Journal of Experimental Botany, Vol. 54, No. 386, pp. 1379–1388, May 2003
DOI: 10.1093/jxb/erg147

Journal of Experimental Botany

RESEARCH PAPER

Quick and reversible inhibition of soybean root nodule growth by nitrate involves a decrease in sucrose supply to nodules

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Received 12 August 2002; Accepted 18 January 2003

Abstract

The upper part of a nodulated soybean root hydroponically cultured in a glass bottle was monitored using a computer microscope under controlled environmental conditions, and the diameter of individual nodules was measured from 10–24 d after planting. The diameter of a root nodule attached to the primary root increased from 1 mm to 6 mm for 2 weeks under nitrogen-free conditions. The increase in diameter of the nodules was almost completely stopped after 1 d of supplying 5 mM nitrate, and was due to the cessation of nodule cell expansion. However, nodule growth quickly returned to the normal growth rate following withdrawal of nitrate from the solution. The reversible depression of nodule growth by nitrate was similar to the restriction of photoassimilate supply by continuous dark treatment for 2 d followed by normal light/dark conditions. In addition, the inhibitory effect of nitrate was partially alleviated by the addition of 3% (w/v) sucrose to the medium. Plant leaves were exposed to ^{11}C or ^{14}C-labelled carbon dioxide to investigate the effects of 5 mM nitrate on the translocation and distribution of photosynthates to nodules and roots. Supplying 5 mM nitrate stimulated the translocation rate and the distribution of labelled C in nitrate-fed parts of the roots. However, the ^{14}C partitioning to nodules decreased from 9% to 4% of total ^{14}C under conditions of 5 mM nitrate supply. These results indicate that the decrease in photoassimilate supply to nodules may be involved in the quick and reversible nitrate inhibition of soybean nodule growth.

Key words: Nitrate inhibition, nodule growth, PETIS, soybean.

Introduction

Soybean plants require a large amount of N, because the seeds contain a high concentration of proteins. In order to obtain the optimum yield of soybean, it is important to use both N_2 fixation in the nodules and inorganic nitrogen assimilation in the roots (Harper, 1974). However, it is well known that the development and N_2 fixation activity of root nodules is depressed when the nodulated roots are exposed to high concentrations of combined nitrogen. Nitrate, a major form of inorganic nitrogen in tilled agricultural soil, strongly inhibits nodulation and N_2 fixation (Gibson and Harper, 1985; Streeter, 1988; Imsande, 1986).

It has been suggested that there are multiple effects of nitrate inhibition, such as the decrease in nodule number,

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nodule mass, and N_2 fixation activity, as well as the acceleration of nodule senescence or disintegration, so nitrate inhibition cannot be explained simply (Streeter, 1988; Harper, 1987). In addition, nitrate inhibition of nodule growth is complex; for example, the effects of nitrate on nodule growth are influenced by nitrate concentration, placement and treatment period as well as legume species (Harper and Gibson, 1984; Davidson and Robson, 1986). Nitrate inhibition is primarily host-plant-dependent and is independent of nitrate metabolism in rhizobia (Gibson and Harper, 1985; Carroll and Mathews, 1990). Many hypotheses are proposed for the cause of nitrate inhibition of nodulation and N_2 fixation, i.e. carbohydrate-deprivation in nodules (Streeter, 1988; Vessey and Waterer, 1992), feedback inhibition by a product of nitrate metabolism such as glutamine (Neo and Layzell, 1997) or asparagine (Bacanamwo and Harper, 1997), and decreased O_2 diffusion into nodules which restricts the respiration of bacteroids (Schuller *et al*., 1988; Vessey *et al*., 1988; Gordon *et al*., 2002).

Local and systemic inhibition by nitrate on nodulation has been recognized for leguminous nodules. The local effect of nitrate inhibition was shown by split-root experiments where strong and rapid nitrate inhibition of nodule growth and N_2 fixation activity is restricted in the nodules attached to the root portions that are in direct contact with nitrate; and no or milder inhibition is induced in the other part of the same root system receiving no nitrate (Tanaka *et al*., 1985). However, some systemic inhibition of nitrate on nodulation and nitrogen fixation has also been observed with a high concentration of nitrate in clover (Silsbury *et al*., 1986) or the continuous supply of 1 mM nitrate to soybean throughout the growth stages (Ohyama *et al*., 1993*a*). In soybean, N_2 fixation activity of the non-nitrate side of a split root system was also inhibited by 30–200 ppm nitrate supply to the other half root after a 20 d treatment, although dry weight of nodules on the non-nitrate side was not inhibited after a 40 d treatment (Tanaka *et al*., 1985).

