Effect of ammonium nitrate on the biosynthesis of leghemoglobin and nitrogen fixation in nodules of soybean plants

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Abstract. The combined nitrogen, ammonium nitrate or potassium nitrate, was used to treat soybean plants four weeks after the seed germination. The ammonium nitrate inhibited the nitrogen fixing activity of nitrogenase, but the ammonium nitrate did not have a direct effect on the nitrogen fixation. The nitrogenase activity was closely related with the concentration of leghemoglobin. The reduction of leghemoglobin contents in cytoplasm of nodules was the limiting factor of the reduction of nitrogen fixation, and the key enzyme for the biosynthesis of heme moiety of leghemoglobin, 4-aminolevulinic acid synthetase, was inhibited by the ammonium nitrate. There were three electrophoretic components in leghemoglobin, and these components were not changed by ammonium nitrate treatment. The inhibitory effect on nitrogen fixation by ammonium nitrate would be released while the ammonium nitrate was exhausted.

Key words: 4-Aminolevulinic acid synthetase; Leghemoglobin; Nitrogen fixation; Nitrogenase; Soybean.

Introduction

It had been known that combined nitrogens inhibit the nitrogen fixation in nodules of pea plants (Oghoghorie and Pate, 1971; Chen and phillips, 1977; Bisseling et al., 1978; Houwaard, 1980), soybean plants (Harper and Cooper, 1971), in Lentil (Wong, 1980). It had also reported that combined nitrogens induced the senescence of root nodules (Chen and Phillip, 1977). It was proposed that a low internal carbohydrate to nitrogen ratio in the presence of combined nitrogen caused the reduction of nodulation and nitrogen fixation (Houwaard, 1980; Wong, 1980). Small and Leonard (1969) and Oghoghorie and Pate (1971) proposed the similar conclusion that a diminished supply of photosynthate to nodules because of the nitrate assimilation reduced the nitrogen fixation.

Leghemoglobin is an oxygen-binding protein with a low Km for oxygen. Therefore, the presence of leghemoglobin increases the rate of oxygen uptake by bacteroids and so increases the nitrogen fixing activity of nitrogenase (Bergersen et al., 1973; Wittenberg et al., 1974; Wittenberg, 1980; Bergersen and Appleby, 1981). It has been demonstrated that the limiting biosynthetic pathway for protoheme formation
in most cells and tissues is the condensation step of succinyl CoA and glycine by δ-aminolevulinic acid synthetase (ALAS) to form δ-aminolevulinic acid (Nadler and Avissar, 1977; Dilworth and Appleby, 1979).

The combined nitrogen, in addition to an inhibitory effect on nitrogen fixation, also inhibited the biosynthesis of leghemoglobin (Bisseling et al., 1978). In this study, it is attempted to figure-out the inhibitory mechanism of combined nitrogen on the biosynthesis of leghemoglobin, and the inter-relationship between the rate of biosynthesis of leghemoglobin and the activity of nitrogen fixation in nodules of soybean plants treated with ammonium nitrate.

Materials and Methods

Isolation and Culture of Rhizobia

*Rhizobium japonicum* was isolated from nodules of soybean plants (*Glycine max.* cv. Shih-Shih), and purified by the regular microbiological techniques (Vincent, 1974). The purified rhizobia were cultured on yeast-mannitol agar for four days, then transferred to a liquid medium. This rhizobial suspension was incubated for two days at 30°C, then it was used directly as an inoculum for soybean seeds.

