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EFFECT OF DIFFERENT NITROGEN SOURCES AND PLANT GROWTH REGULATORS ON GLUTAMINE SYNTHETASE AND GLUTAMATE SYNTHASE ACTIVITIES OF RADISH COTYLEDONS

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Summary. The effect of different nitrogen sources – KNO_3, NH_4NO_3 and NH_4Cl – on glutamine synthetase and glutamate synthase activities of radish cotyledons *(Raphanus sativus* L.) in the presence or absence of light was investigated. The plants were treated with different phytohormones viz. Kinetin (KN), gibberellic acid (GA) and abscisic acid (ABA). In dark ammonia supplementation was effective in promoting glutamine synthetase activity in all the treatments, whereas, KNO_3 was effective only in light in dH_2O, GA and ABA treatments. In dark NH_4NO_3 promoted the glutamate synthase activity in dH_2O while the activity was more with NH_4Cl in KN treated seedlings. GA had no effect, while low concentration of all nitrogen sources promoted the activity in ABA treated seedlings. The activity was less in light as compared to dark grown seedlings Varying levels of NADH-glutamate synthase activity was discernible in all the treatments and addition of ammonia promoted this activity to some extent. It is suggested that different energy status of the seedlings during light/dark or with hormonal treatments may affect the activity of this enzymes differently. However, it is suggested that changes in Fd-GOGAT should be studied before any definite conclusions can be drawn.

***Keywords*:** Radish cotyledons, plant growth regulators, nitrogen sources, glutamine synthetase, glutamate synthase

***Abbreviations*:** GOGAT – glutamate synthase, GS – glutamine synthetase, dH_2O – distilled water, KN – kinetin, GA – gibberellic acid, ABA – abscisic acid

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Introduction

In plant tissue ammonium can be derived either from NO_3^- reduction, uptake of supplied NH_4^+ or photorespiration. An additional internal source of ammonium is the catabolism of amino acids and N-transport compounds. Based on enzymological and inhibitor studies, Miflin and Lea (1976) proposed that the primary pathway for the assimilatory NH_4^+, in plants, both exogenously and endogenously generated, involves the combined activities of glutamine synthetase (GS; EC 6.3.1.2.) and glutamate synthase (GOGAT; EC 1.4.1.14). Much biochemical and genetic evidence available supports this conclusion. For example, it has been shown that the potent inhibitors of GS and GOGAT block NH_4^+ assimilation (Rhodes et al., 1986a). Further, kinetic studies with ^{14}N and ^{15}N indicate that glutamine-amide is generally the first and most heavily labeled product of NH_4^+ assimilation and that glutamine carries a large percentage of the total N-flux of the plant tissue (Rhodes et al., 1986a,b). Further, GS-deficient mutants exhibit rapid accumulation of free ammonia under photorespiratory conditions (Joy, 1988).

GS catalyses the initial assimilation of NH_4^+ into glutamine while GOGAT, is responsible for its transamidation of glutamine-amidonitrogen to the amino position of 2-oxoglutarate, to form two moles of glutamate. Because the two enzymes are dependent on each other for the provision of substrate, their activities constitute a cycle which has been termed as glutamate synthase cycle. Multiple forms of these two enzymes are present in most higher plants. The cytosolic (GS_1) and chloroplastic (GS_2) forms of GS show distinct molecular weights, and are encoded by differentially regulated genes in several species (Beckeret. al., 1992; Sakakibara et. al., 1992). Similarly, two isoforms of GOGAT have been purified and characterized, one dependent on ferredoxin as reductant (Fd-GOGAT; EC 1.4.7.1), whereas, the other utilizes NADH (NADH-GOGAT; EC 1.4.1.4.) (Suzuki et al., 1982).

Studies with barley mutants lacking GS_2, show that the major role of GS_2 in chloroplasts is to deal with the flux of NH_4^+ through the photorespiratory N-cycle (Wallsgrove et al., 1987). Similarly, mutants lacking Fd-GOGAT in chloroplasts are also lethal mutations Similarly, NO_3^- increased the abundance of GS_2 and Fd-GOGAT protein in cultured rice cells (Hayakawa et al., 1990).

