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Regulation of Nitrate Reductase Activity in Soybeans

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REGULATION OF NITRATE REDUCTASE ACTIVITY IN SOYBEANS

by

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VITA

The author, Luke Theodore Curtis, is the elder son of Herbert John Curtis and Elizabeth (Kleinhans) Curtis. He was born January 17, 1959, in Chicago, Illinois.

His elementary education was obtained in the public schools of Chicago, Illinois, and Wilmette, Illinois. His secondary education was completed in 1977 at New Trier High School (East), in Winnetka, Illinois.

In August, 1977, Mr. Curtis entered Valparaiso University in Valparaiso, Indiana. He received his Bachelor of Science in biology and chemistry in May, 1981. In 1980, he worked at the Chicago Botanic Garden (Glencoe, Illinois) as a summer intern.

In August, 1983, Mr. Curtis entered Loyola University of Chicago as a teaching assistant and graduate student. He became a student member of the American Society of Plant Physiologists in January, 1984. Mr. Curtis was one of ten students selected to present research findings at Loyola's Sigma-Xi graduate research forum in 1984. He has also presented posters of his research findings at the UCLA Symposium on Molecular and Cellular Biology (April, 1985, in Keystone, Colorado) and at Loyola's Sigma-Xi graduate research forum in May, 1985.

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ABSTRACT

This study investigates nitrogen nutritional conditions as regulators of nitrate reductase activities in whole soybean plants and cultured soybean cells (callus and suspension cells). Nitrate given to soybean plants was found to enhance enzyme activities about 5-fold relative to plants given no nitrogen. In most cases, glutamine did not repress enzyme activities in the presence of nitrate, and glutamine given as a sole nitrogen source actually enhanced nitrate reductase levels relative to plants provided with no nitrogen. Ten-day old plants given no supplemental nitrate had large stores of nitrate in their plant parts (over 7% by dry weight in their primary leaves) and had NADH or NADPH-linked nitrate reductase activities of about 1 nmole NO_2^-/ minute/ mg. soluble protein. Added nitrate in the plants' media increased leaf nitrate levels about 2-fold and enhanced enzyme activities about 3-fold. These data suggest that the large amounts of nitrate stored in seeds and plant parts may "self-induce" a certain amount of nitrate reductase activity, and additional nitrate in media may further enhance nitrate reductase activities and stored nitrate levels. In cultured soybean cells, nitrate in the presence of a reduced nitrogen source was required to stimulate maximum nitrate reductase activities. Cells given nitrate as a sole nitrogen source soon died. Cells given glutamine as a sole nitrogen source grew well, but had very

low levels of both nitrate reductase activity and stored nitrate. No significant NAD(P)H-linked nitrate reductase activity was found in cultured cells, suggesting that the NAD(P)H-linked enzyme may be under developmental control.

REVIEW OF LITERATURE

Nitrate Uptake in Plants

The amount of available soil nitrogen is often the limiting factor for growth and protein production in plants (1,2). About 2×10^{10} tons of inorganic nitrogen are assimilated by plants annually (3,4). Organic nitrogen in the soil is derived mainly from two sources: 1) ammonia, which is obtained largely from dinitrogen fixation, and 2) nitrate. The predominant form of nitrogen available to field plants is nitrate (5). While most nitrogen in commercial fertilizers is in ammoniacal form, much of the ammonia derived from these fertilizers is oxidized to nitrate by soil bacteria (5-7). High concentrations of ammonia are toxic to plants and to cultured plant cells, whereas nitrate is a relatively safe form of nitrogen even in high concentrations (5). Nitrogen obtained from dinitrogen fixation by bacteria accounts for only about 1% of the nitrogen utilized by plants on a global scale (8). Even in soybeans with well developed nitrogen fixing nodules, dinitrogen fixing accounts for only 30% of the nitrogen assimilation of this species over the course of a lifetime, with the remainder of the plants' nitrogen supplied by nitrate assimilation (9).

Nitrate is utilized by plants via the pathway shown below:

nitrate reductase	nitrite reductase	glutamine synthetase	glutamate synthase

nitrate--->nitrite--->ammonia--->glutamine--->glutamate

NADH or NADPH is used as the electron donor in the reduction of nitrate to nitrite. Nitrite reductase and glutamine synthetase are generally present in much greater activities than nitrate reductase (10). Hence the nitrate reductase step is rate limiting in this nitrogen utilization pathway (10-13).

Nitrogen Nutrient Regulation of Nitrate Reductase Activity

The regulation of nitrate reductase activity by various nitrogen sources has been extensively studied in fungi. Studies with the fungus Neurospora crassa have indicated that nitrate stimulates nitrate reductase activity, while glutamine virtually eliminates nitrate reductase activity even if nitrate is also present in the media (14). Ammonia and other forms of reduced nitrogen also serve as repressors for nitrate reductase in fungi (14-17).

Nitrate reductase regulation has also been investigated in higher plants. It has been established that added nitrate greatly increases nitrate reductase activity in corn (18) and radish (19). Beevers et al. (20)

established that different amounts of nitrate in the media are required for optimal stimulation of nitrate reductase activities in various species of higher plants. Nelson and coworkers (21) found that 15-day old soybeans grown on 15 mM nitrate had 3 times the amount of leaf nitrate reductase activity as the same plants grown on nutrient media containing 3.75 mM urea. Robin and coworkers (22) found that the addition of 50 mM potassium nitrate given at day 5 increased leaf nitrate reductase activities of 8-day old soybeans 5 fold compared to the same plants given no nitrogen. Relatively little is known about the possible repression of higher plant nitrate reductase activities by reduced nitrogen compounds. Ammonia in the media did not repress nitrate reductase activity in radish cotyledons or maize seedlings (23). Ingle and coworkers (19) found that added ammonia in the presence or absence of nitrate increased nitrate reductase activities in radish cotyledons. Radin (24) established that glutamine, glycine, and asparagine strongly inhibited *in vivo* nitrate reductase activities in cotton roots but not in cotton leaves. Factors not directly related to nitrogen nutrition such as light, mineral nutrition, temperature and age of plant also play a role in nitrate reductase regulation in whole plants (4-6,12,25,26).

Nitrate reductase regulatory studies have also been conducted in cell cultures of higher plants. Cultured cells are in a more uniform nutritional milieu than intact plants,

and their biochemical situation is simplified since the cultured cells are undifferentiated and are not arranged in organ systems. Both callus cells (27) and suspension cells (28-30) have been extensively employed for these biochemical experiments. Tissue culture studies in tobacco found that nitrate in the media stimulated nitrate reductase activity while casein hydrolysate or 11 individual amino acids were found to repress nitrate reductase activity even in the presence of nitrate (31). Bayley et al. (32) noted that reduced nitrogen along with nitrate was required for optimal growth and nitrate reductase activities in soybean root cells, although wheat root cells grew rapidly and produced high levels of nitrate reductase activity on media containing nitrate as a sole nitrogen source. Gamborg (28) determined that soybean cells grew poorly on nitrate as a sole nitrogen source although flax, horseradish, and three other higher plant species grew well on nitrate alone. Oaks (29) noted that soybean suspension cells grown on 20 mM glutamine or 20 mM ammonium citrate did not develop significant nitrate reductase activities when transferred to media containing both 25 mM nitrate and 10 mM glutamine or 10 mM ammonium citrate. Nelson et al. (30) determined that soybean cotyledon suspension cultures grown on glutamine as a sole nitrogen source had little in vivo nitrate reductase activities, but transfer to medium containing 25 mM nitrate and 2 mM ammonia greatly increased nitrate reductase activities. Nelson and coworkers also found that the

addition of 10 mM glutamine to suspension cultures containing 25 mM nitrate and 2 mM ammonia did not repress nitrate reductase activities relative to the media containing nitrate and ammonia alone.

Biochemical Aspects of Nitrate Reductase

Most higher plants possess only NADH-linked nitrate reductase activities (33). Certain higher plants possess both NADH and NADPH-linked nitrate reductase activities, including maize, barley, foxtail, Lemma minor, and soybeans (33-36). At least two different nitrate reductase enzymes are present in intact soybean plants. One of the enzymes utilizes NADH as a cofactor and has a molecular weight of 330,000 (37). The other enzyme can utilize either NADH or NADPH as a cofactor (34,38) and has a molecular weight of 220,000 (37). Jolly et al. (37) have separated these two enzymes by DEAE-cellulose column chromatography. In addition, Campbell (39) separated these two enzymes via affinity chromatography on blue-dextran sepharose. Both of these enzymes have pH optimum of 6.5 (37). The NAD(P)H bispecific enzyme has a Km for nitrate of 6 mM (34), while the NADH-linked enzyme has a Km for nitrate of 0.19 mM (40). Some researchers (36,41) have suggested that the NAD(P)H bispecific enzyme is not specific for NADPH at all, but relies on a phosphatase enzyme to convert NADPH to NADH.

Recent evidence suggests the presence of a third soybean nitrate reductase enzyme. This proposed soybean

enzyme is NADH-linked and has a pH optimum of 7.5 which is similar to the nitrate reductase enzyme found in most higher plants including squash (33,42). The Km of nitrate for this enzyme is only 0.1 mM (40), which is very similar to the Km of the corn and spinach nitrate reductase enzymes (37,43). Robin et al. (22) and Nelson and coworkers (21,44) determined that the NADH-linked nitrate reductase activity at pH 7.5 was more inducible by added nitrate than the NADPH-linked activity at pH 6.5.

Mutant soybean plants that lack any constitutive nitrate reductase activities have been developed by Nelson and coworkers (21). These mutant plants show no nitrate reductase activity when grown on urea as a sole nitrogen source, but show considerable "inducible" nitrate reductase activities when nitrate is added to the media (21,22,45). Wild-type plants show considerable nitrate reductase activity when grown on urea as a sole nitrogen source and these activities can be enhanced about 3-fold by addition of nitrate in the media (21,22,45). The activities in the mutant plants grown on nitrate (inducible activity) plus the activities found in wild type plants grown on urea (constitutive activity) equaled the total activity (both constitutive and inducible) found in the wild-type nitrate fed plants (21,45). The constitutive and inducible nitrate reductase activities each accounted for about one-half of the total nitrate reductase activity in 15-day old wild-type soybeans supplied with nitrate (21). Constitutive nitrate

reductase activities (found in wild-type plants given no nitrate in their media) were maximal at about 10 days and later decreased over time (21). Robin et al. (22) determined that nitrate reductase mutant plants supplied with nitrate had very little NADPH-linked activity at a pH of 6.5, but showed a considerable NADH-linked activity at a pH of 7.5. These data suggest that the soybean bispecific NAD(P)H enzyme is a constitutive enzyme that is absent in the mutant plants, and that the NADH-linked enzyme which has a pH optimum of 7.5 is inducible by nitrate in the media. Constitutive and inducible nitrate reductase isozymes have also been obtained from soybean cotyledons which have Km's and pH optima similar to the two enzymes found in soybean leaves (44). Plant breeding work involving crosses between the constitutive mutants and wild-type plants has suggested that the constitutive nitrate reductase is synthesized by a single recessive nuclear gene (44). Nelson and coworkers (30) determined that the NAD(P)H-linked nitrate reductase constitutive enzyme is not present in soybean cotyledon suspension cultures.