The local effect of nitrate inhibition on nodulation and N_2 fixation may be related to the fact that high accumulation of nitrate is restricted in the root parts in direct contact with nitrate (Ohyama *et al*., 1993*a*) and nodules absorb nitrate from the nodule surface and accumulate nitrate mostly in the cortical part (Mizukoshi *et al*., 1995). Systemic inhibition is also not well understood yet, however, the carbon and nitrogen balance or a declining N demand in plants may result in a decreased photosynthate supply from shoot to nodules (Bacanamwo and Harper, 1996, 1997).

It has been reported that the inhibitory effect of 5 mM nitrate on growth and nitrogen fixation activity is reversible during any treatment period from 12 d to 33 d after planting, in the nodules of soybean seedlings hydroponically cultured in a greenhouse (Fujikake *et al*., 2002). In this report, the reversible inhibition on nodule growth imposed by the short-term exposure of 5 mM nitrate was more precisely observed with a computer microscope under controlled environmental conditions. Furthermore, the cause of reversible nitrate inhibition on nodule growth was investigated from the point of changes in the photosynthate supply to the nodules treated with 5 mM nitrate by analysing photosynthates transport and partition by using $^{11}CO_2$ and $^{14}CO_2$ as tracers. In addition, split-root systems were used to examine the local effect of nitrate on the roots that were in direct contact, or not, with nitrate.

Recently, Thorpe *et al*. reported the *in vivo* measurement of $^{11}CO_2$ photoassimilate partitioning within soybean plants (Thorpe *et al*., 1998; Walsh *et al*., 1998*a*, *b*). With the invention of the positron emitting tracer imaging system (PETIS), the real time observation of the two-dimensional distribution of positron emitting elements (e.g. ^{11}C, ^{13}N, ^{18}F, etc.) became possible, and the short-time distribution of ^{13}N in soybean plants was published (Sato *et al*., 1999; Ohtake *et al*., 2001). This method is useful for the analysis of real time movement of photoassimilates in intact soybean plants.

Materials and methods

Plant material and growth conditions

Soybean seeds (*Glycine max* (L.) Merr. cv. Williams) were inoculated with a suspension of *Bradyrhizobium japonicum* (strain USDA 110) and sown in vermiculite. After 10 d, each plant was transplanted to a glass bottle with 800 ml of nitrogen-free nutrient solution (Fujikake *et al*., 2002). The culture solution was continuously aerated by an air pump, and changed three times a week. They were cultivated in a climate chamber (28/18 °C day/night temperature, 55% relative humidity, 228 μmol m^{-2} s^{-1} PPFD, 14 h photoperiod).

Monitoring in vivo *nodule growth by a computer microscope*

The root system of a 10-d-old soybean seedling was fixed on an acrylic board with a ruler using sealing tape, and cultivated hydroponically in a glass bottle with 800 ml nitrogen-free culture solution as described above. Photographs of the nodules on the primary root were taken every day by using a computer microscope (Intel Play QX3 Computer Microscope, Japan) through the bottle with 10× magnification. For nitrate-treated plants, 5 mM $NaNO_3$ was supplied in the culture solution. Control plants were supplied with nitrogen-free culture solution. Observations were repeated several times and the result showed similar patterns, so representative photographs are shown.

Observation of nodule cells morphology by a light microscope

The four largest nodules were sampled from primary roots of three soybean plants in each treatment at 10, 14, 16, and 18 DAP. They were fixed by immersion in 0.05% (v/v) acetic acid and 0.05% (v/v) formalin in 70% (v/v) ethanol at 4 °C for 48 h. They were dehydrated in a graded butanol:ethanol:water series (4:3:3, 5.5:2.5:2, 7:2:1, 8.5:1.5:0, and 10:0:0 by vol.) over a period of 1 h, respectively, then an equivalent volume of melted paraffin was added to the final solution. After incubation overnight at 30 °C, the specimens were completely infiltrated in paraffin at 60 °C over 48 h until butanol was

fully removed. The specimens embedded in paraffin were sliced into 10 μm thick sections and the paraffin was removed by xylene. The sections were stained with 0.05% toluidine blue solution for 8 min and observed with a light microscope (Olympus B201, Japan). Although the data analysis was done with replicated nodule samples, only representative photographs are shown.