The assimilation of inorganic N into amino acids, proteins, and other macromolecules requires provision of carbon skeletons. A cell, therefore, is dependent upon the product of recent photosynthesis or endogenous carbohydrate reserves. It has been shown that in cells lacking readily metabolizable carbohydrate reserves, NH_4^+ assimilation is greatly reduced (Turpin et al., 1991). Many authors reported that NH_4^+ enrichment both to higher plants and algae, causes a decrease in the flow of recent photosynthesis to sucrose and starch (Preiss et al., 1985), and that the supply of reducing equivalents and carbon skeleton may regulate NH_4^+ assimilation (Turpin et al., 1988).

Considering the aforesaid, in the present paper, changes in the levels of glutamine synthetase and glutamate synthase activities, in hormone treated radish seedlings

grown with different concentrations of KNO_3, NH_4Cl and NH_4NO_3 in light and dark conditions, were investigated.

Material and Methods

Seeds of radish *(Raphanus sativus* L) were soaked in dH_2O for 1 h and germinated in the dark on a wet filter paper (Whatman No.1) for 36 h. Seedlings with radicle length of approximately 1 cm, were transferred to sieve culture dishes containing N-free nutrient solution (Doddema and Telkamp, 1979) and phytohormones (KN 20 µM, GA 150 µM, ABA 40 µM). Half of the dishes were then transferred to light (Ca, 20 $\mu M.s^{-2}.m^{-1}$) and the rest were kept in a dark room at constant temperature (25±2 °C). The seedlings were allowed to grow under continuous light/dark conditions for 24 h. Seedlings of equal size were selected and transferred to different concentrations of KNO_3, NH_4Cl and NH_4NO_3 (0, 5, 15, 30, 45, 60, and 90 mM), prepared in fresh nutrient solution containing different phytohormones. For another 17 h the same hormone-pretreated seedlings were incubated with different N-forms in light/dark. The enzyme extract was prepared from the cotyledons of these seedlings for the estimation of both GS and GOGAT activities.

Enzyme extraction

For the preparation of enzyme extract, required number of cotyledons, from seedlings of the above described treatments, were separated off and weighed. The chilled material was homogenized in pre-chilled Tris-HCl extraction buffer (300 mM pH 7.8) containing ethylenediaminetetraacetic acid (EDTA) (100 mM) and mercaptoethanol (5 mM), in a cooled mortar at 4 °C in a cold room. The homogenate was first filtered through 4–5 layers of muslin cloth and then centrifuged at 15000×g for 20 min. The resulting supernatant was passed through a 15 ml column of sephadex G-25 pre-equilibrated with the same extraction buffer. The desalted preparation was then used for the assay of the enzymes.

Assay of glutamine synthetase

GS activity was assayed following the modified method of McCormack et al. (1982). The assay mixture 92.2 ml consisted, of 114 mM imidazole buffer (pH 7.2), 11.4 mM ATP, 45 mM NH_2OH:HCl, 45 mM $MgSO_4.7H_2O$ and the enzyme extract. After 45 min of incubation at 30 °C the reaction was terminated by adding $FeCl_3$ reagent (10% $FeCl_3$ in 0.2 N HCl, 24% TCA and 50% (v/v) HCl mixed in the ratio of 1:1:1). A blank, to which $FeCl_3$ was added prior to the addition of enzyme, served as the control. The precipitated protein was removed by centrifugation. The r-glutamyl hydroxamate in the supernatant was measured at 540 nm. The activity was expressed as nmol glutamyl hydroxamate produced.h^{-1}.cotyledon^{-1}.

Assay of NADH-glutamate synthase

NADH-GOGAT activity was estimated by spectrophotometric assay as described by Chen and Cullimore (1988). The assay mixture in a 3 ml final volume, consisted of 40 mM potassium phosphate buffer (pH 7.5), 10 mM L-glutamine, 10 mM 2-oxoglutarate, 0.14 mM NADH and enzyme extract. The control lacked glutamine, NADH and 2-oxoglutarate. Reaction was started with the addition of enzyme. The oxidation of NADH was observed at 340 nm and the activity is expressed as nmol NADH oxidized.h^{-1}.cotyledon^{-1}.

In all the enzyme assays, optimum pH and conditions for linear rate were determined with respect to substrate concentration and time. The experiments were repeated at least three times and results of one of the replicate experiment are presented.

