THE ROLE OF PHOTOSYNTHESIS AND MINERAL NUTRITION IN NITRATE REDUCTASE IN SOYBEANS

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INTRODUCTION

It has been reported that the process of nitrate reduction is coupled with a photochemical reaction and CO_2 assimilation taking place in the aerial portion of plants[4,10,14,15]. It has also been reported that those enzymes, i. e. nitrate reductase, nitrite reductase, and dehydrogenases, needed for nitrate reduction are localizied within the chloroplast[3,20]. Thus nitrate reduction is closely associated with photosynthesis, at least in some tissues.

In contrast to this, some investigators[8,9,23] have concluded that plant roots, lacking chloroplasts and devoid of illumination, and so unable to carry out photochemical reactions and CO_2 assimilation, still have nitrate reductase activity. Therefore, photosynthesis does not seem to be a necessary requirement for nitrate reduction.

The object of our present studies, in addition to looking into the above two contradictory theories, is to further study; (1). distribution of nitrate reductase in plants, (2). change of nitrate reductase activity in plant life, (3). the effect of malnutrition on nitrate reductase activity, and (4). the effect of the N-source on the nitrate reductase activity.

MATERIALS AND METHODS

1. Plant Culture.

Soybean (*Glycine max*) seeds were soaked for two hours prior to planting in trays of vermiculite well irrigated with nutrient solution. The nutrient solution used for daily irrigation was Haogland's solution, the pH value was adjusted to 7.5 with 0.01 N HCl. Some of the seedlings were grown in the dark, others in the greenhouse until 12 days old and still others until 24 days old. The plant organs (leaves, cotyledons, stems, and roots) were harvested separately.

2. Extraction and Purification of Enzymes.

In order to prepare a chlorophyll free preparation and one that had a minimum of gum— and resin-like materials, we followed the procedure as described by Colowick[7] for making an acetone powder. The dried acetone powder was extracted with ten volumes of cold phosphate buffer (0.2 M, pH 7.5). The crude extracts were purified by a combined technic of calcium phosphate gel adsorption and ammonium sulphate precipitation as described by Evans and Nason[10].

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3. Preparation and Assay of Nitrate Reductase Activity.

The assay systems were prepared as indicated in the accompanying table:

Reaction Mixture	Experimental Thunberg tube A	Control Thunberg tube B
KNO_3, 10^{-2} M	1.00 ml	1.00 ml
Enzyme extract	0.25 ml	—
NADPH, 1.5×10^{-4} M	0.25 ml	0.25 ml
Phosphate buffer	1.25 ml	1.50 ml
FMN, 10×10^{-3} M	0.25 ml	0.25 ml
Final volume	3.00 ml	3.00 ml

* NADP reduced chemically by $Na_2S_2O_4$ (7)

The reaction mixture was incubated for 30 minutes, then the reaction was stopped by adding 2 ml of Uranyl acetate reagent. This was centrifuged and the precipitate discarded. To the supernatant fraction was immediately added 1 ml of N-(naphythyl) ethylenediamine solution (0.02%) to develop the color of NO_2^-. Then after 15 minutes the optical density was determined with a Spectronic 20 at 540 mμ. The optical density of the control was used for correction.

4. Definition of Enzyme Unit and Specific Activity.

One unit of nitrate reductase is defined as that amount of enzyme resulting in the formation of mμ moles of nitrite. Specific activity is expressed as the units of enzyme per milligram of protein. The protein content of the extract was determined by digesting the 10 percent trichloroacetic acid precipitable materials followed by micro-kjeldahl methed.

5. Isolation of Chloroplast.

Twelve day old soybean leaves were harvested from greenhouse plants. The leaves were ground with twice their weight of Tris buffer (0.25 M, pH 7.5) in a mortar and pestle; the homogenate was strained through four layers of cheesecloth, then the filtrate was subjected to sucrose density gradient centrifugation. The purified chloroplast pellets remained in the region of 1.3 M sucrose.