Inoculation and Growth Conditions

Soybean seeds were sterilized with a 0.01% solution of sodium hypochloride solution for five minutes, then rinsed several times with tap water, and once with distilled water. Those seeds were moistened thoroughly with the rhizobial suspension, then sown in pots containing a mixture of one part vermiculite and two parts of sand. The growing conditions for the plants in the growth chamber were temperature, day/night, 28/25°C; photoperiod, light/dark, 14/10 hours; light intensity, 6000 lux; and relative humidity, 75%. Seedlings were supplied with a nutrient solution (Dilworth, 1980) containing 2 mM of ammonium nitrate every other day for a week after seed germination. The small amounts of N supplied by the solution provide a source of N which enhanced nodulation (Harper, 1974). Thereafter, the seedlings were watered with a nitrogen-free nutrient solution. An appropriate amount of ammonium nitrate or potassium nitrate solution were added to vermiculite-sand mixture in pots while the leaf water potential was slightly below −6 bars. The leaf water potential was held more or less at −6 bars. The soybean plants had higher photosynthetic activity while their leaf water potentials were held around at −6 bars, and so, their nodules had higher efficiency in nitrogen fixation (Huang et al., 1975). The water potentials were measured by the thermocouple psychrometer (Huang et al., 1975).

Recovery Experiments

Four weeks after the plants had grown in vermiculite-sand mixtures in pots and supplied with nitrogen-free medium, plants were treated with 8 mM ammonium nitrate for five days. This treatment caused the nodules of plants had negligible leghemoglobin content and nitrogen fixing activity. Then, those plants were transferred to pots with vermiculite-sand mixtures containing various concentrations of ammonium nitrate, i.e., 0, 1, 2, 3, 4, and 5 mM, respectively. Five days after the transferring, the leghemoglobin concentration and the nitrogen fixing activity in detached nodules of plants were determined.

Determination of Nitrogen Fixation in Detached Nodules

Plants were harvested four weeks after the seed emergence. The nodules were detached from the root system of intact plants. The nitrogen fixing activity of nitrogenase in nodules was determined as the method used by Huang
eta. (1975). The gas samples (0.1 ml) were withdrawn at 30 minutes intervals and chromatographed immediately. Acetylene and ethylene were separated by the gas chromatograph (Shimazu, Model 3BF) having a hydrogen ionization detector at an oven temperature of 65°C. A glass column of 0.8 m long and 3 mm i. d. packed with Porapak R was used for the separation. The carrier gas was nitrogen at a flowing rate of 30 ml per minute.

Assay of Nitrate Reductase

One gram of nodules were ground with 1/3 of polyvinylpyrrolidone and 10 ml of 0.02 M K-phosphate buffer (pH 7.5) in a prechilled mortar with pestle. The resulting slurry was squeezed through four layers of cheesecloth and then centrifuged at 10,000 g for 10 minutes. The supernatant was defined as the crude extract. All the operations were run at 4°C. Part of the crude extract was used for the assay for nitrate reductase. The activity of this enzyme was determined as the method described by Scholl et al. (1974).

Assay of \( \delta \)-Aminolevulinic Acid Synthetase (ALAS)

Nodules were also fractionated for the assay of ALAS activity in bacteroids as the method described by Nadler and Avissar (1977), except the bacteroids were broken by the French Press instead of the sonic disruption. All operations were performed at 4°C. The \( \delta \)-aminolevulinic acid (ALA) concentration was determined by the colorimetric reaction with Ehrlich-Hg reagent (Urata and Granick, 1963). The optical density was read at 550 nm, and the molar absorbancy of the color salt was 68,000 (Urata and Granick, 1963).

Assay of Leghemoglobin

Five gram of nodules were homogenized with 50 ml of precooled Drabkins solution (Wilson and Reisenauer, 1963) in a prechilled mortar with pestle. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 10,000 g for 30 minutes. The absorbance of leghemoglobin in the supernatant was read at 540 nm (Wilson and Reisenauer, 1963). The hemoglobin standard was purchased from Sigma Chemical Company.

Gel Electrophoresis of Leghemoglobin

Leghemoglobin for gel electrophoresis was prepared as the method described by Bergersen et al. (1973). The purified leghemoglobin was electrophorezed according to method of Verma et al. (1974).