Results and discussion

Even in the absence of external NO_3^- or NH_4^+, the cotyledons recorded considerably high GS activity, in dark (Fig. 1a). However, upon external addition of NO_3^- and/or NH_4^+, a considerable variation in GS activity was discernible. The concentration-response curves of the three N-sources used revealed that the addition of increasing concentrations of KNO_3 inhibited GS activity while, NH_4Cl promoted it. The activity was intermediate in NH_4NO_3 treatment. In contrast to this, GS activity in light was significantly promoted by all the three nitrogen sources used and maximum promotion was recorded in KNO_3 and NH_4NO_3 (Fig. 1b) In higher plants GS functions to assimilate NH_4^+ generated or mobilized during processes such as seed germination, photorespiration, NO_2^- reduction, N_2 fixation and primary NH_4^+ assimilation from the soil (Miflin and Lea, 1982). The two isoforms of GS have been shown to be localized in different subcellular compartments (chloroplast and cytosol) (Hirel and Gadal, 1981) and are differentially present in various organs. Studies on pea with GS cloned genes have shown that the expression of chloroplastic GS mRNA (GS_2 mRNA) in leaves is regulated by light, in a phytochrome mediated fashion (Tingey et al., 1989), and that the levels of GS_2 mRNA are affected by photorespiratory growth conditions. This suggests a major role of GS_2 in the reassimilation of photorespiratory NH_4^+. In contrast, the GS_1 protein and transcripts are found at relatively high levels in non green tissue, such as roots and etiolated shoots, and its role in the generation of glutamine for intracellular N-transport from cotyledons of germinated seedlings and in N_2 fixing nodules have been highlighted (Coruzzi, 1991). In the present study NH_4^+ supplementation resulted in an increase in GS activity. The similar effects with NH_4^+ on GS activity have also been reported by, Sugiharto and Sugiyama (1992). However, addition of KNO_3 promoted GS activity, only in light while in dark it was inhibited.

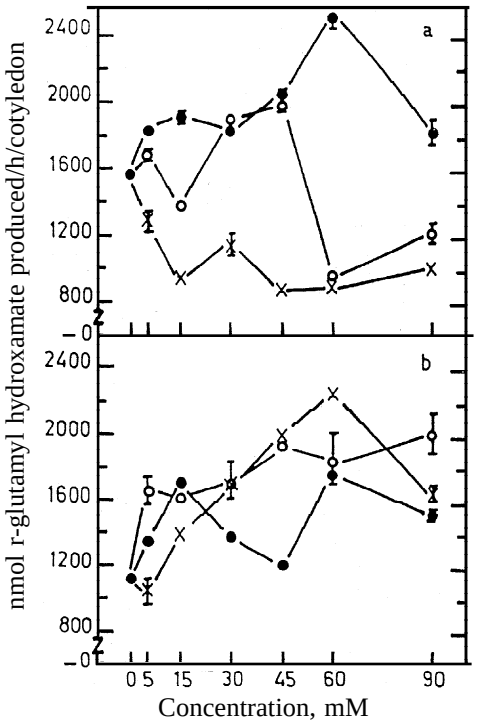

Fig.1. Changes in glutamine synthetase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (o—o) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown dH_2O treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

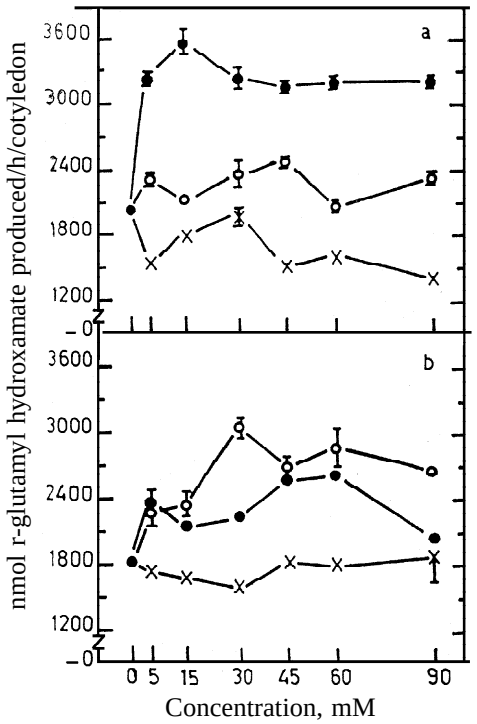

Fig.2. Changes in glutamine synthetase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown KN treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

Redinbough and Campbell (1993) have shown that GS_2 mRNA transcripts accumulate rapidly and transiently in cells exposed to NO_3^- while NH_4^+ treatment had no effects, and suggested that NH_4^+ taken up by the roots is assimilated via GS_1.