RESULTS AND DISCUSSION

1. Distribution and Change of Nitrate Reductase Activity.

The nitrate reductase activity varied with different plant tissues. The enzymes extracted from young leaves of 12 day old green plants had greater nitrate reductase activity than that from any other plant tissues. Generally, the nitrate reductase activity in green plants decreased in the following order: Young leaves (100%)>root (91%)>cotyledon (27.5%)>stem (14.8%). Rapidly metabolizing and growing cells are active in nitrogen metabolism. In contrast to green plants, the root portion of

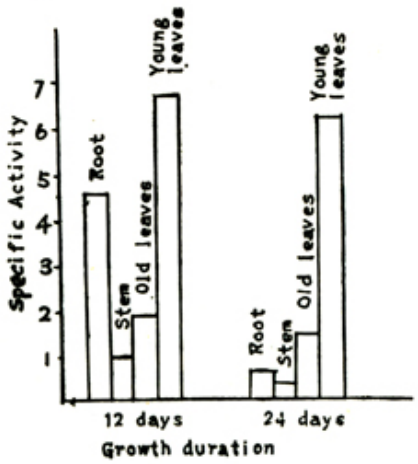
etiolated plants had higher nitrate reductase activity than that of young leaves (Table I). However, the nitrate reductase activity in each portion of a plant decreased with aging. The longer a plant grew, the less nitrate reductase activity it exhibited (Tale II).

Table I. Distribution of Nitrate Reductase in Plants

Plant tissues	Enzyme*			
	Protein, mg/3 ml		Specific activity	
	in light	in dark	in light	in dark
Young leaves	23.86	18.12	6.9	3.3
Cotyledon	21.35	14.43	1.9	0.28
Stem	16.32	12.93	1.0	0.40
Root	16.78	12.04	4.9	4.50

* The enzyme extracts were fraction III.

Table II. Change of nitrate reductase activity in plant lite.



2. Effect of Illumination on Nitrate Reductase Activity.

If potted plants grown in a greenhouse were moved back to the dark, the nitrate reductase activity decreased sharply. For examples, plants grown in the darkness for 24 hours showed their nitrate reductase was 46.4% of the control, and after 48 hours it was 20% of the control, and after more than 80 hours the nitrate reductase activity could hardly be assayed. If plants that had been kept in darkness for 48 hours were moved back again into the light, the nitrate reductase activity was restored within a few hours (Figure I).

3. Effect of Chloroplast and NAD^+ (or $NADP^+$) on the Nitrate Reductase Activity.

In the light, if the reaction mixture in addition to enzyme extracts and nitrate,

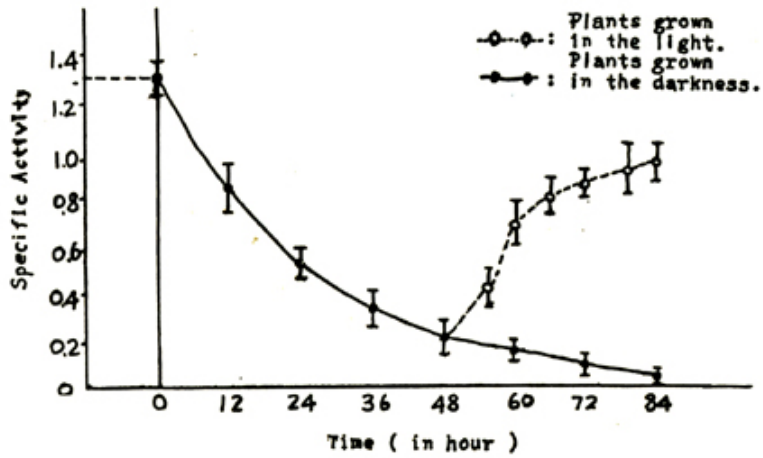


Figure I. Effect of illumination on nitrate reductase activity.