Light/dark treatments

To examine the relationship between nitrate inhibition and the decrease in phyotosynthate supply to nodules, prolonged dark treatment on nodule growth was examined. Soybean plants were grown with nitrogen-free culture solution until 21 DAP. Sets of four plants were treated with light/dark conditions and 0 mM and 5 mM nitrate treatments: (a) plants were grown under a 14 h photoperiod for light/dark conditions with 0 mM or 5 mM nitrate; (b) plants were continuously grown under dark conditions with 0 mM or 5 mM nitrate. After 2 d, all treatments were stopped and the plants cultivated under 14 h photoperiod light/dark conditions with nitrogen-free culture solution (0 mM nitrate) for the following 5 d.

Sucrose addition to the medium

At 12 DAP, four replicated plants were treated with sucrose addition and nitrate treatments: (a) plants were grown with nitrogen-free culture solution, (b) plants were grown with nitrogen-free culture solution containing 3% (w/v) sucrose, (c) plants were grown with culture solution containing 5 mM nitrate, (d) plants were grown with culture solution containing 5 mM nitrate and 3% (w/v) sucrose. The treatments were continued for 3 d and the culture solution was changed every day.

Visualizing real time ^{11}C translocation

The positron emitting radioisotope ^{11}C atoms were produced by a ^{14}N (p, α) ^{11}C nuclear reaction by bombarding a target nitrogen gas with 10 MeV protons at a current of 1 μA using the TIARA (Takasaki Ion Accelerators for Advanced Radiation Application) AVF cyclotron. The $^{11}CO_2$ was produced from the ^{11}C atoms and the oxygen present in the target chamber. At 24 DAP, the primary roots of soybean plants was cut off by scissors below the level of the primary root nodules and the lateral roots were split into two parts to form a split-root system. Control plants were supplied with nitrogen-free culture solution to both root portions. Nitrate-treated plants were separated into two patterns: (a) 5 mM nitrate was supplied to both sides of the split root system for 3 d, (b) 5 mM nitrate was supplied to only one side of the split root system for 3 d. Three plants were grown per treatment. At 29 DAP, $^{11}CO_2$ (50 MBq) was supplied to the first trifoliolate leaves for 10 min, and ^{11}C movement to the root was monitored using a PETIS for 120 min. After 120 min of analysis by PETIS, the radioactivity of a whole plant was observed with a Bio-imaging analyser (BAS-1500, Fuji Film, Japan), and ^{11}C translocation from the leaves to various parts of the plant was observed.

Quantitative analysis of ^{14}C partitioning

In the first experiment, whole shoots of four 22 DAP plants per treatment were enclosed in plastic bags (Gas sampling bag 10 l, 350×400 mm, GL Sciences, Japan), and each plastic bag was connected to a closed gas circuit system with an air pump. The $^{14}CO_2$ was generated in a test tube by injecting 5 ml of 10% (v/v) $HClO_4$ into the test tube containing 370 kBq of ^{14}C-labelled sodium carbonate. Plants were exposed to the $^{14}CO_2$ for 120 min. After this time plants were immediately dried in an oven at 80 °C, the plants were separated into leaves, stems, roots, and nodules. The samples about 5 mg DW of each part were bleached and dissolved in 170 μl of 30% (v/v) H_2O_2 plus 330 μl of 60% (v/v) $HClO_4$. The radioactivity (DPM: disintegrations per minute) in these parts was determined using a Liquid Scintillation Counter (LSC-6000, ALOKA, Japan). Control plants were grown with nitrogen-free culture solution throughout the experiment. Nitrate-treated plants were supplied with 5 mM nitrate from 1 d before ^{14}C supply.

