concerning the effects of nitrogen fertilization on alkaloid content have given somewhat inconsistent results, but in a majority of cases additional nitrogen was found to raise the alkaloid concentration, with increase ranging from 25% to 300%".

Source of nitrogen plays an important role in the net effect of nitrogen supply on alkaloid production. Martin *et al.* (1974) during a study on reed canary grass, reported that ammonium source of N produced a greater concentraion of alkaloids than did a nitrate source, while grass supplied with both source of nitrogen (ammonium & nitrate) had an intermediate alkaloid concentration. The increase in the alkaloid content due to ammonium and not due to nitrate can be due to increased production of putrescine. Slocum (1984) reported that putrescine accumulates in the plants maintained with ammonium as nitrogen source. It has recently been reported that putrescine plays a central role in the biosynthesis of tropane alkaloids (Lette, 1990). Since ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) leads to the production of putrescine, it seems that any factor influencing these two enzymes wil effect the net production of alkaloids (Fig. 1).

Due to the contradictions in the literature about the effect of nitrogen fertilizer this experiment was carried out to see the effect of different sources of nitrogen (only nitrate, both nitrate and ammonium and ammonium only) on the growth and alkaloidal content of the plant. Moreover the putrescine level and the activities of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) were also studied to understand the possible mechanism through which nitrogen supply effects the alkaloid biosynthesis in *A:acuminata*.

MATERIAL AND METHODS

Plant material

Authentic seeds of *Atropa acuminata* provided by Pakistan Forest Institute, Peshawar, were grown in the green house of Plant Science Department, University of Reading, England. After germination the seedlings were transferred to sand and fed with complete nutrient solution for 12 months. The plants were then cut 3 cm above ground and were allowed to continue growing. When the regenerated plants were approximately 6 cm in height they were divided into 4 groups each containing 10 plants. One group was kept as control, the other three groups were fed with 10-fold more nitrogen than that of control. The source of nitrogen was only nitrate ($NO3^-$) in group 2, both nitrate and ammonium (NH_4^+ + NO_3^-) in group 3 and only ammonium (NH_4 Cl) in group 4.

Alkaloid extraction

Dry powdered plant material (0.5g) was shaken with 15 ml of 0.2M H_2SO_4 and was left for 1 hr to remove most of the chlorophyll pigments. After filtration, the solution was made alkaline by additon of 1 ml of concentrated NH_3 solution and extracted into 15 ml Et_2O. Each step was repeated twice in order to ensure complete extraction. The combined ether extracts were evaporated and the residue used for further analysis (Wagner and Zgainski, 1984)

Alkaloid analysis

The absorptiometric method (Freeman, 1954) was used for the determination of total alkaloids. An aliquot containing 0.05-0.15 mg of alkaloids was evaporation to dryness, and then nitrated by addition of 0.2-0.25 ml of fuming HNO_3. After evapn the residue was taken up in a

small volume of dimethylformamide and transferred to a 5 ml calibrated flask. 20% aq. tetraethyl- ammonium hydroxide (0.187 ml) was added, the final volume made up to the 5 ml mark with dimethylformamide. The absorbance was measured at 540 nm. A calibration curve was obtained using authentic alkaloid marker from which the alkaloid percentage was calculated.

High performance liquid chromatography (HPLC)

HPLC was used for both the identification of individual alkaloids and for their quantitative analysis. The procedure of Robert and Svendon (1976) was used with some modification. HPLC was carried out on a 5 m Partisil column, eluted isocratically with diethylamine - MeOH (19:1) containing 1% diethylamine, with a flow rate of 1.5 ml min^{-1} and detected at 254 nm. Plant samples were prepared in MeOH and were passed through Extrelute (Merck) before injection.