Recent work using gel electrophoresis with cereal nitrate reductase enzymes suggests that two NADH-linked nitrate reductase isozymes are present in barley (47). The activity of the slower migrating barley nitrate reductase enzyme was greatly increased by the addition of nitrate to the nutrient media, while the activity of the faster migrating enzyme was unaffected by added nitrate in the

media. Hence the slower migrating enzyme can be considered to be inducible in nature and the faster enzyme can be considered to be a constitutive form of nitrate reductase.

Location of Nitrate Reductase in Plants

Nitrate reductase enzymes are found in almost every type of higher plant cell (48,49). Nitrate reductase has been isolated from the root cells of various plant species (34,50). However, much of the nitrate reductase activity in higher plants is present in the leaves (5,12,42). Evans and Nason (34) noted much higher nitrate reductase activities in young soybean leaves as opposed to soybean roots. Nitrate reductase in leaves is believed to be a soluble cytoplasmic enzyme (6,12).

Transport of Nitrate and Other Nitrogenous Compounds

The nitrate absorbed by the plant must be transported up the xylem so that it can be reduced in the leaves. It has been established that nitrate and the amide amino acids glutamine and asparagine are the major carriers of organic nitrogen in the sap of most higher plants (51). Pate et al. (52) reported that nitrate in the media did not significantly decrease the concentration of glutamine or asparagine in the xylem or phloem of Lupinus albus. In soybeans and some other nitrogen fixing legumes ureides are important transporters of nitrogen in the xylem sap (53-55). McClure and Israel (54) found that mature, nodulated soybean

plants given no supplemental nitrate transport most of their xylem nitrogen as ureides, while in unnodulated plants or in plants given supplemental nitrate the primary xylem nitrogen transporters were nitrate and amino acids.

Nitrate Levels in Plants and their Relationship to Nitrate Reductase Activities

Several studies have investigated the relationship between nitrate in the leaves and enzyme activities of nitrate reductase and other enzymes involved in nitrogen metabolism. Thibodeau and Jaworski (56) and Harper and Hageman (9) have determined that young field grown soybeans have large amounts (over 0.75% by dry weight) of stored nitrate in their leaves during the early stages of growth. These researchers also noted that both the levels of stored nitrate in the leaves and the leaf nitrate reductase activities declined as the plant aged, while nitrogenase activities increased over time as nodules became more developed. Harper and Hageman (9) suggested that lower nitrate levels in older soybean plants are at least partially responsible for the decline in specific nitrate reductase levels over time. Barneix *et al.* (57) found that added nitrate in the media increased both leaf nitrate levels and leaf nitrate reductase activities in barley. Barneix and associates also suggested that nitrate may accumulate in the young plant far in excess of its need for nitrogen or its ability to assimilate it via the nitrate

reductase pathway. This stored nitrate can later be utilized by the plant when the level of available nitrogen in the soil is low. Shaner and Boyer's (58) work with water-stressed maize leaves suggested that nitrate flux may be a more important stimulator of nitrate reductase activity than the actual level of nitrate in the leaves. Since nitrate reductase is believed to be a cytoplasmic enzyme, it has been suggested that only the nitrate entering or leaving the cytoplasm may be involved in the regulation of nitrate reductase activity (57). The nitrate stored in vacuoles or other morphologically isolated compartments may thus have little or no effect on the regulation of nitrate reductase activities (57).

Changes in Nitrate Assimilation Over the Life Cycle of a Plant

Studies have also been conducted that measure the relative levels of nitrate reduced over the life cycle of a plant. Nitrate serves as nearly the sole source of nitrogen in soybeans whose nodules have not fully developed (9). Hence nitrate reductase plays an especially vital role in the young soybean. Nitrogenase activities develop later in the life of the plant, although nitrate reductase levels still remain significant. In nodulated soybean plants nitrogenase and nitrate reductase activities seem to be inversely related. Added nitrate in the soil stimulated nitrate reductase activities but decreased nitrogenase

activities in the nodule bacteria (59). Neyra and coworkers (60) noted that while nitrogenase activities dropped sharply in Phaseolus vulgaris at time of flowering, nitrate reductase activities could be greatly stimulated at this time by the presence of nitrate in the soil.

MATERIALS AND METHODS

Whole Plant Nitrate Reductase and Nitrate Studies

Soybean seeds were grown in a growth chamber which received artificial illumination for 16 hours a day at 22° C. The seeds were germinated in vermiculite and were watered with distilled water for 10 days. Subsequently, the plants were irrigated with Hoagland's nutrient media (61) modified to contain the following nutrients as sole nitrogen sources: 1) no nitrogen, 2) 50 mM KNO_3, 3) 10 mM glutamine, and 4) 50 mM KNO_3 and 10 mM glutamine. The nutrient media contained 5 mM $CaCl_2$ instead of 5 mM $Ca(NO_3)_2$.

Cotyledons were cut at the time of nutrient media irrigation in the cotyledon cutting experiments. Random samples of soybean primary leaves were harvested at various time intervals after irrigation. The leaves were homogenized with a mortar and pestle containing a cold extraction buffer similar to that used by Jolly *et al.* (37), which contained 25 mM KPO_4^- at pH 6.5, 1 mM EDTA, 10mM cysteine, and 0.2% insoluble polyvinyl polypyrrolidone. This homogenate was then centrifuged at 10,000 xg to remove insoluble proteins and cellular debris. Nitrate reductase levels were assayed by a method similar to that of Hageman and Reed (42). The assay was stopped by the addition of zinc acetate as described by Scholl *et al.* (62). For the pH 6.5 NADH or NADPH-linked assays the assay mixture contained 80

mM KNO_3, 25 mM KPO_4^- at pH 6.5 and 0.1 mM of either NADH or NADPH. For the pH 7.5 NADH-linked assays the assay mixture contained 10 mM KNO_3, 25 mM KPO_4^- at pH 7.5, and 0.1 mM of NADH. A lower concentration of KNO_3 was used when assaying for the pH 7.5 NADH-linked enzyme since this enzyme has a much lower Km for nitrate than the pH 6.5 optimum NAD(P)H bispecific enzyme (37). Nitrite was determined using the colorimetric reagents, 1% sulfanilamide in 20% HCl and 0.02% N-1-Napthylethyelenediamine-2HCl as described by Hageman and Reed (42). Protein levels of the homogenized leaves were determined by the method of Lowry et al. (63), using bovine serum albumin as a standard protein. The specific nitrate reductase activities were calculated in terms of umoles NO_2^- produced/ minute/ mg. soluble protein.

Experiments measuring nitrate reductase activities in squash were also conducted to contrast with the soybean studies. Whole plant squash nitrate reductase assays were conducted in a manner similar to the soybean assays but with the following modifications: 1) cotyledon tissue rather than primary leaf tissue was harvested for assaying nitrate reductase activities, 2) plants were given nutrient media at 7 days instead of 10 days, as the cotyledons became fully developed by 7 days, 3) the extraction buffer contained 100 mM KPO_4^-, 1 mM EDTA, and 0.2% insoluble polyvinyl polypyrrolidone as described by Smarrelli and Campbell (64), 4) the NADH-linked and NADPH-linked assays were run only at

a pH of 7.5, with 10 mM KNO_3. The NADH-linked enzyme in squash is known to have a pH optimum of 7.5 (33).

Seeds and other plant parts used for nitrate determinations were initially ground in liquid nitrogen. These cells were then homogenized by boiling in 1 M NaOH for 10 minutes. The homogenates were diluted and nitrate levels determined by the chromotropic acid method of West and Ramachandran (65).

Cultured Cell Experiments

Cells for callus cultures were obtained from primary leaves 8 to 12 days old. The leaves were sterilized by immersion in a mixture containing 10% Chlorox and 0.1% sodium dodecyl sulfate for 10 minutes followed by immersion in 70% ethanol for 5 minutes. The culture medium for the agar plates was similar to that of the B5 medium developed by Gamborg *et al.* (48), with the following modifications: 1) 1 mg/ liter of napthaleneacetic acid was used instead of 2,4 dichlorophenoxyacetic acid, 2) 0.2 mg/ liter of kinetin was added to the medium, and 3) 1 mM of citrate adjusted to pH 5.5 with KOH was added to increase the buffering capacity of the medium. The vitamins, kinetin, and glutamine used in tissue culture work were filter sterilized, while all of the other medium components were sterilized by autoclaving. The B5 media contained 2 mM NH_4^+ and 25 mM KNO_3 as nitrogen sources. Calluses were allowed to form on the B5 plates for approximately 3 weeks. These calluses were then repeatedly

subcultured onto fresh B5 media at approximately 10 day intervals. All callus tissue used in the callus experiments or used to make suspension cultures had undergone at least 6 subculture passages and this tissue was very friable and fairly uniform in appearance. The callus tissue were grown in a growth chamber at a temperature of 25°C, and were under artificial light for 12 hours a day and were under darkness for the remaining 12 hours.

Callus cells used for the nitrate reductase "induction" experiment were grown for the last 2 subcultures on nutrient medium which contained 15 mM KCl plus 10 mM glutamine as a sole nitrogen source. All callus tissue used had been last subcultured 5 days earlier; this relatively short subculturing period was used so that the plant cells would be uniformly treated and well nourished. At day 0 some randomly selected cells were harvested for nitrate reductase assays. Also at day 0 callus tissue was placed on nutrient media containing the following sources of nitrogen and potassium nutrition: 1) 15 mM KCl (no nitrogen), 2) 25 mM KNO_3, 3) 10 mM glutamine and 15 mM KCl, 4) 25 mM KNO_3 and 10 mM glutamine, and 5) 25 mM KNO_3 and 2 mM NH_4^+. Three pieces of callus tissue from different and randomly selected cell lines were placed onto 3 sterile petri plates containing approximately 50 ml. of nutrient medium. The weights of the calluses were determined on an analytical balance and in every case the weight of the callus was between 0.75 and 1.00 grams. A different set of 3 plates

was made for each time and nutritional condition. These were then placed in a growth chamber and harvested after 1, 3, 5, 7, and 14 days. The 14-day calluses were subcultured at 7 days onto 5 plates containing about 50 ml of fresh medium. The fresh weights of the callus tissue were determined to calculate the relative growth of the callus tissue. The callus tissue from each experimental condition were pooled together so that a representative sample could be obtained. The callus tissue was then homogenized by grinding in liquid nitrogen and extraction buffer. Nitrate reductase assays (NADH and NADPH-linked at pH 6.5 and NADH-linked at pH 7.5) and protein assays were made following a procedure similar to that used for the soybean leaf tissue.

A soybean callus "repression" assay was conducted in a manner similar to that of the callus "induction" assay but with the following modifications: 1) prior to the experiment the callus cells were grown on B5 nutrient media containing 25 mM KNO_3 and 2 mM NH_4^+. All of the calluses used in this experiment were last subcultured 5 days earlier as in the "induction" assay. 2) All of the nitrogen nutrition media used in the "induction assay" was used with the exception of the B5 media, which was omitted.