containing both chloroplasts and NAD^+ (or $NADP^+$) are incubated in a water bath for 30 minutes at 31°C, greater amounts of nitrites are formed. But if either chloroplast or NAD^+ (or $NADP^+$) are omitted from the reaction mixture, only 3.3% to 10% of the nitrite is formed as compared with that of the control. In the dark, the control which lacked chloroplast and NAD^+ (or $NADP^+$) and the sample being investigated which contained both chloroplast and NAD^+ (or $NADP^+$) showed very little nitrite production. This means that nitrate reductase activity in the dark is not so much as that in the light (Figure II). From the above observations we know that; (a). Light plays an important role in the process of nitrate reduction in green leaves. (b). Chloroplast and NAD^+ (or $NADP^+$) accompanied with illumination are essential requirements for nitrate reduction. Since illumination caused chloroplasts to carry out a photochemical reaction, and NAD^+ (or $NADP^+$) was photochemically reduced to NADH (or NADPH) in the process of the photochemical reaction. It may be concluded that one of the correlated functions of illumination, chloroplasts and NAD^+ (or $NADP^+$) is to produce electron donors NADH (or NADPH) for nitrate reduction. Therefore, if chemically reduced NADH (or NADPH) insted of NAD^+ (or $NADP^+$) is added to the enzyme reaction mixtures, the nitrate reductase activity even in the dark shows up vigorously.

4. Effect of Carbohyrate on the Nitrate Reductase Activity.

Enzymes extracted from the etiolated plants grown in a nutrient solution containing nitrate showed higher nitrate reductase activity, if sucrose was present in the nutrient solution. Enzymes extracted from green plants did not show increasing nitrate reductase activity when sucrose was added (Table III). If etiolated plants were grown for a few days in a nutrient solution containing nitrate but lacking sucrose, larger amounts of the nitrate ion were found to have accumulated in the root. Prolonged growth up a 14 or 21 days, showed the etiolated plant leaves to have

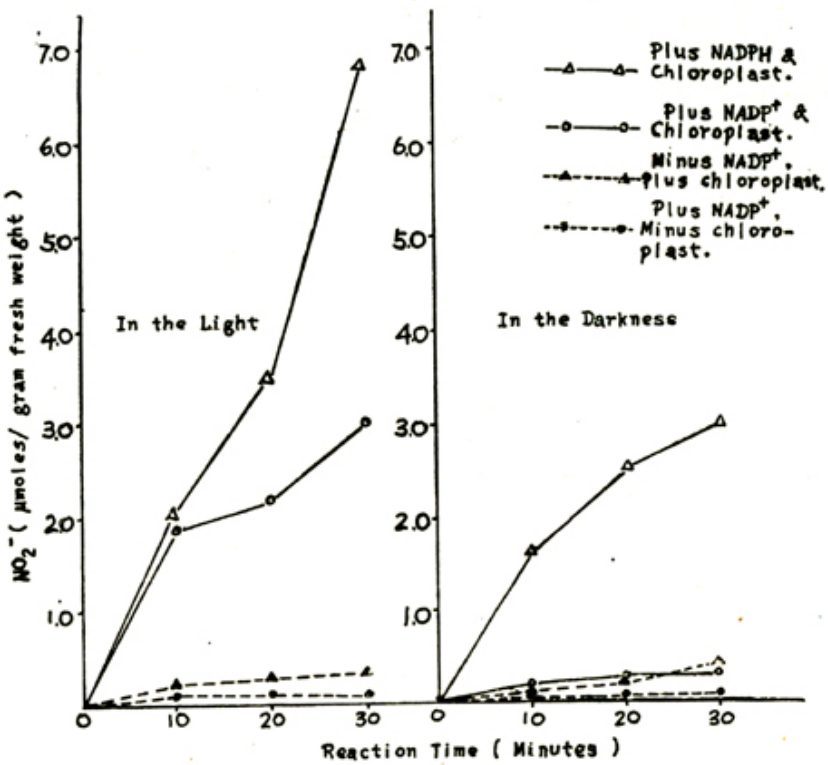


Figure II. The correlating effect of chloroplast, NADP+, and illumination on the nitrate reductase activity.