ODC and ADC extraction

Fresh plant material (5 g) was macerated for 15 min with pestle and mortar at below 5° with 15 ml extraction buffer and 5 g sand. The extraction buffer was 0.1 M K-Pi, pH 7.0 containing; 1mM diNa EDTA, 2mM dithiothreitol (DTT), 0.1 mM pyrodoxal phosphate (PLP) and 0.3 mM phenylmethylsulphonylfluoride (PMSF). The extract was centrifuged at 15000 rpm for 20 minutes. The residue was discarded. Each assay contained 225 μl enzyme (200 μl enzyme +25μl of other test compound) + 25 μl L-ornithine hydrochloride (cold) 5 mM containing 0.05 μCi D, L [1-^{14}C] ornithine. 25 μl 2M NaOH was spread on a small strips of filter paper and was fastened to the top of assay tube. After 2 hr of incubation at 30° 0.2 ml of 1 mM HCl was added and left for 1 hr to allow distillation of the 14 CO_2. The paper was placed in 4 ml of scintillation fluid (2 M NaOH: Hisafe 3; 1:4 by vol.) and was counted for 10 min.

Putrescine

Dry powdered plant material (1 g) was boiled in water and was made up to 5 ml. 100 ml of this extract was chromatographed on Whatman No.1 paper, using the solvent system n-butanol-EtCOMe-aq. NH_3-H_2O (5:3:1:1). Putrescine was detected with ninhydrin and the concentrations determined semi-quantitatively from the size and intensities of the spots compared with the standard (Smith, 1963).

Statistical analysis

All plants were separately harvested and the dry wts of roots, stems and leaves determined separately. Alkaloid analysis was carried out twice on each sample and the two measurements per plant were averaged. All the percentage data were subjected to arcsine transformation. An analysis of variance was performed using 'Minitab' statistical package (Ryan,1982).

RESULTS

The experiment was carried out to determine the effect of different sources (nitrate only, both nitrate & ammonium and ammonium only) of added nitrogen on growth and alkaloid production of the plants. The dry weight as well as alkaloid content were determined for each part separately and the results are given in Fig. 2. The results for total alkaloids produced per plant are given in Fig. 3. Roots and leaves samples were analysed to determine the activities of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) and putrescine level. The effect of nitrogen on ODC and ADC activities and putrescine level are given in Figs.4 and 5.

Effect on total biomass

The total biomass of the plants increased when more nitrogen was available. One-year-old plants grown under different sources of added nitrogen (10-fold more than that of control) for three months show an increase in the biomass in all cases. The increase in total biomass was 24.7% of control in plants treated with nitrate, 27.2% of control in the plants treated with both ammonum and nitrate and 22.6% in the plants treated with only ammonium as the source of nitrogen. The increase was significant in both stems and leaves of the plants and there was no significant change in the biomass of roots as compared to that of control in all three cases (Fig.2).

Effect on alkaloid concentration

Plants (grown during first year with normal dose of nitrogen) when treated during second year, for three months with different sources of added (10-fold more than that of control) nitrogen gave higher concentration of alkaloids in whole plant as compared to that of control. The increase in alkaloid concentration was 26.6% of control due to added nitrate, 35.6% of control due to added ammonium & nitrate and 34.7% of control due to added only ammonium. The increase was significant only in the stems of the plants and there was no significant change in the alkaloid concentration of roots and leaves of plants as compared to that of control. The results for the effect of various sources of added nitrogen on alkaloid concentration of plants are given in Fig. 3.

Total alkaloid produced per plant were also calculated and it was significantly high as compared to that of control in all three cases of added nitrogen. The increase in total alkaloid produced per plant was 57.4% of control due to added nitrate, 73.6% of control due to added ammonium & nitrate and 65.9% of control due to added only ammonium.