For the nitrate determinations in soybean callus tissue, callus was grown for 6 subcultures on media containing 15 mM KCl plus 10 mM glutamine as a sole nitrogen source. Random samples of callus were placed onto the 5

different nutrient media using the same protocol as in the callus "induction" assay. Three days later the callus tissue was harvested and weighed. Callus tissue was ground in liquid nitrogen and the cells were further digested by boiling in 1 M NaOH for 10 minutes. This homogenate was analyzed for nitrate by the method of West and Ramachandran (65).

For suspension cultures, callus cells were placed into liquid nutrient medium containing 15 mM KCl plus 10 mM glutamine as a sole nitrogen source. The initial density of the callus in the suspension cultures was about 0.05 g. cells/ ml medium. The suspension cultures were placed on an automatic shaker at 90 rpm under ambient temperature. These cells were subcultured into fresh liquid B5 media every 10 days and the large cell fragments were decanted or filtered. To confirm lack of bacterial or fungal contamination in the the suspension cultures, a sterile loop was used to streak small amounts of the suspension cultures onto plates containing "L" media (1.5% agar, 0.5% yeast extract, 1.0% NaCl, and 1.0% tryptone at pH 7.5, similar to the media developed by 66). Lack of growth on "L" plates indicated no detectable contamination in the suspension cultures. Cell growth was determined by measuring the packed cell volume of the suspension cultures. Cultures that increased in packed cell volume by more than 2.5 fold in a week were considered viable.

Viable soybean suspension cells which were grown on liquid media containing glutamine as a sole nitrogen source for at least 2 subcultures were used in a suspension "induction" assay of nitrate reductase activities. These cells were pelleted at 2000 xg and transferred to liquid media containing the following nitrogen and potassium sources: 1) 15 mM KCl (no nitrogen), 2) 25 mM KNO_3, 3) 15 mM KCl and 10 mM glutamine, 4) 25 mM KNO_3 and 10 mM glutamine, and 5) 25 mM KNO_3 and 2 mM NH_4^+. One 100 ml flask of sterile nutrient media was prepared for each experimental condition. Each flask received cells from 3 parent cell lines which were randomly chosen. The packed cell volumes for each of these flasks were measured and in each case the initial density was 0.7% to 1.0% cm^3 cells/ ml media. The cells in the flasks were harvested at 1, 3, and 6 days after inoculation, pelleted at 2000 xg., and their final packed cell volumes recorded. The cells were homogenized by grinding in liquid nitrogen and extract buffer. Nitrate reductase assays (NADH and NADPH-linked at pH 6.5 and NADH-linked at pH 7.5) and protein assays were performed by methods similar to those employed for whole plants.

Materials

Soybean seeds (*Glycine max* var. Williams 82) were obtained from Funk Seeds in Bloomington, Illinois. All chemicals used in nutrient media or assay mixtures were of reagent grade or better. Distilled water was used for assay

mixtures and distilled and deionized water was used for nutrient and tissue culture media. Nitrate levels in the distilled water, nutrient media lacking potassium nitrate, and a solution of distilled water soaked in vermiculite overnight were below 0.03 mM.

Data and Statistics

In all of the enzyme, protein, and nitrate assays two to four replicates of each sample were made. Means and standard errors were calculated for all of the data. An ANOVA (ANalysis Of VAriance) and a Student-Neuman-Keuls test were performed on the data in order to determine if the differences were statistically significant (67,68).

RESULTS

Whole Plant Soybean Nitrate Reductase Regulation Studies

Figures 1-4 and Tables I-III show changes in leaf nitrate reductase activities at various times after irrigation with various nutrient media. NADH and NADPH-linked activities were very similar under equivalent time, nutrient and cut/uncut cotyledon conditions. Maximum soybean enzyme activities were usually obtained at 48 or 72 hours after irrigation with nutrient media.

Figures 1 and 2 and Table I show that nitrate in the medium greatly increased nitrate reductase activity; NADH-linked activity increased about 5 fold from 0 to 48 hours. Adding glutamine to the nitrate nutrient media did not significantly repress enzyme activities at two days; NADH-linked activities also increased nearly 5 fold from 0 to 48 hours. Glutamine given as a sole nitrogen source did not suppress nitrate reductase activity to any extent; NADH-linked activities actually increased about 3 fold from 0 to 48 hours. The plants that were given no nitrogen in their media showed a 1.5 fold increase in NADH-linked activities from 0 to 48 hours. Initial nitrate reductase activities at time of nutrient media irrigation were approximately 0.75 nmoles NO_2^-/ minute/ mg. soluble protein.

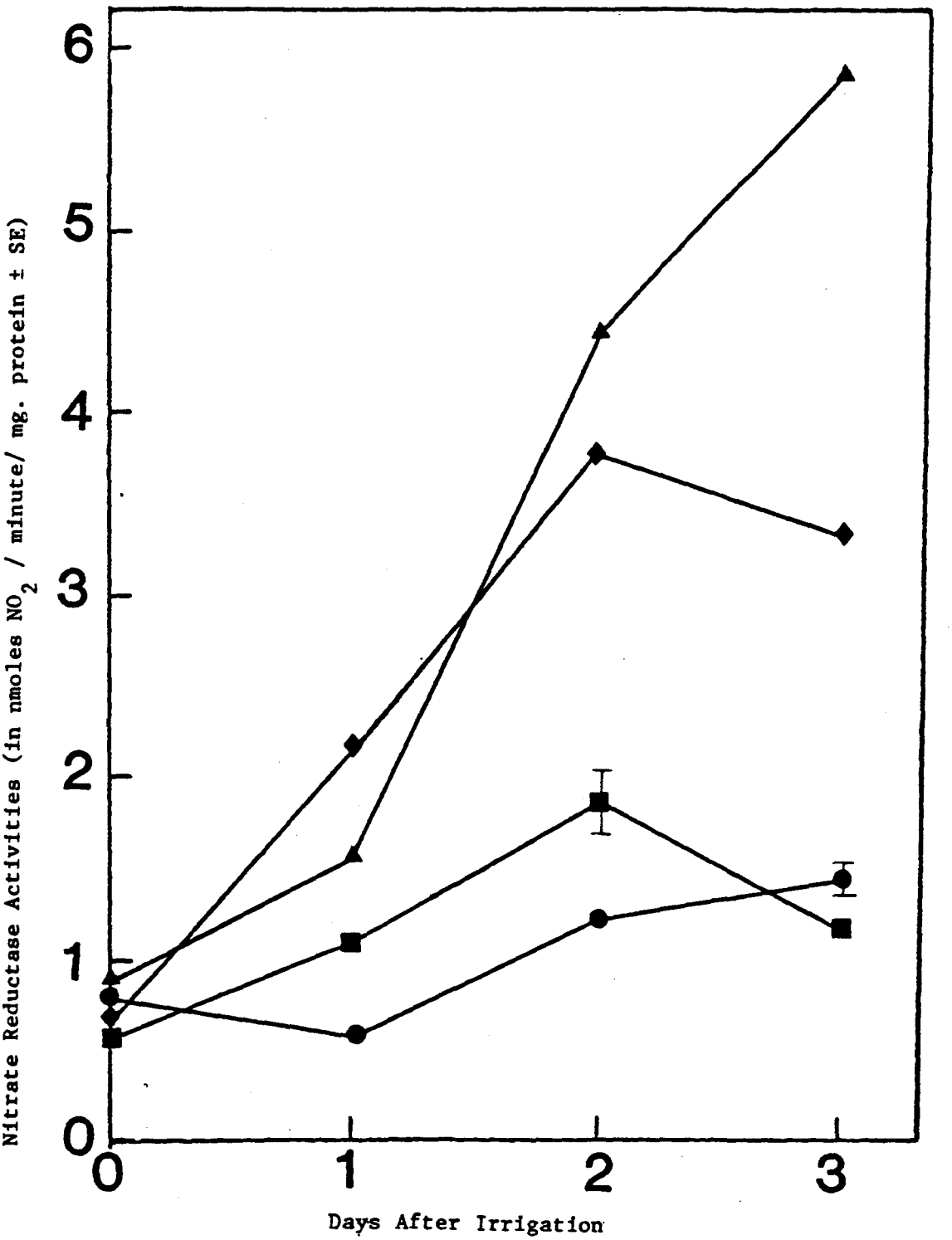

Figure 1. Soybean Primary Leaf NADH-linked Activities: Plants with Intact Cotyledons Irrigated at 10 days. (● =no nitrogen, ▲=KNO_3, ■ =Gln, ◆=KNO_3 + Gln, media as described in Materials and Methods)

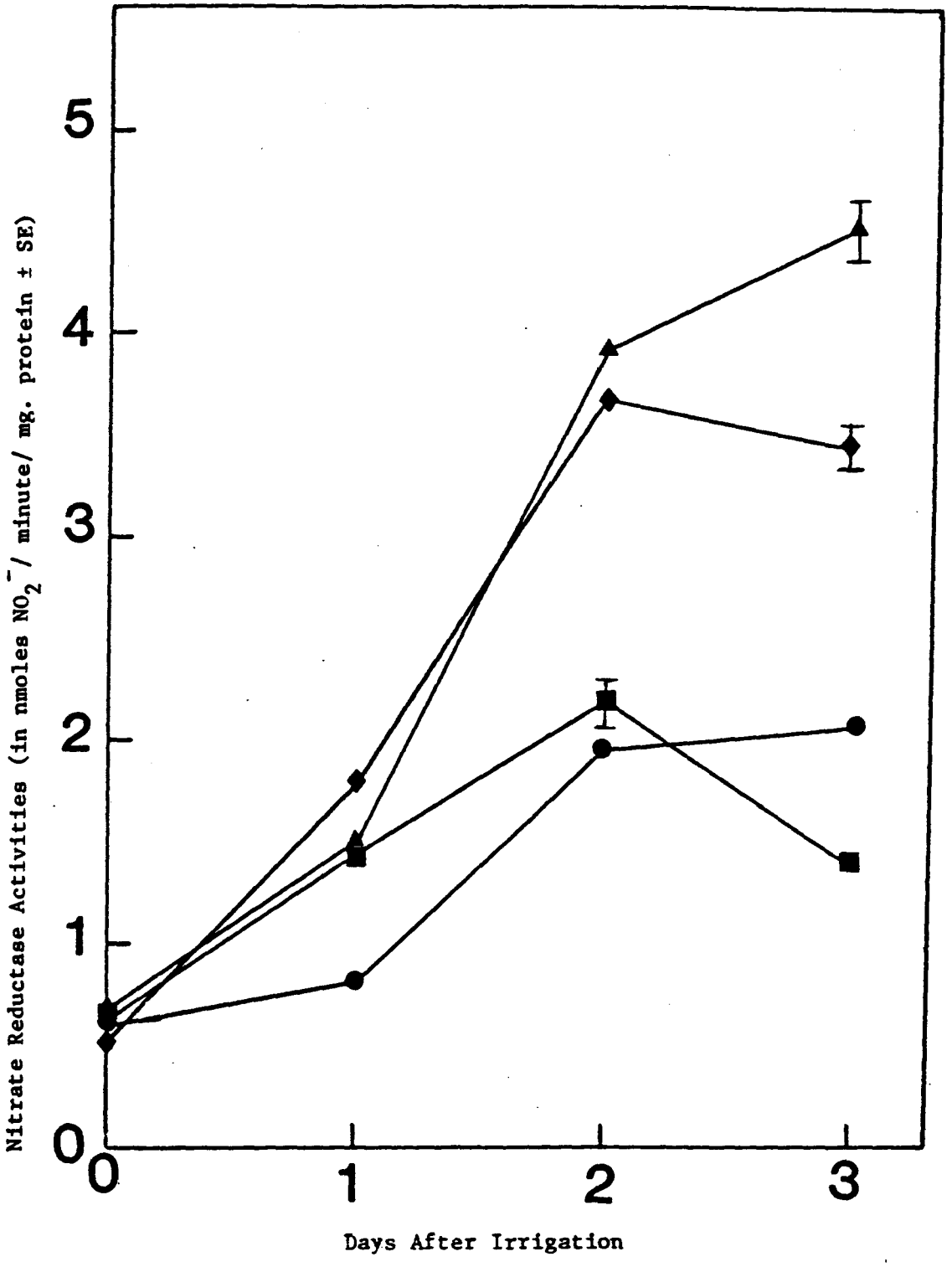

Figure 2. Soybean Primary Leaf NADPH-linked Activities: Plants with Intact Cotyledons Irrigated at 10 days. (● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆ =KNO_3 + Gln, media as described in Materials and Methods)