For the comparisons of the effect of N source on plant growth, four weeks after the seed germination, part of soybean plants which had been supplied with a medium free from ammonium nitrate were treated with 8 mM ammonium nitrate for five days as the N source for plants, and another part of plants were remained untreated with ammonium nitrate, so the nitrogen fixed in nodules was the solely N source for plants. Those plants which were treated or untreated with ammonium nitrate were then harvested for the measurements of nitrogenase activity, concentrations of leghemoglobin, organic matters, chlorophyll, and dry weight of leaf and root systems.

Assay of Organic Compounds

The crude extract from nodules that was likely the crude extract obtained from nodules for assay of nitrate reductase was deproteinized by 16% of trichloroacetic acid and centrifuged at 5,000 g for 10 minutes after standing still two hours. The resulting supernatant was used for the determination of soluble carbohydrate, amino acid, and protein contents. The carbohydrate, amino acid, and protein concentrations were determined by anthrone (Kochler, 1952), ninhydrin (Moore and Stein, 1954), and Lowry method (Cooper, 1977), respectively.
Chlorophyll Determination

The extraction and determination of chlorophyll were according to the method of Mayfield and Huff (1986).

Dry Weight Determination

Plants were washed several times with tap water, and dried in an oven with 80°C for twenty-four hours. The leaves and root systems were then separated and their dry weights were determined.

Results

The nitrogen fixing activity (acetylene reduction) of nitrogenase was reduced about 73%, and the activity of nitrate reductase was increased about 3.5 folds in nodules of soybean plants at five days after the plants had treated with ammonium nitrate, but the nitrogenase activity was still increasing significantly, and the nitrate reductase remained steadily low activity in nodules of plants untreated with ammonium nitrate. Obviously, the longer periods of ammonium nitrate treated to plants, the lower activity of nitrogenase, and the higher activity of nitrate reductase were detected (Fig. 1). The inhibitory effect of combined nitrogen on the nitrogen fixing activity was changed with the concentrations of combined nitrogen treated to plants (Fig. 2). The higher concentration of combined nitrogen treated to plants, the lower nitrogen fixing activity was measured. In addition to the concentration, the different forms of combined nitrogen also caused various levels of inhibitory effect on nitrogen fixation. As the data shown in Fig. 2, ammonium nitrate enforced much more severe influence than nitrate on the nitrogen fixation. The nitrogenase in nodules of soybean remained about 71%, 54%, 36% and 8.6% activity while plants were treated with ammonium nitrate at concentrations 2, 4, 6, and 8 mM, respectively.

However, the nodules remained about 92%, 73%, 67%, and 43% activity while plants were treated with potassium nitrate at concentrations of 4, 8, 12, and 16 mM, respectively. The ways of reduction of nitrogenase activity in nodules of soybean plants treated with ammonium nitrate or with K-nitrate were closely related with the reduction of leghemoglobin concentration. The higher nitrogen fixing activity was observed while there was higher concentration of leghemoglobin in nodules, and vice versa (Fig. 2).

The inhibition of combined nitrogen on the nitrogen fixation and the reduction of leghem-
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Fig. 2. Leghemoglobin concentration and nitrogenase activity in nodules of soybean plants treated with ammonium nitrate or potassium nitrate. Four weeks after seed germination, plants were treated with various concentrations of ammonium nitrate or potassium nitrate for five days, then root-nodules were harvested for measurements. Leghemoglobin concentration, ○; nitrogenase activity, ▲, in nodules of plants treated with ammonium nitrate at concentrations 2, 4, 6, 8 mM; Leghemoglobin concentration, ○, nitrogenase activity, ▲, in nodules of plants treated with potassium nitrate at concentrations 4, 8, 12, 16 mM.

Fig. 3. Recovery experiments. Leghemoglobin concentration and 26%, 46% and 93% of nitrogenase activity were restored after those plants were released from the treatment of 8 mM ammonium nitrate and transferred to vermiculite-sand mixture supplied with 5 mM, 3 mM, and 0 mM of ammonium nitrate, respectively.