In the second experiment, the primary root of the plant below the level of the primary root nodules was cut off by scissors and lateral roots were split into two identical parts at 25 DAP. The plants were treated with two nitrate treatments: (a) both sides of the split root system were filled with nitrogen-free culture solution, (b) one side of the split root system was filled with nitrogen-free culture solution, the other with a culture solution containing 5 mM nitrate. Whole shoots of 28 DAP plants were exposed to $^{14}CO_2$ for 120 min. The underground parts of plants were separated into the primary roots, primary root nodules, lateral roots, and lateral root nodules. The radioactivity in these parts was analysed as above.

Results

Effects of nitrate on nodule growth

Soybean nodules grew steadily for 2 weeks in a nitrogen-free nutrient solution with 0 mM nitrate (Fig. 1Aa). The initial diameter of nodules was about 1.1 mm at 10 DAP, and the final diameter was 6.0 mm at 24 DAP (Fig. 1Ba). When 5 mM nitrate was supplied after 10 DAP, nodule growth was quickly suppressed from the day after nitrate addition, although the roots grew more vigorously (Fig. 1Ab). The initial diameter of nodules was 1.3 mm at 10 DAP, and the final diameter was only about 1.6 mm at 24 DAP (Fig. 1Bb). In addition, nodule growth was inhibited by nitrate treatment from 15 d to 20 d but was quickly restored by changing the nitrate concentration in the culture solution from 5 mM to 0 mM at 20 d (Fig. 1Ac, Bc).

The morphology of typical nodule slices of soybean observed by an optical microscope are shown in Fig. 2A. It is conspicuous that the infected cells of nodules became larger under nitrogen-free conditions from 10 to 18 DAP (Fig. 2Aa). Under 0 mM nitrate conditions, the average cell diameter of all three types of cells, i.e. infected cells, uninfected cells, and inner cortex cells, increased from 10 to 18 DAP (Fig. 2Ba). On the other hand, the infected cells of nodules remained small with 5 mM nitrate in the culture solution for the same 9 d period (Fig. 2Ab, Bb). Cell growth recovered rapidly after 2 d of 0 mM nitrate treatment following 3 d of 5 mM nitrate treatment (Fig. 2Ac, Bc).

Effect of dark treatment and sucrose addition on nodule growth

Under 14 h photoperiod light/dark conditions, the nodule growth of soybean plants was significantly depressed after 2 d exposure to 5 mM nitrate (Fig. 3). Although nodule growth of the plants grown with nitrogen-free culture solution under continuous dark conditions maintained growth for the first day, it was depressed by the second day. The nodules of the plants grown with 5 mM nitrate under dark conditions was rapidly and completely inhibited