Table I. Soybean Primary Leaf NADH-linked Nitrate Reductase Activities in plants with intact cotyledons (in nmoles NO_2^-/ minute/ mg protein ± SE)

Media	Time after Irrigation 0 Hours	48 Hours	Fold increase 0 to 48 Hours
No Nitrogen	0.82±0.08	1.22±0.07	1.22
KNO_3 (50 mM)	0.88±0.04	4.36±0.03	4.91
Glutamine (10 mM)	0.57±0.00	1.88±0.21	3.30
KNO_3 (50 mM) + Glutamine (10 mM)	0.65±0.04	3.78±0.00	5.82

Figures 3 and 4 and Table II illustrate changes in nitrate reductase activity in plants that had their cotyledons cut at the time of nutrient media irrigation. Maximum nitrate reductase activity occurred in plants given nitrate, either alone or with glutamine. Glutamine did not suppress nitrate reductase in the presence of nitrate, for the glutamine and nitrate fed plants show higher enzyme activities than the plants given nitrate as a sole nitrogen source. For the plants fed glutamine and nitrate, NADH-linked enzyme activities rose 6.7 fold from 0 hours to 72 hours. Relatively little change in activity was noted for the plants with cut cotyledons that were given no

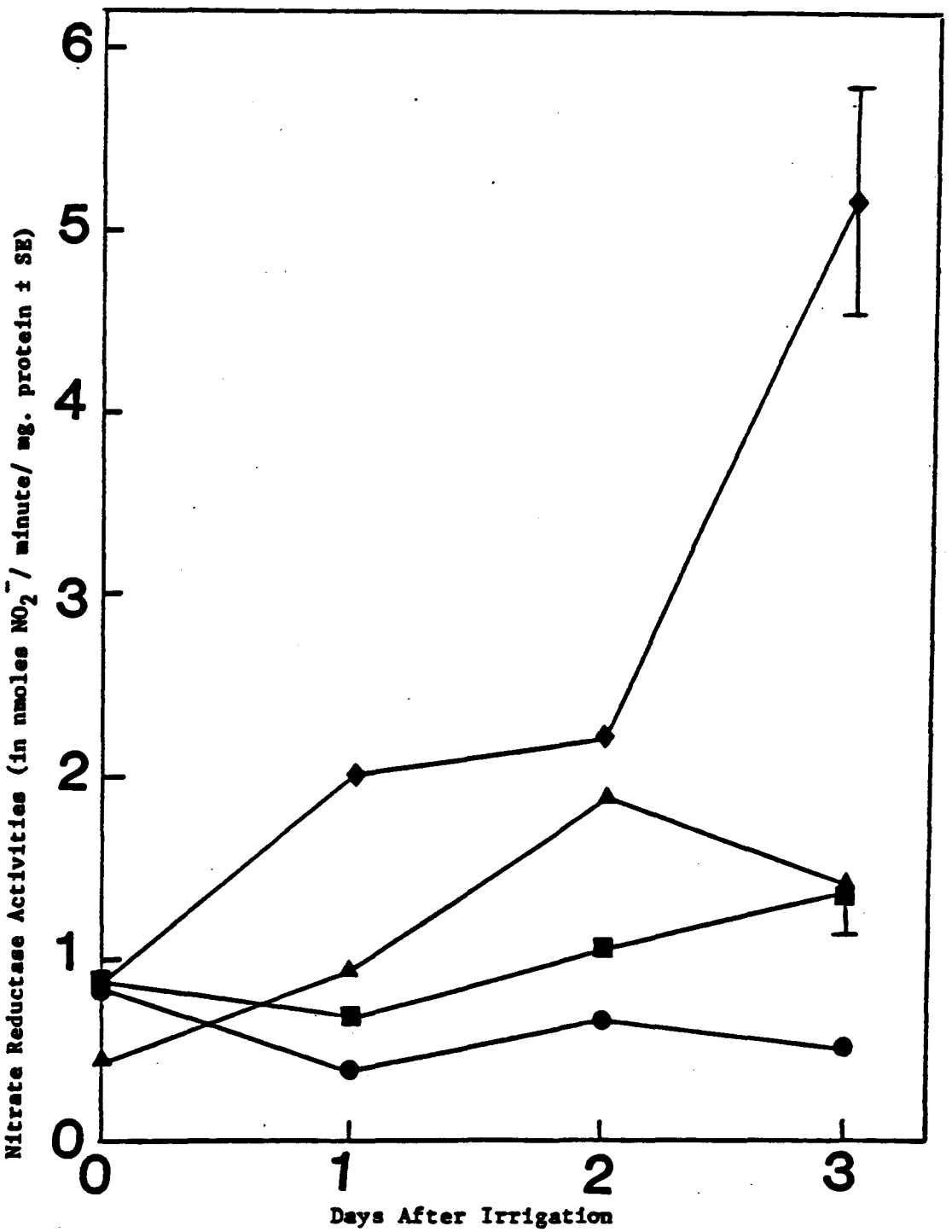

Figure 3, Soybean Primary Leaf NADH-linked Activities: Plants with Cut Cotyledons Irrigated at 10 days. (● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆ =KNO_3 + Gln, as described in Materials and Methods)

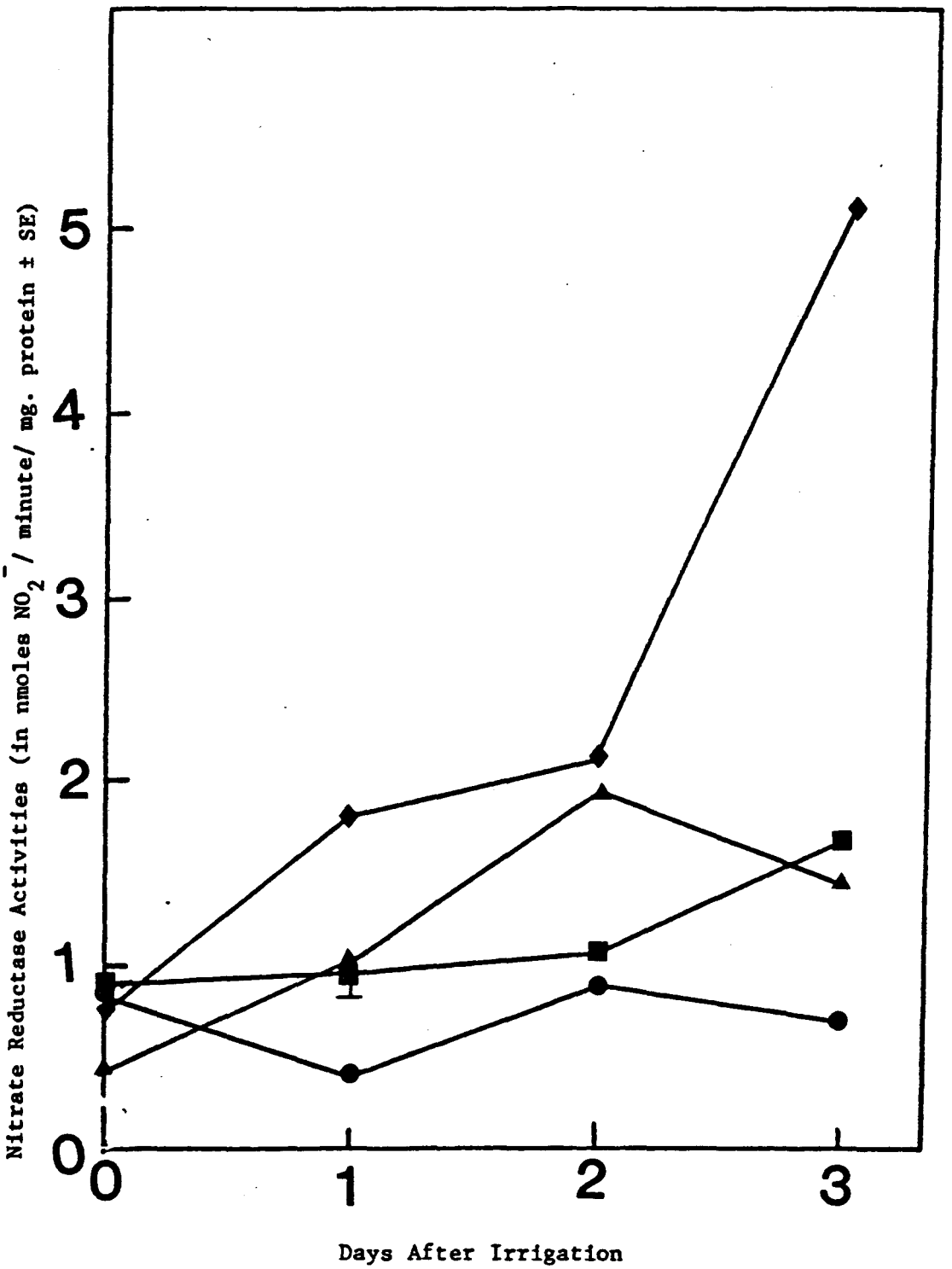

Figure 4. Soybean Primary Leaf NADPH-linked Activities: Plants with Cut Cotyledons Irrigated at 10 days. (● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆ =KNO_3 + Gln, as described in Materials and Methods)

nitrogen or glutamine media; their enzyme activities remained between 0.6 and 1.1 nmoles NO_2^-/ minute/ mg. soluble protein throughout the experiment. Table III demonstrates that nitrate reductase activities at 48 hours for plants with their cotyledons intact were about twice as great as for plants with their cotyledons cut that received similar sources of nitrogen in their nutrient media.

Table II. Soybean Primary Leaf NADH-linked Nitrate Reductase Activities in plants with cut cotyledons.