Effect on enzyme activity

Roots and stems of plants were analysed to determine the effect of different sources of added nitrogen on ODC and ADC activity. The ADC activity in the roots of plants fed with added nitrogen in the form of only nitrate did not show any significant change as compared to that of control. The ADC activity in roots of plants fed with added nitrogen in the form of both ammonium & nitrate and only ammonium increased by 31.4% and 48% of control respectively. The ADC activity in the leaves of plants fed with added nitrogen in all three forms showed an increase as compared to that of control. The increase was 98.9% of control in the plants fed with nitrate only, 287% of control fed with both ammonium & nitrate and 286% of control in the plants fed with added nitrogen in the form only ammonium. The ODC activity in the roots of plants fed with added nitrate only, both nitrate and ammonium and ammonium only increased by 26%, 74.3% and 80% of control respectively. The increase in ODC activity of the leaves of plants fed with only nitrate was 308% of control 391% of control in plants fed with both ammonium & nitrate and 375% in the plants fed with added nitrogen in the form of only ammonium. The results for the effect of different sources of added nitrogen on enzyme activity are given in Fig. 4.

Effect on the putrescine level

Putrescine level increased due to added nitrogen in all three cases. The increase in the roots of the plants fed with nitrate only was 25% of control, while in the roots of plants fed with both ammonium &nitrate and ammonium only the increase was 143.5% and 238% of control respectively. In the plants fed with nitrate only,

both ammonium & nitrate and ammonium only, the increase in the leaves was 125%, 159% and 273.4% respectively. The results for the effect of different sources of added nitrogen on putrescine level are given in Fig. 5.

DISCUSSION

These results agree with the generally accepted idea that nitrogen fertilization increases the total biomass. They also agree with the idea that nitrogen supply influences the growth of the aerial parts of plants more than the growth of the roots. In the present experiment there was no change in the biomass of roots. These results are in agreement with those reported by Klemn (1966) that there is a tendency for higher nitrogen levels to inhibit root growth. For the stems of the plants these results are partially in contrast with those of Klemn (1966) who reported that with an increasing supply of nitrogen the shoot elongation is enhanced. However, in these results though the greatest increase was in the biomass of stems as compared to the other parts of the plant as well as that of control in all experiments; the effect on height of plants was not so prominent. The results suggest that the increased biomass of stems due to added nitrogen in *Atropa acuminata* plants is due to the increased side branches as well as increased diameter of the stems and not due to the height of the plants.

In general these results suggest that ammonium nitrate gave better results for crop yield as compared to that of nitrate or ammonium only (Fig.2) which is in agreement with that of Brewer and Hiner (1950) who worked on *Atropa belladona* and reported similar results.

These results also agree with the conclusion of Gershenzon (1984) that additional nitrogen can raise the alkaloid concentration. A significant increase of 26.6%-35.6% was recorded (Fig. 3) when added nitrogen was applied during second year of growth for a 3 month duration. A high alkaloid concentration was observed when the nitrogen was supplied in the form of both ammonium and nitrate (35.6% of control) or only ammonium (34.7% of control) as compared to those plants which were supplied with only nitrate (26.6% of control). This is in agreement to the results reported by Martin *et al.* (1974) for reed canary grass that ammonium source of nitrogen gave a greater alkaloid concentration than did a nitrate source. A very interesting point was observed during analysis of individual organs of the plants fed with added nitrogen, that the highest increase in alkaloid concentration was in the stems of the plants and it had no effect on alkaloidal content of roots and leaves. It suggests that the stems of the plants which are normally considered to have low alkaloid content should not be ignored, particularly in plants fed with added nitrogen.

These results also show that, although added nitrogen has got little effect on alkaloid concentration, the total alkaloid produced per plant plant increases significantly due to increase in the biomass of plants. The best results (increase of 73.6% of control) were achieved in the plants fed with nitrogen in form of both ammonium and nitrate for a duration of three months.