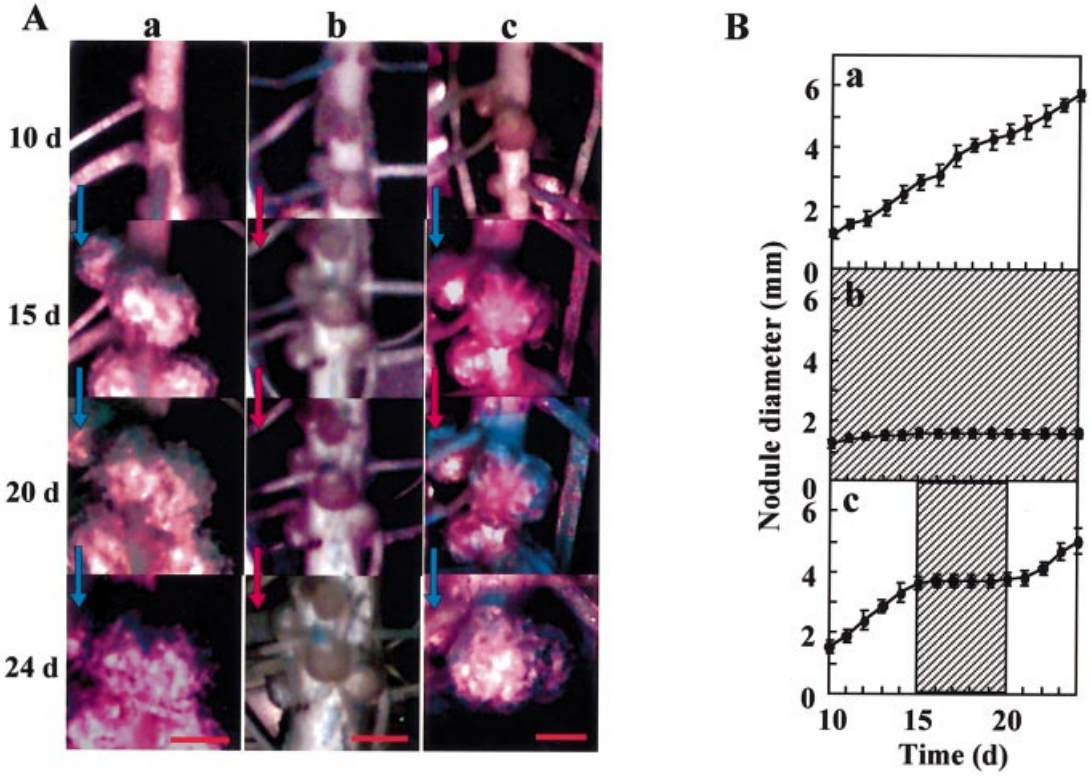

Fig. 1. (A) Growth response of soybean nodules to 0 mM (blue arrows) or 5 mM (red arrows) nitrate application in the culture solution. (a) Nodule growth with nitrogen-free medium. (b) Nodule growth with continuous 5 mM nitrate supply in the medium. (c) Nodule growth with 5 mM nitrate from 15–20 DAP. Bars=2 mm. (B) Changes of nodule diameter. The slanted lines indicate the times of 5 mM nitrate treatments. Results show the mean value with SE of the three replicated plants.

during the 2 d treatment. When the plants were returned to the nitrogen-free culture solution under 14 h photoperiod light/dark conditions, all treatments recovered nodule growth compared with the 14 h photoperiod light/dark with nitrogen-free conditions.

3% (w/v) sucrose supply to the culture solution promoted the nodule growth of plants with 0 mM nitrate after the third day (Fig. 4). In addition, the nodule growth of 5 mM nitrate-fed plants was partially alleviated by the addition of sucrose. After the 3 d treatments, the sucrose concentration in nodules was 23 mg g^{-1} DW and 15 mg g^{-1} DW with 0 mM and 5 mM nitrate, respectively. The 3% sucrose supply increased it to 33 mg g^{-1} DW (0 mM nitrate) and 58 mg g^{-1} DW (5 mM nitrate) in nodules.

Effects of 5 mM nitrate supply on ^{11}C translocation

In both control 0 mM nitrate (Fig. 5Aa) and 5 mM nitrate-fed plants (Fig. 5Ab), ^{11}C assimilated in the first trifoliate was translocated both upward to the young developing leaf buds and downward to the whole root system. However, very little was translocated to the developed trifoliates and primary leaves. The radioactivity of ^{11}C in apical developing leaf buds appeared to be high in plants with 0 mM nitrate compared with 5 mM nitrate-fed plants. However, ^{11}C activity was relatively higher in the lower part of the roots in plants with 5 mM nitrate compared with plants with 0 mM nitrate. PETIS was used to monitor the real time movement of ^{11}C to the roots (Fig. 5B). The movement of ^{11}C from the base of the root started at about 40 min and reached the root tips at 101–120 min. The accumulation of ^{11}C radioactivity in nodules appeared to be higher than that of ^{11}C radioactivity in roots in all plants (Fig. 5B). In the roots of the plant with 0 mM nitrate, initial transport of ^{11}C to the lower roots was relatively slow reaching the root tips after 101–120 min (Fig. 5Ba). Supplying 5 mM nitrate promoted the transport rate of ^{11}C in whole roots (Fig. 5Bb). Split lateral roots in direct contact with 5 mM nitrate accumulated ^{11}C at a higher rate than the roots cultivated with 0 mM nitrate solution in the same plant (Fig. 5Bc). The initial arrival time of ^{11}C at the point of R-1 (basal root) was 34 min in the plant with 0 mM nitrate and the mean speed through the stem was about 5 mm min^{-1} (Fig. 5Ca). The arrival time was 30 min in the plant with 5 mM nitrate and mean speed through the stem was about 8 mm min^{-1} (Fig. 5Cb). The ^{11}C arrived at point N (nodule) at 43 min and point R-2 (roots under point N) at 65 min in –N plants. On the other hand, the ^{11}C arrived at point N at 38 min and point R-2 at 54 min in plants with