(in nmoles NO_2^-/ minute/ mg. protein ± SE)

	Time after Irrigation		
Media	0 Hours	48 Hours	Fold increase 0 to 48 Hours
No Nitrogen	0.84±0.03	0.64±0.02	0.81
KNO_3 (50 mM)	0.45±0.03	1.90±0.03	4.22
Glutamine (10 mM)	0.87±0.10	1.04±0.01	1.19
KNO_3 (50 mM) + Glutamine (10 mM)	0.75±0.04	2.20±0.02	2.92

Table III. Comparision of NADH-linked Activities 48 Hours After Irrigation with Nutrient Media: Plants with intact cotyledons vs. plants with cut cotyledons. (activities in nmoles NO_2^-/ minute/ mg. soluble protein ± SE)

Media	Cotyledons Uncut	Cotyledons Cut	Fold increase Uncut vs. Cut
No Nitrogen	1.22±0.07	0.64±0.02	1.91
KNO_3 (50 mM)	4.36±0.03	1.90±0.03	2.29
Glutamine (10 mM)	1.88±0.21	1.04±0.01	1.81
KNO_3 (50 mM) + Glutamine (10 mM)	3.78±0.00	2.20±0.02	1.72

Various ANOVA and Student-Neuman Kuels tests (alpha=0.001) obtained from data from the soybean whole plant experiments (Figures 1-4, Tables I-III) clearly indicate that: 1) nitrate alone or with glutamine significantly increased NADH or NADPH-linked nitrate reductase activities in plants with intact or cut cotyledons, 2) for soybeans with cut cotyledons NADH and NADPH-linked enzyme activities were significantly higher in plants given nitrate and glutamine as opposed to nitrate alone, 3) for soybeans with intact cotyledons NADH and NADPH-linked enzyme activities were statistically similar at most time intervals in plants given nitrate alone as opposed to plants given both nitrate and glutamine; enzyme

activities were significantly higher at 3 days in plants given nitrate alone as opposed to those plants given both nitrate and glutamine, 4) plants with their cotyledons intact had significantly higher activities of both enzymes.

The Relationship between Primary Leaf Nitrate Reductase Activities and Nitrate Levels in Plant Parts

Nitrate comprises over 1% of the dry weight of various soybean seeds (Table IV). The amounts of nitrate stored in various organs of 10-day old soybeans given no supplemental nitrogen are also significant, with nitrate comprising 1.6% to 7.5% of the dry weight of various soybean plant organs (Table V). Table VI notes that NADH and NADPH-linked nitrate reductase levels of approximately 1.0 nmole NO_2^-/ minute/ mg. soluble protein were present 48 hours after the plants were irrigated with no nitrogen or glutamine nutrient media. Forty-eight hours after irrigation with no nitrogen or glutamine nutrient media the plants had nitrate stores comprising 1.2% to 5.2% of the dry weight of their plant organs. The plants that were given nitrate, either alone or with glutamine, showed enhanced levels of both nitrate reductase activities and nitrate levels compared to the plants given no supplemental nitrate. Forty-eight hours after nutrient media irrigation, NADH-linked enzyme activities were 3.2 fold higher and leaf nitrate levels were 2.9 fold higher in plants given nitrate as opposed to plants given no nitrogen. The increases in NADH and NADPH-linked

activities and nitrate levels in the plant parts were statistically significant (alpha=0.001).

Table IV. Nitrate Levels Stored in Seeds

Expressed in terms of weight % NO_3^- ± SE

Cultivar	% NO_3^- Fresh weight	% NO_3^- Dry weight
Williams (Soybean)	1.34±0.05	1.48±0.06
Prize (Soybean)	2.02±0.11	2.24±0.13
4 Health Food Store Varieties of Soybeans	1.50 to 1.69	1.67 to 1.90
Squash (Buttercup)	0.89±0.06	1.01±0.07

Table V. Stored Nitrate in Soybean Plant Organs in 10-day old plants irrigated with distilled water.

Expressed in terms of weight % NO_3^- ± SE

Williams cultivar used

Plant Part	% NO_3^- Fresh weight	% NO_3^- Dry weight
Primary Leaves	1.16±0.07	7.48±0.45
Cotyledons	0.39±0.03	4.47±0.34
Stems	0.17±0.01	1.61±0.12
Roots	0.14±0.02	2.00±0.27

Table VI. Nitrate Reductase Activities and Nitrate Levels in Intact Soybeans supplied with Nutrient Media at 10 days.

Media	Primary Leaf Nitrate Reductase Activities (nmoles NO_2^-/ min./ mg. protein)		NO_3^- levels Expressed as weight % NO_3^- per dry weight of plant part listed below			
	NADH-linked	NADPH-linked	Primary Leaves	Cotyledons	Stems	Roots
0 Hours After Irrigation with Media						
No Nitrogen	0.55	0.46	7.00	3.53	1.61	2.13
KNO_3 (50 mM)	0.74	0.74	6.67	4.78	1.61	1.99
Gln (10 mM)	0.52	0.85	7.97	4.78	1.89	2.56
KNO_3 (50 mM)+ Gln (10 mM)	0.70	0.86	8.61	4.89	1.13	1.99
48 Hours After Irrigation with Media						
No Nitrogen	0.85	0.88	4.86	2.62	1.23	1.28
KNO_3 (50 mM)	3.20	2.70	14.2	4.90	5.59	9.37
Gln (10 mM)	1.04	1.17	5.18	3.30	1.89	1.70
KNO_3 (50 mM)+ Gln (10 mM)	2.16	2.51	10.0	5.92	4.64	6.11

Studies of the Regulation of the Three Different Soybean Nitrate Reductase Activities

Table VII notes that all three enzyme activities are fairly similar in terms of levels of specific enzyme activity among the plants given nitrate either alone or with glutamine. However, enzyme activities of the NADH-linked enzyme at pH 7.5 were less than one-third of the other two enzymes in the plants given no nitrogen or glutamine media. The nitrate media increases NADH-linked pH 7.5 enzyme activities about 10 fold relative to the no nitrogen media, although nitrate in the media increased activities only about 4 fold for the other two enzymes.

Table VII. Comparison of 3 Nitrate Reductase Activities in Soybean Primary Leaves (activities in nmoles NO_2^-/ minute/ mg. protein ± SE)

12 day old plants- irrigated 48 hours earlier with nutrient media.

Type of Nitrate Reductase Activity	Media	Nitrate Reductase Activity	Fold Increase Relative to No Nitrogen Plants
NADH-linked	No Nitrogen	0.78±0.03	1.00
pH 6.5	KNO_3 (50 mM)	2.97±0.04	3.81
80 mM KNO_3	Gln (10 mM)	0.87±0.02	1.12
in assay	KNO_3(50 mM)+ Gln (10 mM)	1.59±0.04	2.05
NADPH-linked	No Nitrogen	0.67±0.03	1.00
pH 6.5	KNO_3 (50 mM)	2.75±0.11	4.10
80 mM KNO_3	Gln (10 mM)	0.78±0.08	1.16
in assay	KNO_3(50 mM)+ Gln (10 mM)	1.68±0.08	2.49
NADH-linked	No Nitrogen	0.22±0.01	1.00
pH 7.5	KNO_3 (50 mM)	2.30±0.09	10.45
10 mM KNO_3	Gln (10 mM)	0.13±0.06	0.59
in assay	KNO_3(50 mM)+ Gln (10 mM)	1.14±0.01	5.20

Regulation of Nitrate Reductase in Squash Cotyledons

Nitrate in the media greatly stimulated nitrate reductase activities in squash cotyledons (Figure 5). Specific NADH-linked nitrate reductase activities (NADH-linked at pH 7.5) increased about 27 fold from 0 to 24 hours in the plants given nitrate as a sole nitrogen source. Glutamine in the presence of nitrate inhibited squash enzyme activities somewhat, activities increasing only about 10 fold in the first 24 hours after induction for the glutamine and nitrate fed plants. Enzyme activities below 0.25 nmoles NO_2^-/ minute/ mg. soluble protein were recorded in the cotyledons at the time of nutrient media irrigation. For the plants given no nitrogen or glutamine media, enzyme activities decreased more than 10 fold from 0 to 48 hours after irrigation.

Nitrate Reductase Regulation and Nitrate Levels in Cultured Soybean Cells

The callus "induction" assay (Figures 6, 7) showed that nitrate in the presence of reduced nitrogen stimulates NADH-linked nitrate reductase activities. Maximum activity was noted from 1 to 3 days (data statistically significant at alpha=0.001). Callus cells fed nitrate as a sole nitrogen source showed an increase in activity at day 1 but this activity quickly declined over time to levels below 0.2 nmoles NO_2^-/ minute/ mg. soluble protein. The cells fed nitrate as a sole nitrogen source failed to grow very

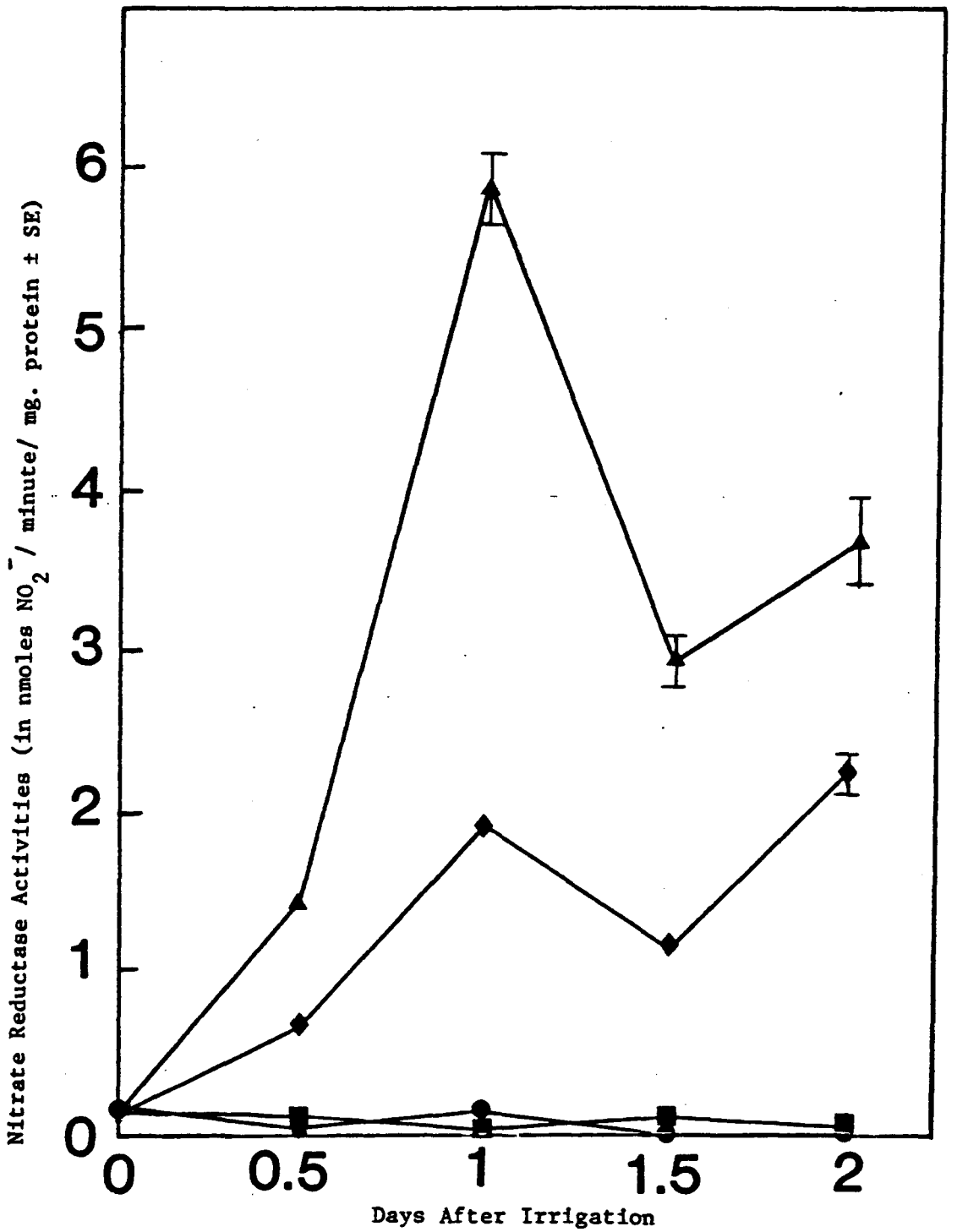

Figure 5. Squash Cotyledon NADH-linked Activities: Plants Irrigated at 7 days.
(● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆ =KNO_3 + Gln, as described in Materials and Methods)