The preliminary results regarding the putrescine level are in agreement with those of Slocum (1984) who reported that putrescine accumulates in the plants maintained with only ammonium. It can be seen in Fig 5 that putrescine level was increased by only 25% of control in the roots of plants fed with added nitrate but the increase in the plants fed with both ammonium & nitrate and ammonium only was 143.5% and 238% of control respectively. The high activities of ODC and ADC with ammonium and not with nitrate support the above idea, since these two

enzymes are responsible for the production of putrescine. A possible explanation for the results reported by Martin *et al.* (1974) as well as for results obtained in this study that alkaloid increase is more due to added ammonium as compared to that of nitrate is the putrescine (accumulated due to ammonium supply) which plays an important role in the biosynthesis of tropane alkaloids (Leete, 1990).

CONCLUSION

Added nitrogen has litle effect on alkaloid concentration but the alkaloid produced per plant increases significantly due to increase in the biomass of the plant. The best results can be achieved by supplying nitrogen in the form of ammonium nitrate rather than as nitrate or ammonium ion.

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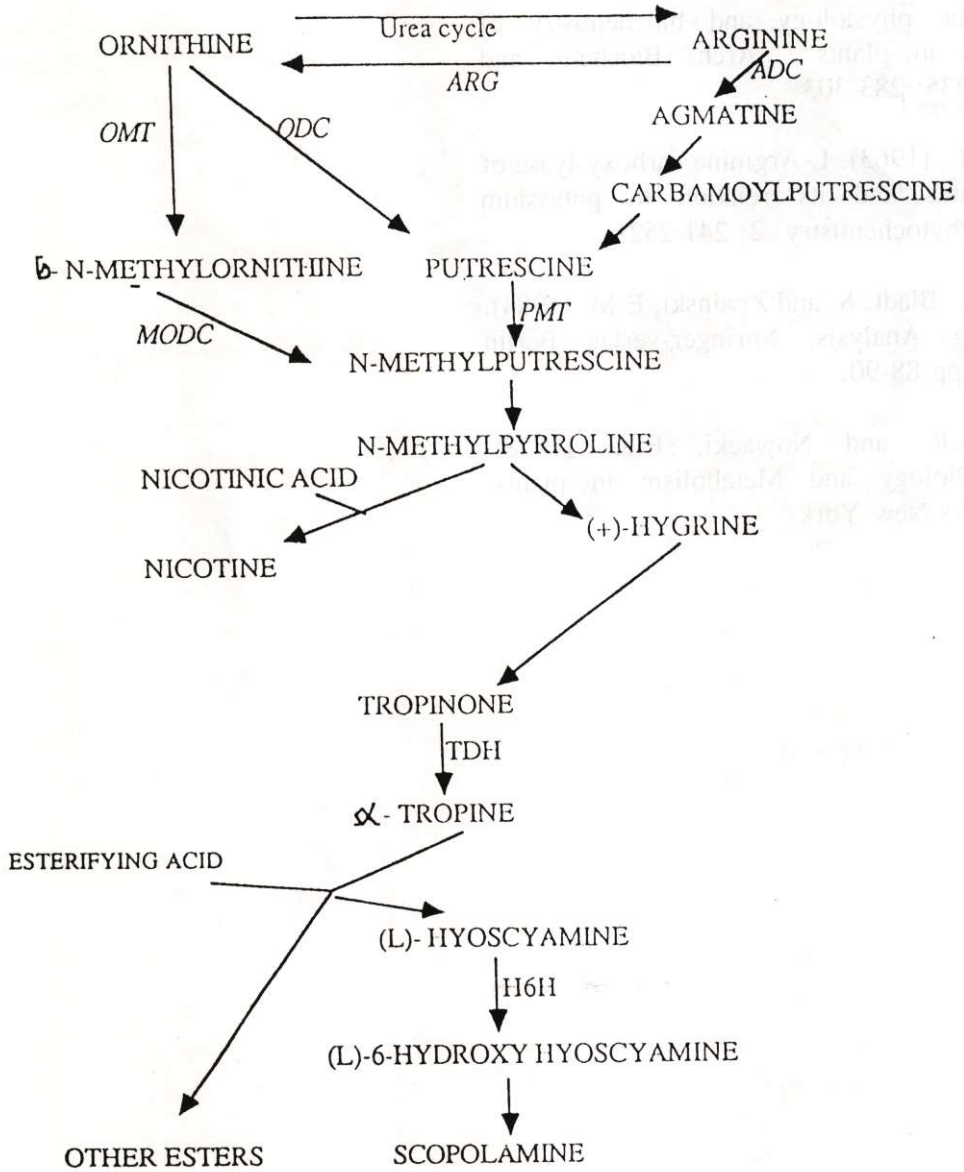

Fig. 1.