accumulated a large amount of NH_4^+ but no NO_3^-, and these made the leaves become brown to dark brown in color, and they finally withered, and looked like they had been burned. These studies indicate that: (a). Nitrate absorbed by the root were reduced to NH_4^+, but the etiolated plants were deficient in sucrose, and none of the TCA cycle acids demanded for amination were formed, so that NH_4^+ accumulated and poisoned the plants. (b). NADH (NADPH) is essential for nitrate reduction. NADH (NADPH) may be derived either from the processes involing photochemical reactions or from oxidation of carbohydrates. Since etiolated seedlings were unable to obtain photochemically reduced NADH (NADPH) by the photochemical reactions, they could gain the required electron donors and α-keto acids from the oxidation of sucrose via the TCA cycle, so that the presence of carbohydrates in the nutrient solution greatly favored nitrate reduction in the etiolated seedlings. (c). Light grown plants apparently are endowed with sufficient amounts of chemical energy in form of sugars, organic acids, ATP, and reduced pyridine nucleotides to ensure maximal rate of nitrate reduction. Therefore, the addition of sucrose did not alter the rate of nitrate utilization. (d). In contrast to that of green plants, the root portions of etiolated plants had higher nitrate reductase activity than that in young leaves.

Table III. Effect of Sucrose and/or nitrate on the Nitrate Reductase Activity.*

Nutrient component	Plant tissue	Protein, mg/3 ml		Specific activity	
		dark	light	dark	light
Sucrose+Nitrate	Young leaves	18.12	23.86	3.3	6.9
	Root	12.04	16.78	4.5	4.9
	Stem	12.93	16.32	0.4	1.0
	Cotyledon	14.43	21.35	0.28	1.9
Nitrate, without sucrose	Young leaves	15.43	24.95	0.86	6.7
	Root	10.59	16.32	1.00	4.4
	Stem	05.76	15.69	0.08	1.14
	Cotyledon	17.88	21.67	0.06	1.9
Sucrose, without nitrate	Young leaves	12.84	20.07	0.38	1.18
	Root	10.44	21.37	0.62	0.6
	Stem	06.12	15.46	0.07	0.37
	Cotyledon	16.50	21.36	0.01	0.35
Without sucrose, or nitrate	Young leaves	10.08	20.17	0.32	1.10
	Root	08.28	21.78	0.04	0.56
	Stem	02.64	15.60	0.04	0.35
	Cotyledon	17.30	16.59	—	0.30

* The enzyme used in this experiment was the crude extract. The units of consumption and production indicated above are mμ moles.

5. The Effect of Plant Acids on Nitrate Reductase Activity.

As previous studies have shown the electron donors required for nitrate reduction may be derived form the catablism of carbohydrates. So that if one of the catabolic intermediates of the TCA cycle, such as α-ketoglutaric acid, succinic acid, or malic acid, instead of sucrose or NADH (or NADPH) is added to the nitrate reductase reaction mixture, it is found that nitrates are consumed and the nitrites are produced at the same time. There is a greater amount of nitrate consumption and nitrite production if these experiments are carried out under illumination (Table IV).

Table IV. Plant Acids and Nitrate Reductase Activity*

		Plant acid, H-donor (3×10^{-2}M)		
		α-ketoglutarte	succinate	malate
Light	NO_3^- consumed	173	145	121
	NO_2 formed	139	105	111
Dark	NO_3^- consumed	72	68	54
	NO_2^- formed	55	49	39

* The enzyme used in this experiment was the crude extract. The units of consumption and production are indicated above by mμ moles.