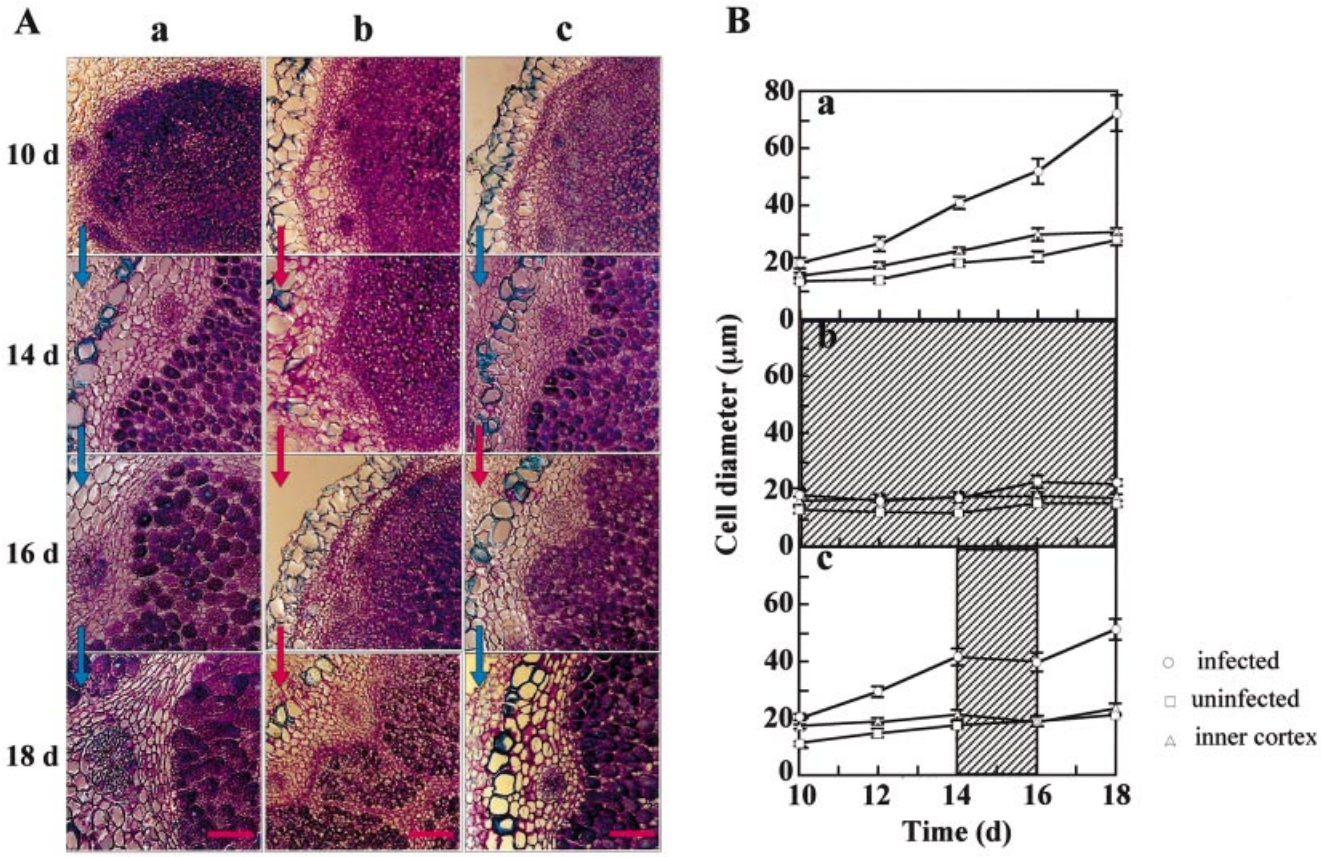

Fig. 2. (A) Effect of 0 mM nitrate (blue arrows) or 5 mM (red arrows) nitrate treatments on the structure of soybean nodule cells. (a) Typical soybean nodules cultivated with nitrogen-free culture solution for 10 d. (b) Nodules cultivated with culture solution containing 5 mM nitrate from 10–18 DAP. (c) Changes in nodule cells by 5 mM nitrate treatment from 14–16 DAP. All samples were observed with the same magnification. Bars=100 μm. (B) changes in the diameter of infected cells (circles), uninfected cells (squares) and inner cortex (triangles) cell types. The slanted lines indicate the times of 5 mM nitrate treatments. Bars indicate standard error of 10 values from three independent nodules. Results from each treatment were obtained from three separate plants.