Pathway showing possible routes of tropane alkaloid biosynthesis.

ARG, Arginase; ODC Ornithine decarboxylase; ADC arginine decarboxylase;

OMT, ornithine δ-N-methyltransferase; MODC, δ-N-methylornithine decarboxylase;

PMT, putrescine N-methyl transferase; MPO, N-methylputrescine oxidase;

TDH, tropinone dehydrogenase; H6H hyoscyamine-6-B-hydroxylase

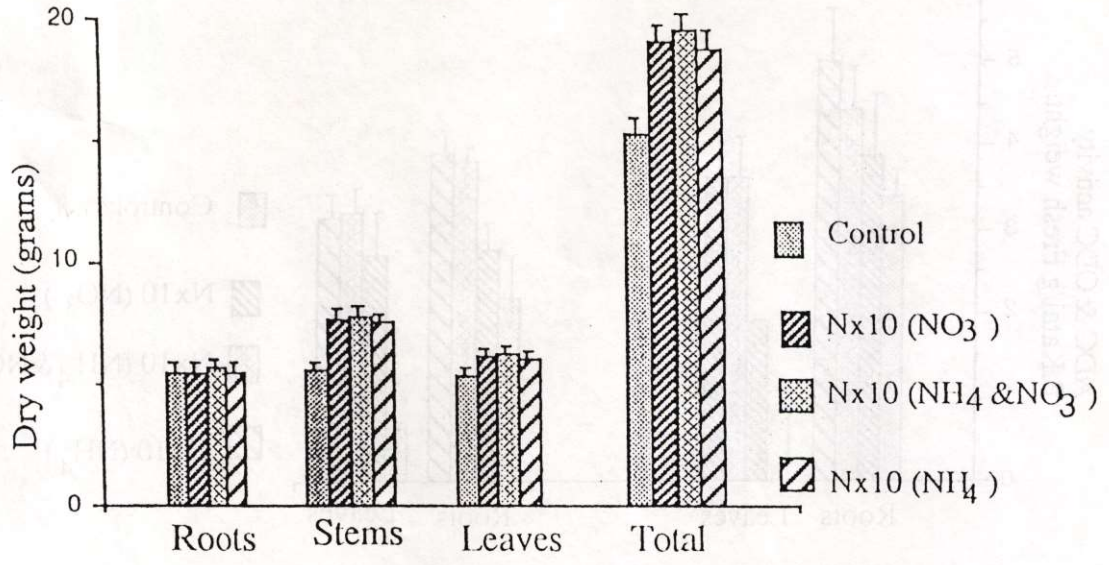

Fig. 2: Effect of different sources of added nitrogen (x10) on biomass in different organs of *Atropa acuminata*.