In the absence of any form of N in the medium, KN treatment promoted GS activity in light as well as in dark. Supplementing these seedlings with NH_4Cl significantly promoted GS activity (Fig. 2a) while, addition of KNO_3 decreased the activity. In NH_4NO_3 supplementation, slight promotion in activity was discernible. In light grown KN-treated seedlings, NH_4NO_3 and NH_4Cl addition promoted GS activity while, KNO_3 was not effective (Fig. 2b). Treatment of seedlings with GA also promoted GS activity in the absence of N in the medium. Both NH_4NO_3 and NH_4Cl further promoted the activity while, KNO_3 inhibited it (Fig. 3a). Like dH_2O-control, here also, KNO_3 supplementation was most effective in light while, NH_4NO_3 and NH_4Cl showed promotion only at higher concentrations (Fig. 3b). Abscisic acid promoted GS activity

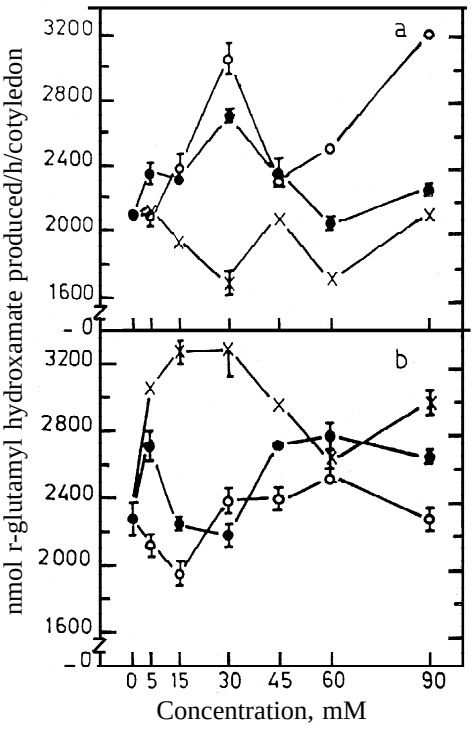

Fig.3. Changes in glutamine synthetase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown GA treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

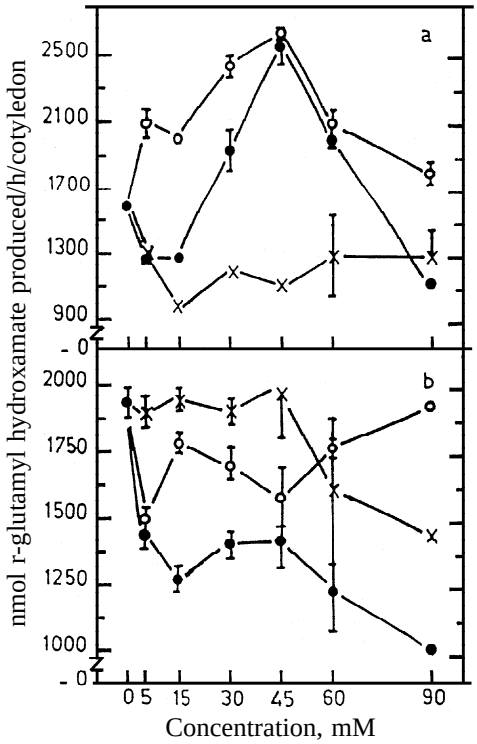

Fig. 4. Changes in glutamine synthetase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown ABA treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

only in light in the absence of N in the medium. In dark, both NH_4NO_3 and NH_4Cl were able to enhance GS activity and KNO_3 inhibited it (Fig. 4a). In light grown seedlings, on the other hand, KNO_3 promoted GS activity which is similar to the promotion recorded in dH_2O-control and GA grown seedlings (Fig. 4b).