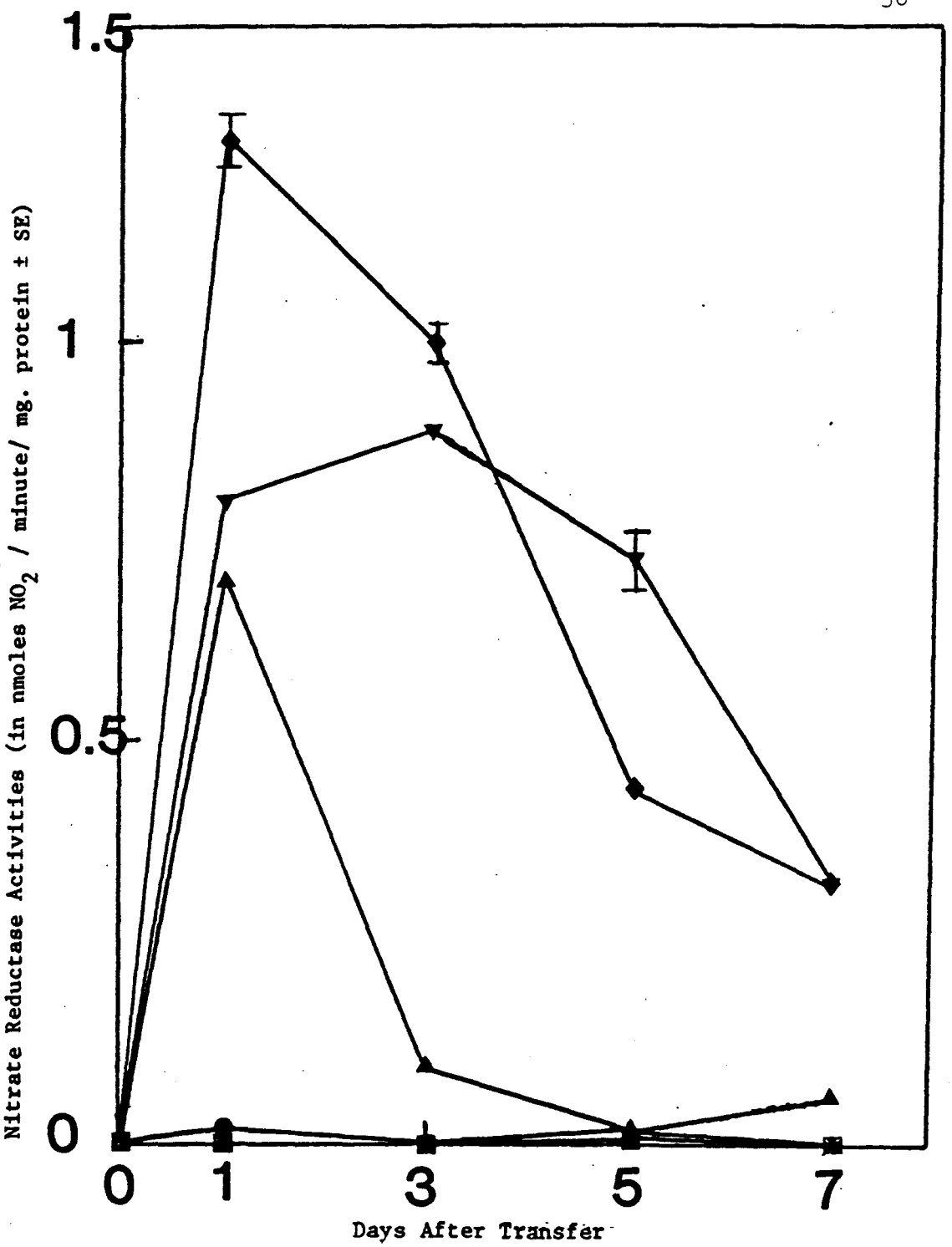

Figure 6. NADH-linked Activities at pH 6.5 in Soybean Callus: Cells Initially Grown on 10 mM Glutamine as a sole nitrogen source. (●+=no nitrogen, ▲ =KNO_3, ■=Gln, ◆ =KNO_3 + Gln, ▼ =KNO_3+NH_4 , media as described in Materials and Methods)

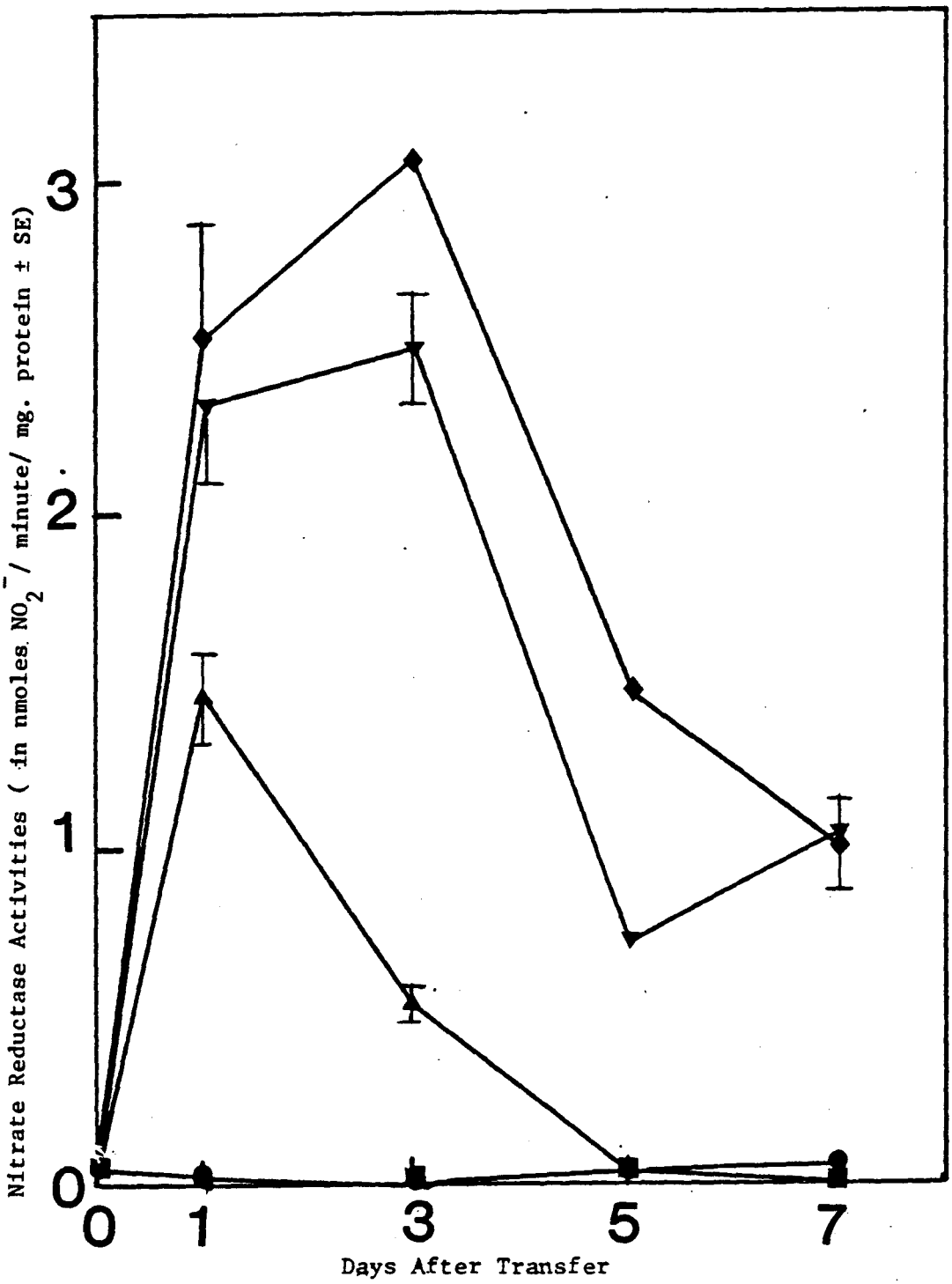

Figure 7. NADH-linked Activities at pH 7.5 in Soybean Callus: Cells Initially Grown on 10 mM Glutamine as a sole nitrogen source. (●=no nitrogen, ▲=KNO_3, ■=Gln, ◆=KNO_3 + Gln, ▼ =KNO_3+NH_4^+, media as described in Materials and Methods)

rapidly and died within 2 weeks. The cells fed no nitrogen also grew poorly and died within 2 weeks. However, the cells given glutamine or nitrate along with a reduced nitrogen source were healthy and increased in mass at least 4.5 fold over the two week period of the experiment (data not shown). The cells fed no nitrogen or glutamine had nitrate reductase activities below 0.1 nmoles NO_2^-/ minute/ mg. soluble protein (Figures 6, 7), whereas in the intact plants nitrate reductase activities as high as 0.75 nmoles NO_2^-/ minute/ ml. protein or more were noted even when no nitrate was present in the media (Figures 1-4). The callus repression assay (Figures 8, 9) shows that NADH-linked nitrate reductase activities declined the least in the cells given nitrate along with glutamine.

Glutamine grown callus cells accumulated nitrate stores comprising 0.099 to 0.147% of fresh weight after they were placed on media containing nitrate with or without a reduced source of nitrogen (Table VIII). Cells that were placed on the glutamine media had only 0.004% nitrate on a fresh weight basis.