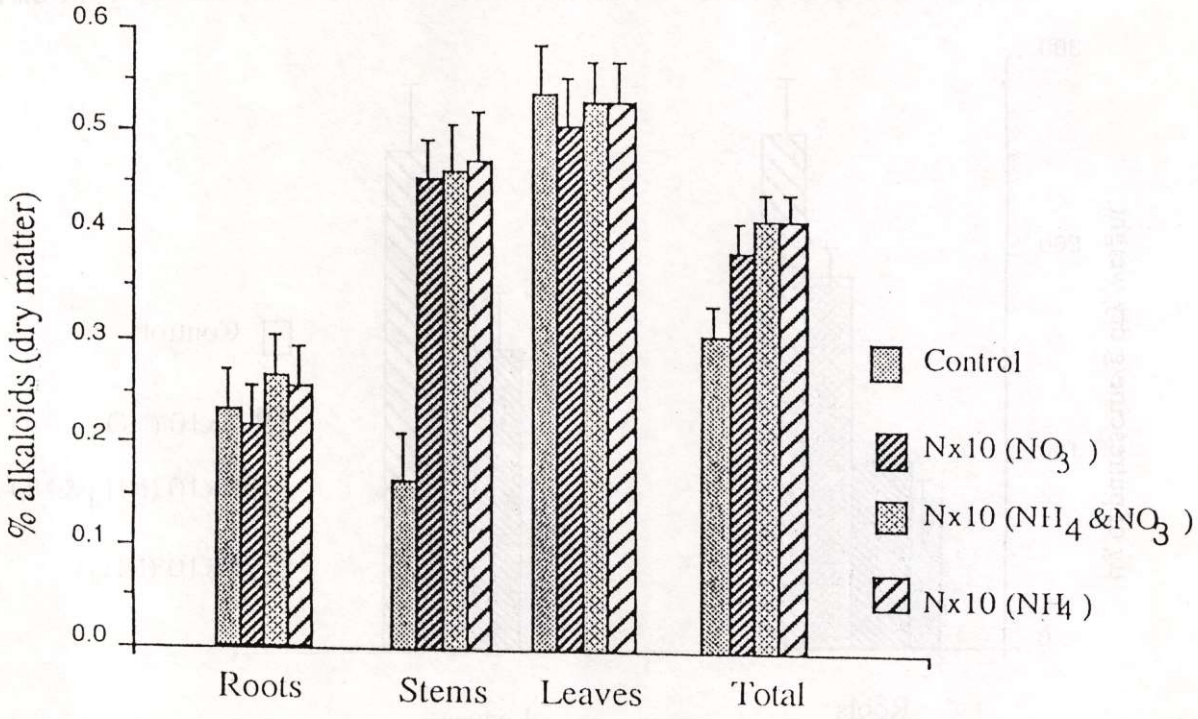

Fig. 3: Effect of different sources of added nitrogen (x10) on alkaloid content in different organs of *Atropa acuminata*.