The enzyme ALAS is a limiting enzyme for heme biosynthesis in bacterioids of nodules of soybean plant (Dilworth and Appleby, 1979). This enzyme activity was also inhibited by the treatment of ammonium nitrate (Fig. 4). The longer treatment of ammonium nitrate to plants, the lower activity of ALAS was detected. There was negligible activity could be measured five days after the plants had treated with ammonium nitrate (Fig. 4). The close interrelationships among ALAS activity, leghemoglobin concentration, and the nitrogenase activity in nodules of plants untreated or treated with ammonium nitrate are shown in Figs. 4A and 4B, respectively. The leghemoglobin concentration in nodules of treated plants was decreased as the ALAS activity reduced, and
According to their mobility, there were slow, moderate, and fast electrophoretic components (Fig. 5). These three electrophoretic components of leghemoglobin could not be changed by the ammonium nitrate treatment to plants, and the electrophoretic patterns of leghemoglobin obtained from the nodules of plants untreated and treated with ammonium nitrate were identical (data not shown). Besides, the electrophoretic patterns of a mixed leghemoglobins, i.e., the leghemoglobins extracted from untreated and treated plants were mixed, were looked likely the patterns of leghemoglobin obtained from the untreated plants (Fig. 5).

Although the leghemoglobin concentration and the nitrogenase activity were reduced by the ammonium nitrate, a higher concentration of proteins, free amino acids were measured in nodules of plants treated with ammonium nitrate for five days four weeks after the seed germination (Fig. 4A).

The nitrogenase activity was decreased as the leghemoglobin concentration reduced (Fig. 4B). However, the ALAS activity, leghemoglobin concentration, and the nitrogenase activity in nodules of untreated plants remained slightly increasing four weeks after the seed germinated (Fig. 4A).

There were three components of leghemoglobin could be detected by gel electrophoresis.
Table 1. Comparison of the nitrogenase activity and concentrations of leghemoglobin and other organic matters in nodules of soybean plants untreated or treated with ammonium nitrate

Data shown in Table are the average values of triplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogenase activity ( \text{mmolC}_2\text{H}_4/\text{g nodule 30 min} )</th>
<th>Leghemoglobin ( \text{mg/g nodule} )</th>
<th>Protein ( \text{mg/g nodule} )</th>
<th>Free amino acid ( \text{mg/g nodule} )</th>
<th>Soluble sugar ( \text{mg/g nodule} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.8</td>
<td>11.5</td>
<td>14.2</td>
<td>5.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Ammonium nitrate ( (8\text{ mM}) )</td>
<td>0.1</td>
<td>0.2</td>
<td>16.9</td>
<td>6.5</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 2. The chlorophyll concentrations and some morphological parameters of soybean plants treated with ammonium nitrate

Data shown in Table are the average values of triplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll ( \text{mg/g dry wt} )</th>
<th>Leaf area ( \text{cm}^2/\text{plant} )</th>
<th>Leaf dry weight ( \text{g/plant} )</th>
<th>Root dry weight ( \text{g/plant} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.26</td>
<td>130.72</td>
<td>0.35</td>
<td>0.40</td>
</tr>
<tr>
<td>Ammonium nitrate ( (8\text{ mM}) )</td>
<td>3.63</td>
<td>141.84</td>
<td>0.45</td>
<td>0.43</td>
</tr>
</tbody>
</table>

germination (Table 1). Besides, those plants treated with ammonium nitrate grew better than those untreated plants. A higher concentration of chlorophyll in leaves, larger leaf area, and heavier leaf and root dry weights were measured from those treated plants (Table 2). However, a lower concentration of soluble sugars was detected in nodules of plants treated with ammonium nitrate (Table 1).