5 mM nitrate. The mean speed of ^{11}C movement in the roots from R-1 to N (upper part of roots) and from N to R-2 (lower part of roots) was about 3 mm min^{-1} in both cases for the plants with 0 mM nitrate, and about 4 mm min^{-1} and about 6 mm min^{-1} in 5 mM nitrate plant, respectively.

Effects of nitrate on ^{14}C partitioning to each organ

In the plants with 0 mM nitrate, ^{14}C radioactivity in nodules was about five times higher than that in the roots (Fig. 6A). The ^{14}C radioactivity in nodules treated with 5 mM nitrate for 1 d was almost half of the control nodules with 0 mM nitrate. The activity in nodules was further decreased to about 25% of control plants at 3 d after supplying 5 mM nitrate (data not shown). The distribution of ^{14}C to each organ is shown in Fig. 6B. In nodules treated with 0 mM nitrate, the distribution of ^{14}C was about 9.1% of total activity; however, it was only 4.3% after 1 d of 5 mM nitrate supply. By contrast, ^{14}C distribution in roots increased from 5.2% to 9.1% following the addition of 5 mM nitrate. There were no differences in the distribution to shoot organs between the plants with 5 mM and 0 mM nitrate.

In the second experiment, ^{14}C radioactivity was measured in plants when both sides of split lateral roots were cultivated with 0 mM nitrate solution (Fig. 7Aa) and one side of the roots was in direct contact with culture solution containing 5 mM nitrate (Fig. 7Ab). The radioactivity of primary root nodules was less than half that of 0 mM nitrate plants after being supplied with 5 mM nitrate for 3 d, although there was no differences in that of the primary root between 0 mM and 5 mM nitrate-treated plants (Fig. 7A). In lateral roots, the radioactivity increased about twice in both sides of the split roots whether directly in contact with 5 mM nitrate or not. However, the radioactivity was only decreased in lateral root nodules exposed to 5 mM nitrate. The distribution of ^{14}C in soybean root system is shown in Fig. 7B. In 0 mM nitrate plants, the lateral roots shared about 20% of the total ^{14}C accumulated in the root, but lateral roots then became >40% after supplying with 5 mM nitrate for 3 d. Although

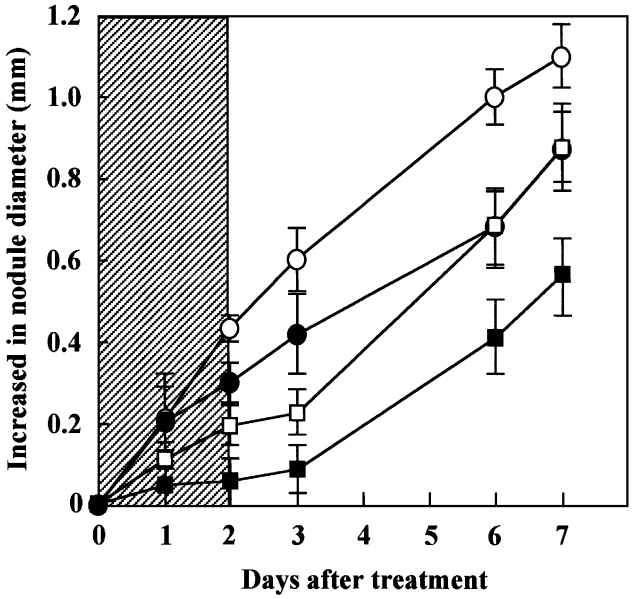

Fig. 3. Nodule growth of soybean plants grown with 5 mM nitrate and light/dark treatments as follows: grown with nitrogen-free culture solution under 14 h photoperiod light/dark conditions (open circles) or under continuous dark conditions (filled circles), grown with a culture solution containing 5 mM nitrate under 14 h photoperiod light/dark conditions (open squares) or under continuous dark conditions (filled squares). Bars indicate standard error of 20 values (five nodules from four independent plants). The slanted lines indicate times of treatments.