From the above mentioned experiments, it was clear that in dark, NH_4^+ supplementation was effective in promoting GS activity while NO_3^- was effective in light. N-assimilation is among the most energy-intensive processes in plants, requiring the transfer of 2 electrons for NO_3^- converting to NO_2^-, 6 electrons for NO_2^- converting to NH_4^+ and 2 electrons and 1 ATP per NH_4^+ converting to glutamate. In light, the ATP and reductant supply, necessary for these reactions, are generated photochemically (Anderson and Done, 1977), whereas, the dark-grown plants may divert a significant proportion of reductant from mitochondrial electron transport (Bloom et al., 1992). During dark NO_3^- assimilation, shoots of higher plants (Bloom et al., 1989) and algae

(Weger and Turpin, 1989) evolved CO_2 sufficiently faster than they consumed O_2, presumably because the TCA cycle or the OPP pathway catabolized the substrate and transported some electrons to NO_3^- and NO_2^- rather than to O_2. These results indicated that in dark, shoots expend up to 25% of their respiratory energy on N-assimilation (Bloom et al., 1989), while studies with NH_4^+ assimilation indicated that nearly 14% of carbon catabolism is coupled to NH_4^+ absorption and assimilation (Bloom et al., 1992). The lower activity of GS during KNO_3 supplementation in dark may, therefore, indicate that the availability of ATP and reductant may be limited when compared to light-grown seedlings. It is only in KN-treated and KNO_3-fed seedlings that KNO_3 was not able to promote GS activity in light (Fig. 4b). In an earlier work (Sood et al., 2000) it was shown that nitrate reductase activity was inhibited by KN treatment and thus, the decreased assimilation of NO_3^- to NH_4^+ may inhibit GS activity.

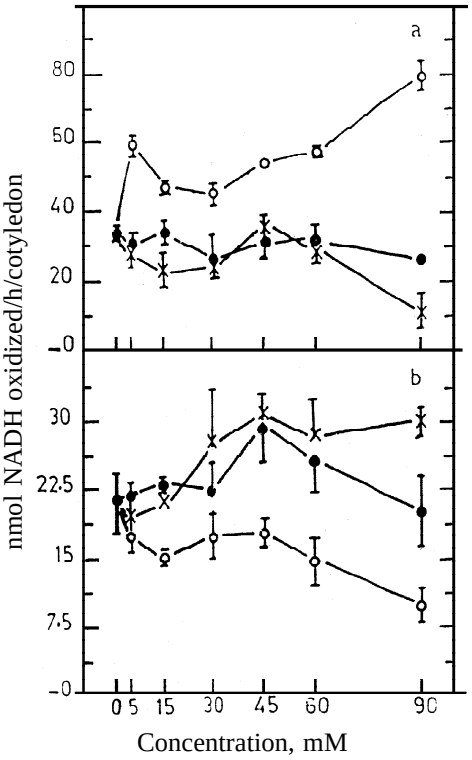

Fig. 5. Changes in glutamate synthase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown dH_2O treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

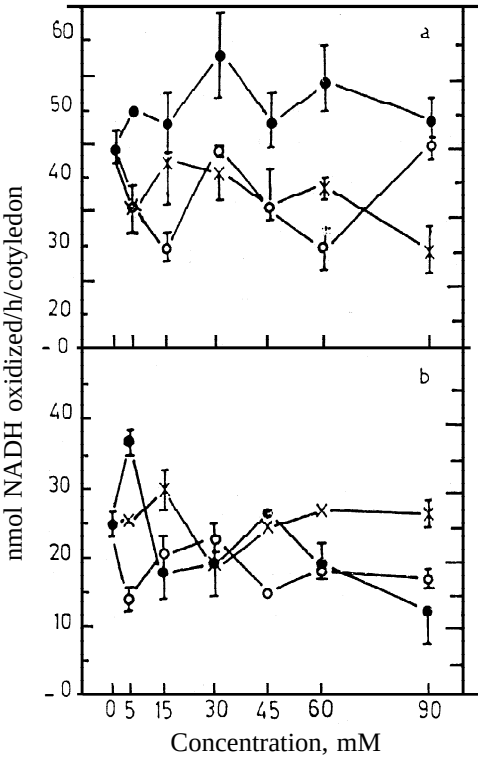

Fig. 6. Changes in glutamate synthase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown KN treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

Glutamate synthase, is the second enzyme of "glutamate synthase cycle". Although, in higher plants, Fd and NADH dependent GOGAT have been reported, it is suggested that NADH-GOGAT may be more important in N-metabolism during the earlier growth period and in dark. In the present study, considerable NADH-GOGAT activity was recorded in the absence of N in the medium (Fig. 5). Supplementation with NH_4^+ or NO_3^- alone did not affect the activity to a larger extent. However, addition of NH_4NO_3 in dH_2O-control seedlings promoted NADH-GOGAT in dark (Fig. 5a). In contrast to this, KN-treated seedlings, in dark, recorded higher NADH-GOGAT activity when NH_4Cl was the N-source, while KNO_3 and NH_4NO_3 had slightly inhibitory effects (Fig. 6a). No considerable effect of N-source was evident in dark-grown GA-treated seedlings (Fig. 7a), while in ABA-treated seedlings, all N-sources promoted the activity at low concentrations (Fig. 8a).