Discussion

The nitrogen fixing (acetylene reduction) activity of nitrogenase in nodules of soybean plants was significantly inhibited by combined nitrogen (Figs. 1, 2 and 4). The ammonium nitrate had much more harmful effect than the K-nitrate on the nitrogen fixation (Fig. 2). The different levels of inhibition on nitrogen fixation caused by ammonium nitrate or by potassium nitrate were due to the forms of combined nitrogen rather than the concentrations of combined nitrogen treated to plants. However, the nitrate and ammonia equally inhibited the nitrogen fixation had also been reported (Harper and Cooper, 1971). It had been reported or reviewed in papers that nitrogenase biosynthesis could be inhibited by the ammonia (Tubb and Postgate, 1973; Nicholas and Deering, 1976; Eady et al., 1978; Brill 1980; Yates and Eady, 1980; Dixon et al., 1981; Eady, 1981). However, in this study, like the report of Bisseling et al. (1978), ammonium nitrate could reduce the concentration of leghemoglobin in nodules (Table 1, Figs. 2 and 4), and the changes of nitrogen fixation was closely matched with changes of leghemoglobin concentration, and the changes of leghemoglobin concentration were intimately related with the change of ALAS activity (Fig. 4). Therefore, the repression of ammonium nitrate on the nitrogen fixing activity of nitrogenase was probably due to the inhibitory effect of ammonium nitrate on the activity of ALAS, and that, consequently reduced the biosynthesis of leghemoglobin in nodules.

The nitrate reductase in nodules was induced and the activity of this enzyme was promoted by ammonium nitrate, but very low activity of this enzyme was detected in nodules of soybean plants untreated with ammonium nitrate (Fig. 1). The activity of nitrate reductase and nitrog-
enate could not have function simultaneously. This indicates that nitrate reduction reaction and nitrogen fixation cannot carry on simultaneously in nodules of plants treated with ammonium nitrate under the nitrogen.

The leghemoglobin in nodules of soybean plant composed of three components (Fig. 5). Although the concentration of leghemoglobin was decreased by the ammonium nitrate, the electrophoretic components were not changed by the treatment of ammonium nitrate to plants (Fig. 5). This suggests that certain enzyme(s), such as ALAS, involving in biosynthesis of the heme moiety of leghemoglobin, but not the gene(s) regulating biosynthesis of this enzyme(s), are inactivated or inhibited by ammonium nitrate. Ammonium nitrate decreased the heme concentration in nodules of pea plants has also been reported by Bisseling et al. (1978).

The soybean plants treated with 8 mM of ammonium nitrate grew better than those untreated plants (Tables 1 and 2). Besides, the concentrations of organic nitrogenous compounds, such as proteins and amino acids, were about 19% and 30% higher in nodules of the treated plants than in those of untreated ones (Table 1). This might be that quantity of N2 fixed by nitrogenase was less than that of N supplied to plants in form of ammonium nitrate, or the N in form of ammonium nitrate was preferentially utilized and assimilated by plants. However, the concentration of soluble sugar in nodules of treated plants was 39% lower than in those of untreated ones. This suggests that the total energy cost for the uptake and assimilation of ammonium nitrate is more than those for nitrogen fixation and assimilation of fixed nitrogen.

Literature Cited


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硝酸銅處理大豆其根瘤血紅素之合成對固氮作用之影響

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大豆種子發芽後四週開始處理硝酸銅或硝酸鉀，此兩種氮化學肥料均可抑制根瘤之固氮作用，惟此種抑制作用是可逆的現象。當大豆從高濃度之硝酸銅移至低濃度之硝酸銅生長環境時，其根瘤固氮作用可恢復和減少。

硝酸銅及硝酸鉀均亦可抑制根瘤血紅素合成酵素如 δ-aminolevulinic acid synthetase (ALAS)之活性，及減少血紅素之合成。由於根瘤血紅素濃度之減低，固氮酶活性因而減少。硝酸銅雖然對 ALAS 活性以及血紅素含量有影響，但是對血紅素電泳圖譜並沒有改變之現象。