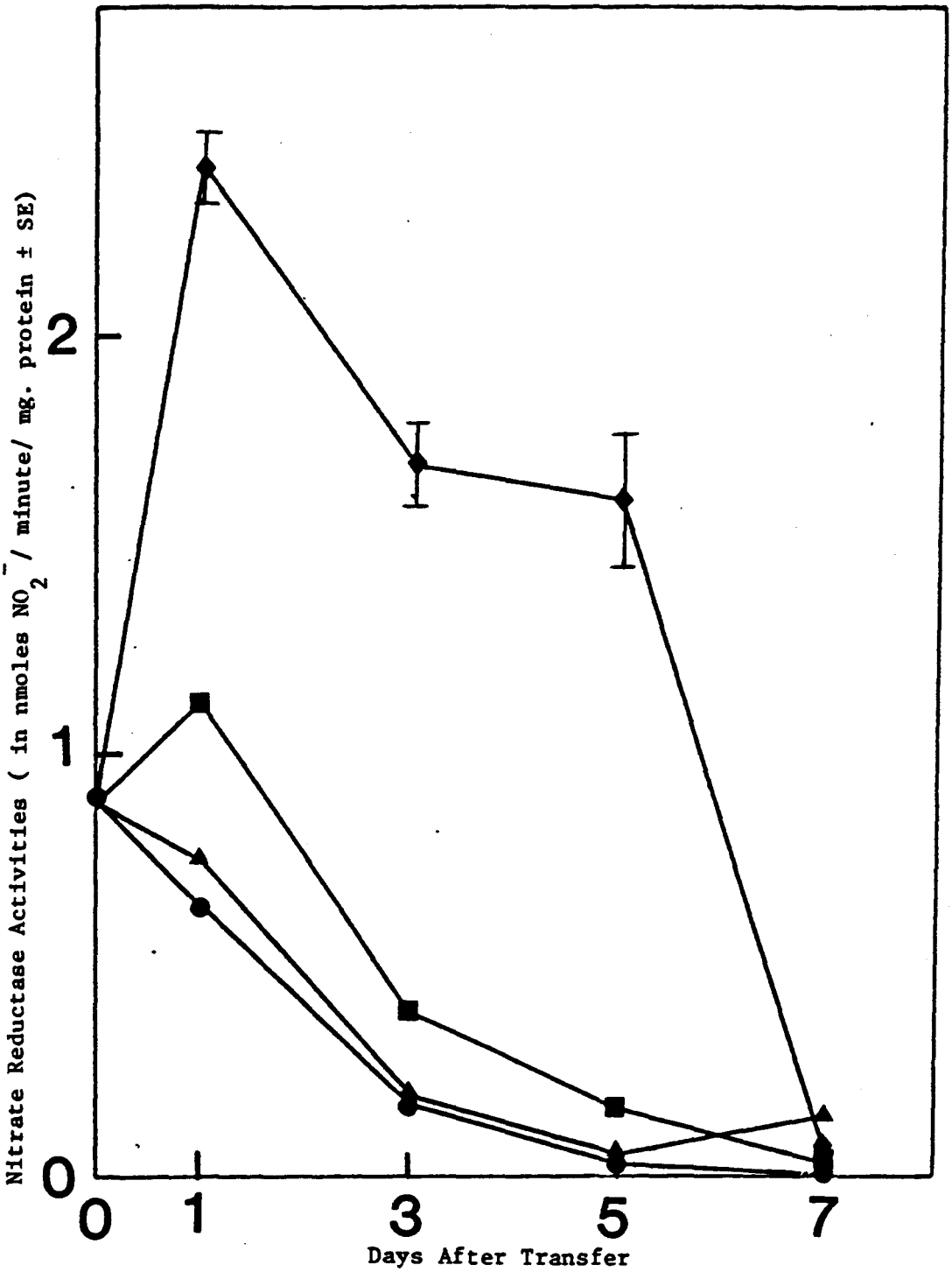

Figure 8. NADH-linked Activities at pH 6.5 in Soybean Callus: Cells initially Grown on 25 mM KNO_3 and 2 mM NH_4^+ as Nitrogen Sources. (● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆ =KNO_3 + Gln, media as described in Materials and Methods)

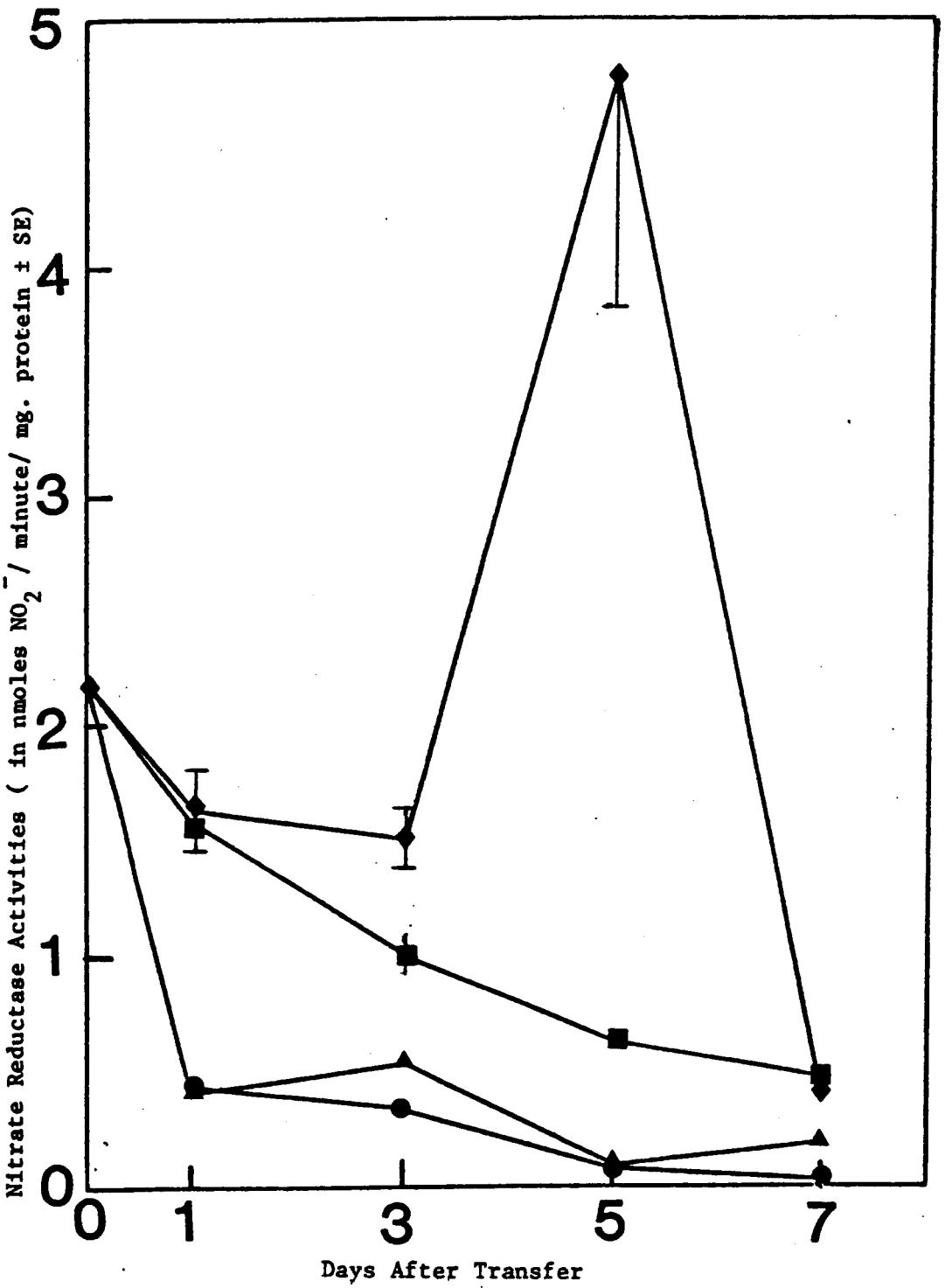

Figure 9. NADH-linked Activities at pH 7.5 in Soybean Callus: Cells Initially Grown on 25 mM KNO_3 and 2 mM NH_4^+ as Nitrogen Sources. (● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆=KNO_3 + Gln, media as described in Materials and Methods)

Table VIII. Nitrate Levels in Soybean Callus

Callus initially grown on 10 mM Gln for 6 subcultures
Callus placed on nutrient media containing different sources of nitrogen 72 hours before nitrate assay.

Media	% NO_3^- (fresh weight) ± SE
KNO_3 (25 mM)	0.099±0.015
Gln (10 mM)	0.004±0.004
KNO_3 (25 mM) + Gln (10 mM)	0.107±0.002
KNO_3 (25 mM) + NH_4^+ (2 mM)	0.147±0.008

Results from the suspension culture "induction" experiment were quantitatively and qualitatively very similar to that of the callus assay (Figures 10, 11). Nitrate in the presence of a reduced nitrogen source such as glutamine or ammonia stimulated NADH-linked nitrate reductase activities (data were statistically significant at alpha=0.001). Enzyme activities reached a peak of about 1.0 nmole NO_2^-/ minute/ mg. protein at 3 days in the cells given nitrate along with ammonia or glutamine. Nitrate reductase activities below 0.25 nmoles NO_2^-/ minute/ mg. soluble protein were noted in the cells fed glutamine or no nitrogen. A moderate and temporary increase of pH 7.5 NADH-linked activities was noted in soybean cells fed glutamine as a sole nitrogen source. Little growth was noted within the no nitrogen or nitrate only fed cells. The