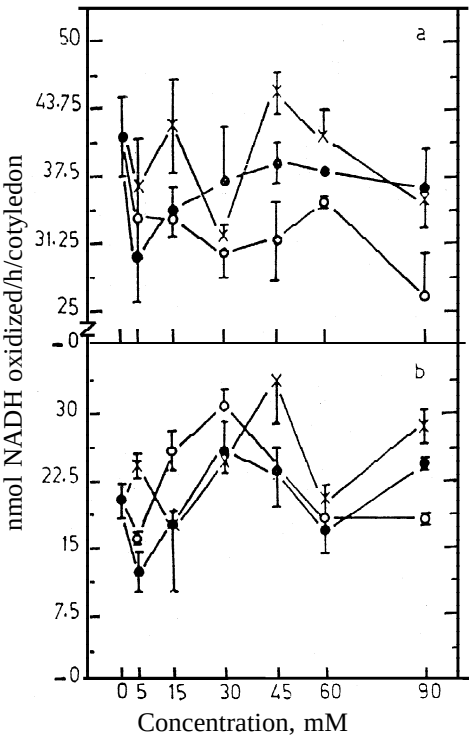

Fig. 7. Changes in glutamate synthase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown GA treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

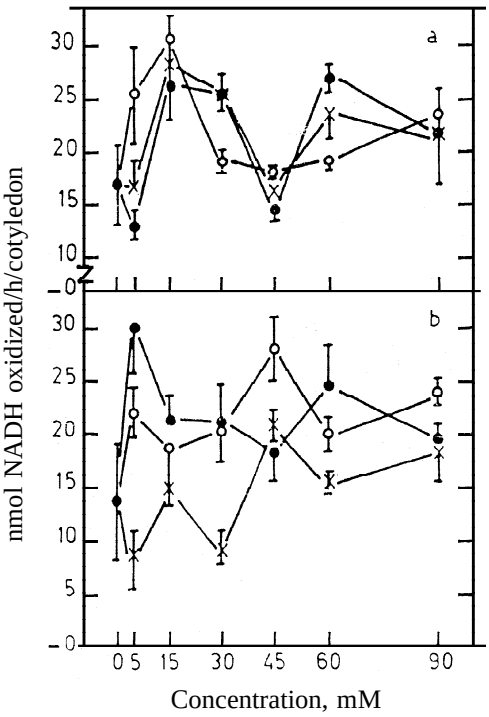

Fig. 8. Changes in glutamate synthase activity with different ambient concentrations of KNO_3 (X—X), NH_4Cl (•—•) and NH_4NO_3 (o—o) in the cotyledons of (a) dark grown and (b) light grown ABA treated radish seedlings. Vertical bars represent ±SD (wherever bars are absent, SD is so small that it is within the symbol).

In light-grown seedlings, NADH- GOGAT activity was inhibited when compared to dark-grown seedlings. In dH_2O-control seedlings, both KNO_3 and NH_4Cl promoted NADH-GOGAT activity while NH_4NO_3 showed inhibitory effects (Fig. 5b). In KN treatment, addition of NH_4NO_3 inhibited NADH-GOGAT to some extent while in the other two N-sources no clear trend was discernible (Fig. 6b). As in dark grown seedlings, the effect of all the three N-sources was unclear in light grown GA treated seedlings (Fig. 7b). The activity of NADH-GOGAT was promoted by both NH_4NO_3 and NH_4Cl in ABA-treated seedlings while, KNO_3 was effective only at higher concentrations (Fig. 8b). From the above mentioned changes in NADH-GOGAT activity no clear conclusions can be drawn. This may, perhaps, be due to the fact Fd-GOGAT activity was not studied and it is desirable that changes in Fd-GOGAT are also monitored. However, it appears that addition of NH_4^+ promoted NADH-GOGAT activity to some extent. Earlier work has also shown that NH_4^+ can induce the expression of NADH-GOGAT in maize seedlings (Handa et al., 1985), in the roots of alfalfa (Groat and Vance, 1982) and in the cotyledons of *Phaseolus vulgaris* (Leon et al.,1990).

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