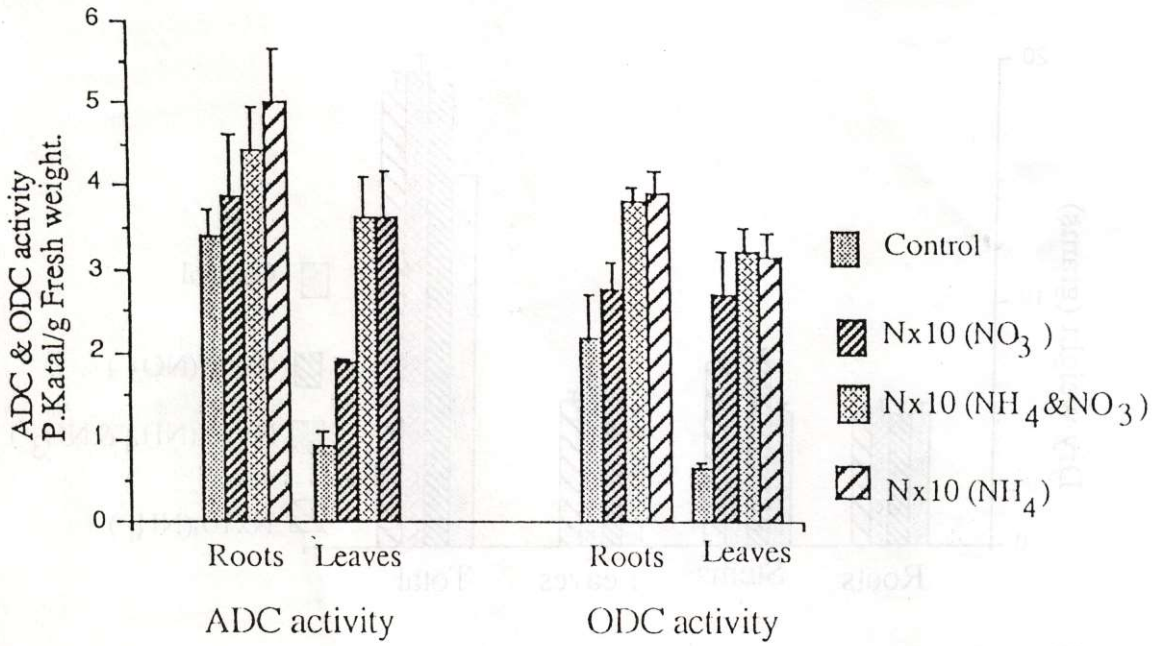

Fig. 4: Effect of different source of added nitrogen (x10) on ADC and ODC activity in roots and leaves of *Atropa acuminata*.

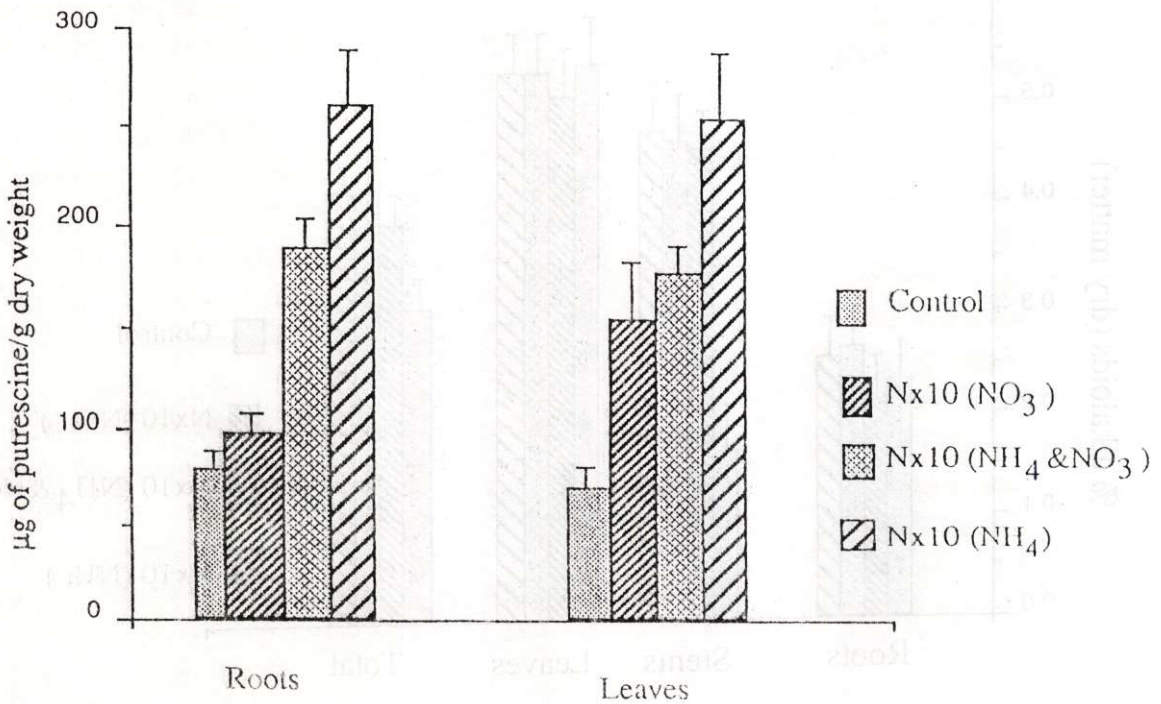

Fig. 5: Effect of different sources of added nitrogen (x10) on Putrescine level in roots and leaves of *Atropa acuminata*.