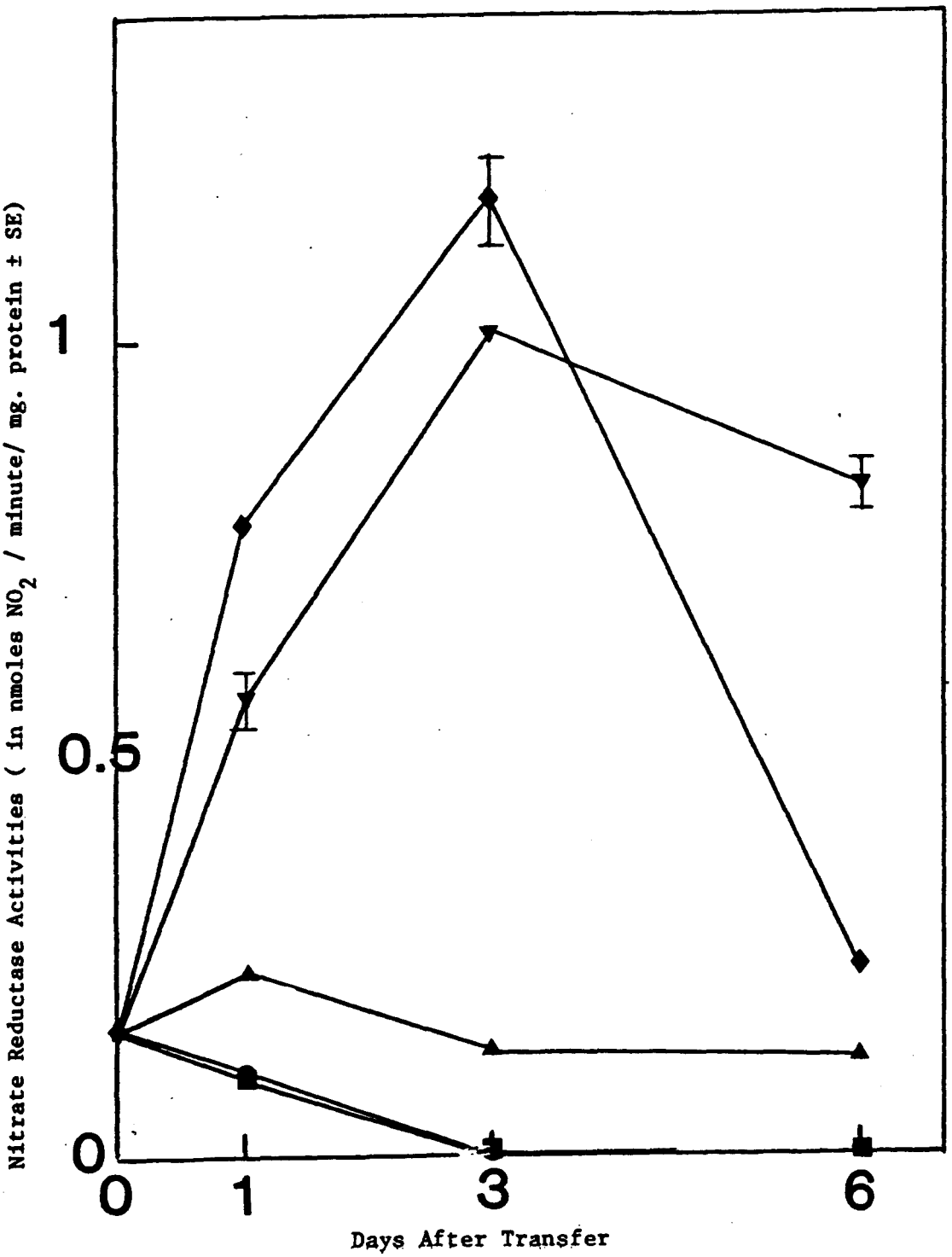

Figure 10, NADH-linked Activities at pH 6.5 in Soybean Suspension Cells: Cells Initially Grown on 10 mM Glutamine as a sole nitrogen source. (● =no nitrogen, ▲ =KNO_3, ■ =Gln, ◆ =KNO_3+Gln, ▼ =KNO_3 + NH_4^+, media as described in Materials and Methods)

glutamine, glutamine and nitrate, and ammonia and nitrate fed cells all grew at least 2.4 fold in 6 days as measured by their packed cell volumes.

No measureable NADPH-linked nitrate reductase activities were noted for soybean callus or suspension cells, although NADH and NADPH-linked activities were approximately equal for the whole plants (Figures 1-4). The NADH-linked activity levels show similar responses to nutrients in the media for the callus (Figures 6, 7) and suspension (Figures 10, 11) cultures.

DISCUSSION

Nitrate Reductase Regulation in Intact Plants and Cultured Cells

Figures 1-4 indicate that nitrate in the media greatly enhances soybean nitrate reductase activities. Squash nitrate reductase activities were also enhanced by exogenous nitrate (Figure 5). This nitrate stimulation of nitrate reductase is consistent with other higher plant studies (5,6,12,18,20), and with studies done with fungi (14-17). Glutamine in the presence of nitrate did not significantly inhibit soybean nitrate reductase activities. The activities in the plants with cut cotyledons (Figures 2 and 4) were generally higher with both nitrate and glutamine present in the nutrient media than with glutamine alone. The plants given nitrogen free nutrient media still showed considerable nitrate reductase levels. Glutamine as a sole nitrogen source actually stimulated nitrate reductase activity; NADH-linked activities increased more than 3-fold from 0 hours to 48 hours after nutrient media irrigation (Table I). The reduced nitrogen and/or carbon skeletons provided by glutamine in the media may stimulate synthesis of nitrate reductase and other enzymes. In this study, glutamine appears to play a much different role in the regulation of nitrate reductase in fungi. Dunn-Coleman and coworkers (14) found that Neurospora crassa given 5 mM

glutamine and 5 mM nitrate had only 5% of the nitrate reductase activity of those provided with 10 mM nitrate. In squash, glutamine in the presence of nitrate inhibited nitrate reductase activities to a much greater extent than in soybeans but to a much lesser extent than in fungi (Figure 5).

Table VII and Figure 5 indicate that the pH 7.5 NADH-linked enzyme is present in relatively low amounts in squash cotyledons and soybean primary leaves given no supplemental nitrate. The pH 7.5 NADH-linked activities increased 10-fold in soybean primary leaves and 27-fold in squash cotyledons by the addition of nitrate. The NADH-linked pH 7.5 enzymes in soybeans and most higher plants seem to be similar in that their "constitutive" activities in the absence of added nitrate are very low, but these activities can be greatly enhanced by added nitrate in the media.

In cultured cells grown initially on glutamine as a sole nitrogen source maximum NADH-linked nitrate reductase levels were obtained in cells given both nitrate and a reduced nitrogen source such as ammonia or glutamine (Figures 6, 7, 10, 11). In cells initially grown on B5 media containing 25 mM nitrate and 2mM ammonia, enzyme activities declined the least in cells given both nitrate and glutamine (Figures 8, 9). These data are consistent with other studies which found that soybean cells cannot grow well or produce high levels of nitrate reductase when

given nitrate as a sole nitrogen source (28,32). In the callus cells given nitrate as a sole nitrogen source (Figures 6 and 7), nitrate reductase activities were high at day 1 but quickly declined. The nitrate fed callus cells probably produced high levels of nitrate reductase at day 1 since these cells had been grown on 10 mM glutamine and still had considerable stores of reduced nitrogen. After day 1 these cells, which were given nitrate as a sole nitrogen source, exhausted their stores of reduced nitrogen and could not grow or produce high levels of nitrate reductase. Low nitrate reductase activities were also noted in the cells given no nitrogen or glutamine as a sole nitrogen source. Hence, it can be concluded that both nitrate and a reduced nitrogen source are required for maximal levels of nitrate reductase activities in cultured soybean cells.

Glutamine fed cells experienced similar increases in NADH-linked activities when placed on media containing 25 mM nitrate along with either 2 mM ammonia or 10 mM glutamine (Figures 6, 7, 10, 11). Nelson and coworkers (30) utilized soybean cotyledon suspension cultures initially grown on 10 mM glutamine media and found that 10 mM added glutamine to media containing 25 mM nitrate and 2 mM ammonia did not reduce nitrate reductase activities. These findings suggest that either 10mM glutamine or 2 mM ammonia were good sources of reduced nitrogen to supplement media containing 25 mM nitrate, and they also indicated that neither glutamine or

nitrate seem to significantly inhibit nitrate reductase activity. However, Oaks (29) found that soybean suspension cultures initially grown on 20 mM glutamine or 20 mM ammonium citrate developed no nitrate reductase activity when subcultured onto media containing both 25 mM nitrate and either 10 mM glutamine or 10 mM ammonium citrate. Perhaps the very high concentrations of reduced nitrogen given these cells suppressed the development of nitrate reductase activity.

Increases in nitrate reductase activities in nitrate fed cells were qualitatively and quantitatively similar (Figures 6, 7, 10, 11) for both the callus cultures and the suspension cultures, suggesting that nitrate reductase regulation is similar in both callus and suspension cultures. NADH-linked activities for callus cells given both nitrate and reduced ammonia in the "induction" experiment (Figures 6, 7) were about twice as great at pH 7.5 than at pH 6.5. These data indicate that in callus cultures the pH 7.5 NADH-linked enzyme activities may be more "inducible" by nitrate in the media than the pH 6.5 NADH-linked enzyme (Figures 6, 7). These data are consistent with the data obtained in whole plants (Table VII). Additional work with suspension cultures is needed to determine if the NADH-linked nitrate reductase activities are higher at pH 7.5 than at pH 6.5.

The absence of NADPH-linked activity in cultured soybean leaf cells in this experiment and in the soybean

cotyledon cells used by Nelson and coworkers (30) suggests that the bispecific NADH or NADPH-linked enzyme is under some form of developmental control and is produced only in intact plants and not in cultured cells. Soybean urease, another critical enzyme for soybean nitrogen metabolism, is found in two forms in intact plants but only in one form in cultured cells (69).

Relationships between Nitrate Concentrations and Nitrate Reductase Activities in Whole Plants and Cultured Cells

The high levels of nitrate stored in soybean seeds and in plant parts of 10 day old soybeans (Tables IV & V) suggest that this endogenous nitrate may play a role in "self-inducing" nitrate reductase activities. The higher nitrate reductase levels in soybeans with intact cotyledons (Table III) also suggest that nitrate or other stored forms of nitrogen in the cotyledons may play a critical role in stimulating nitrate reductase activity. Relatively high nitrate and enzyme levels are found in young soybeans given no exogenous nitrate and even higher enzyme and nitrate levels are found in soybeans given exogenous nitrate (Table VI). These data suggest that the large amount of nitrate stored in soybeans may "self-induce" nitrate reductase activities and that additional nitrate in the media may further enhance enzyme activities above this "self-induced" level. Perhaps the endogenous nitrate in the soybean

may be able to stimulate activity of the "constitutive" enzyme described by numerous sources (16,21,44,45).

The very low levels of nitrate reductase activity (Figures 6, 7) and stored nitrate (Table VIII) in callus cells grown on glutamine as a sole nitrogen source suggest that these cells have little or no nitrate stores in which to "self-induce" nitrate reductase levels (Table VI). These data indicate that the constitutive enzyme activity in glutamine-fed cultured soybean cells may be very low. Nelson et al. (30) also reported no constitutive nitrate reductase in soybean cotyledon suspension cells.

General Conclusions

Additional understanding of nitrate reductase regulation in higher plants could lead to the development of better plants capable of more efficient utilization of soil nitrates. Some studies relating nitrate reductase levels and crop yields have been made, but the correlations have not been high enough to elicit much response from plant breeders (5,11,13,70). Croy and Hageman (13) found that different levels of nitrate reductase in corn cultivars accounted for only 35% of the variation in yield. Table VI indicates that nitrate reductase activities increased as the amounts of stored nitrate in the plant parts increased. Other studies (9,57,58) have suggested that increases in nitrate levels in plant parts or increases in nitrate flux in the leaves may serve to enhance nitrate reductase

activities. Further understanding of the relationship between nitrate flux and stored nitrate on nitrate reductase activities could lead to improvements in nitrate uptake in field grown plants.

Some studies of seasonal patterns of nitrate reductase and other enzymes relating to nitrogen metabolism have been conducted in higher plants such as soybeans (9,56). Further understanding of nitrate reductase regulation in higher plants may enable researchers to develop improved fertilization or other cultural practices that may increase yields in field grown plants. Neyra et al. (60) noted that seasonal patterns of nitrogenase levels declined after flowering in Phaseolous vulgaris, while nitrate reductase activities increased just after flowering. Application of 40 kg NO_3^-/ha during the flowering period significantly increased nitrate reductase activities and bean yields almost doubled.

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Approval Sheet

The thesis submitted by Luke Theodore Curtis has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted as partial fulfillment of the requirements for the degree of Master of Science.

7/24/85
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