6. The Effect of N-source on Nitrate Reductase Activity.

The nitrate reductase activity is affected by different kinds of N-source. Generally, nitrates are better substrates than nitrites or ammonium salts for nitrate reductase. If the ammonium salts are used as a N-source for plants, plants show a lower nitrate reductase activity, and at the same time plants tested qualitatively with the Nessler's reagent show that a large quantity of ammonium ions have accumulated in the plants. No matter what kinds of N-salts are used as a N-source, the nitrate reductase activity decreases with the increasing age of the plants (Table V).

Table V. N-source and Nitrate Reductase Activity

N-source	Growth duration in days	Enzyme		
		Total activity	Protein, mg per ml	Specific activity
NO_3^-	12	166.6	32.8	6.9
	24	154.3	24.5	6.3
NO_2^-	12	100.3	17.0	6.0
	24	91.2	15.2	5.9
NH_4^+	12	50.3	14.4	2.78
	24	16.73	0.82	2.04

7. The Effect of Malnutrition on the Nitrate Reductase Activity.

If any one of the macro- or micro-elements is left out of the nutrient solution, plants not only show deficiency symptoms but also have a lower nitrate reductase activity (Table VI). The elements arranged in the following order show their decreasing effects on enzyme activity:

Mg^{++}, Fe^{++}, Mo^{++}, $S(SO_4^{-2})>Ca^{++}$, $P(PO_4^{-3})$, Mn^{++}, $K^+>Zn^{++}$, Cu^{++}.

Table VI. Effiects of Inorganic Ions on Nitrate Reductase Activity.

Elements	Enzyme activity		
	Control*	Expt.*	% of control
Complete medium	72		100
— K^+		51	71
— P (PO_4^{-3})		45	63
— S (SO_4^{-2})		40	56
— Ca^{++}		41	57
— Fe^{++}		37	51
— Mg^{++}		33	46
— Mn^{++}		50	70
— Zn^{++}		56	78
— Mo^{++}		38	53

* (a). The enzyme used here for assaying was the crude extract from the leaves of 24 day old green plants. (b). The enzyme activity expressed here is the specific activity. (c). The complete medium was prepared according to Haogland's Solution.

Plants deficiency in Mg^{++}, Fe^{++}, Mo^{++}, and S have greater ill effects on the nitrate reductase activity than when of any other elements are lacking, because Mg^{++} and Fe^{++} have a direct or indirect effect on chlorophyll synthesis[13]. Previous studies have showed that "if there is no photosynthesis, there is no nitrate reduction". Molybdenum plays an important role in the electron transfer system[2], so that if plants are deficient in Mo^{++}, the activity of nitrate reductase will be retarded. S (in the form of SH) is an important component of cysteine which is related to the active site of nitrate reducase[3], it there is no S in the plants the nitrate reductase can not function.

SUMMARY

1. Enzymes extratced from root portions of etiolated plants in contrast to that of green plants had higher nitrate reductase activity than that extracted from young leaves.
2. Nitrate reductase activity decreased with aging.
3. Nitrate reductase activity in etiolated plants was not so vigorous as that in green plants. If light grown plants were transferred to the dark, the nitrate reductase activity decreased to 80% or more within 48 hours, and after more than 80 hours in the dark the nitrate reductase activity could hardly be assayed. But nitrate reductase activity was recovered within a few hours, if the plants were returned to light.
4. Light plays an important role in nitrate reduction in geen plants, but light itself has no direct effect on it. Reduced pyridine nucleotide (NADPH) enhances nitrate reductase activity under otherwise unfavorable conditions.
5. Etiolated plants have protein synthesis capacity, if both sucrose and nitrate are dissovled in the nutrient solution.
6. Among the various kinds of N-salts, nitrate was shown to be the best N-source for plants.
7. The elements involved in chlorophyll and protein synthesis, or electron transfer have greater effects on nitrate reductase activity than other elements.

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