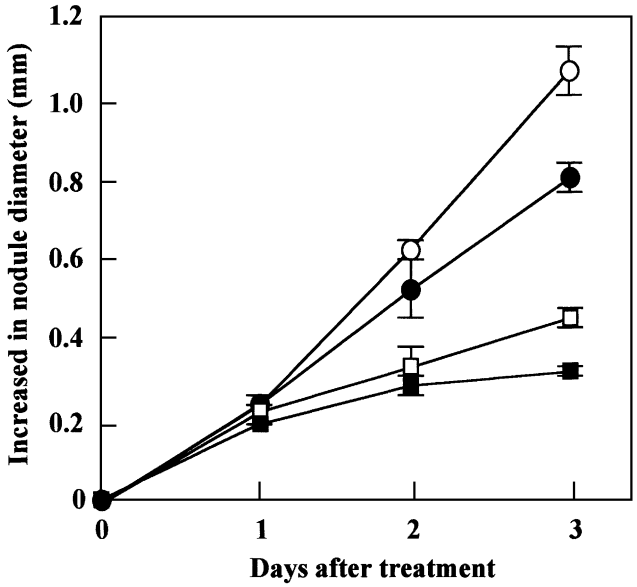

Fig. 4. Effect of 3% (w/v) sucrose addition on nodule growth of soybean plants. Plants grown with a nitrogen-free culture solution containing 3% (w/v) sucrose (open circles) or not (filled circles), and plants grown with a culture solution containing 5 mM nitrate and 3% (w/v) sucrose (open squares) or containing only 5 mM nitrate (filled squares). Bars indicate standard error of 20 values (five nodules from four independent plants).

the primary root nodules were exposed to the air during the treatment period, the distribution of ^{14}C decreased from about 50% to 20% after the 5 mM nitrate treatment.

Discussion

It was confirmed that 5 mM nitrate almost completely depressed nodule growth at 1 d after nitrate treatment and rapidly recovered after nitrate withdrawal under controlled environmental conditions (Fig. 1). A similar response was observed irrespective of nodule sizes and treatment periods of 5 mM nitrate (Fujikake *et al*., 2002). Because the depression was reversible, it is assumed that disintegration or senescence has not been started by the 5 mM nitrate treatment. Cell expansion of nodule cells, especially the infected cells was suspended by the presence of 5 mM nitrate (Fig. 2). Soybean nodules are classified as determinate type nodules. Cell division and nodule organogenesis is completed in the early stages of development, and then nodule growth (increase in nodule diameter) is mainly brought about by cell expansion to form the functional nodule (Gresshoff, 1993). Hence, it is reasonable to suggest that the growth inhibition of established soybean nodules by nitrate is accounted for by the cessation of cell expansion.

In the present study, nodule growth in soybean was also inhibited by 2 d of dark treatment, however, growth was recovered following a return to 14 h photoperiod light/dark conditions (Fig. 3). This response was similar to the reversible nitrate inhibition of nodule growth, and nodule growth was synergistically inhibited with combinations of both 5 mM nitrate and dark conditions. On the other hand, nodule growth was enhanced by a 3% (w/v) sucrose addition to the culture solution and the depressive effect of 5 mM nitrate was partially alleviated by sucrose addition. The increase in sucrose concentration in the nodules following a 3% sucrose supply suggests that the added sucrose is incorporated into the nodules. The possibility can be excluded that a high accumulation of sucrose in nodules may act as an osmoticum to support cell expansion, because the highest sucrose accumulation in nodules (58 mg g^{-1} DW) was equivalent to about 0.8% sucrose (w/v) in the fresh nodules, which was lower than the external solution. The 3 d treatments with the same molar concentrations (88 mM) of mannitol or KCl, caused about a 40% depression of nodule growth compared with control plants either with 0 mM or 5 mM nitrate (data not shown).

Noel *et al*. (1982) reported that acetylene reduction activity in soybean nodules declined 1 d after 10 mM nitrate treatment and reverted to the control level a few days after nitrate withdrawal. The same was true for nodule growth with 5 mM nitrate treatment (Fig. 1). The long-term application of 5 mM nitrate from 7 d to 27 d after planting for soybean (cv. Williams) markedly increased the dry weight of stems (396%), leaves (204%) and roots (163%), but severely depressed nodule dry weight (5%)

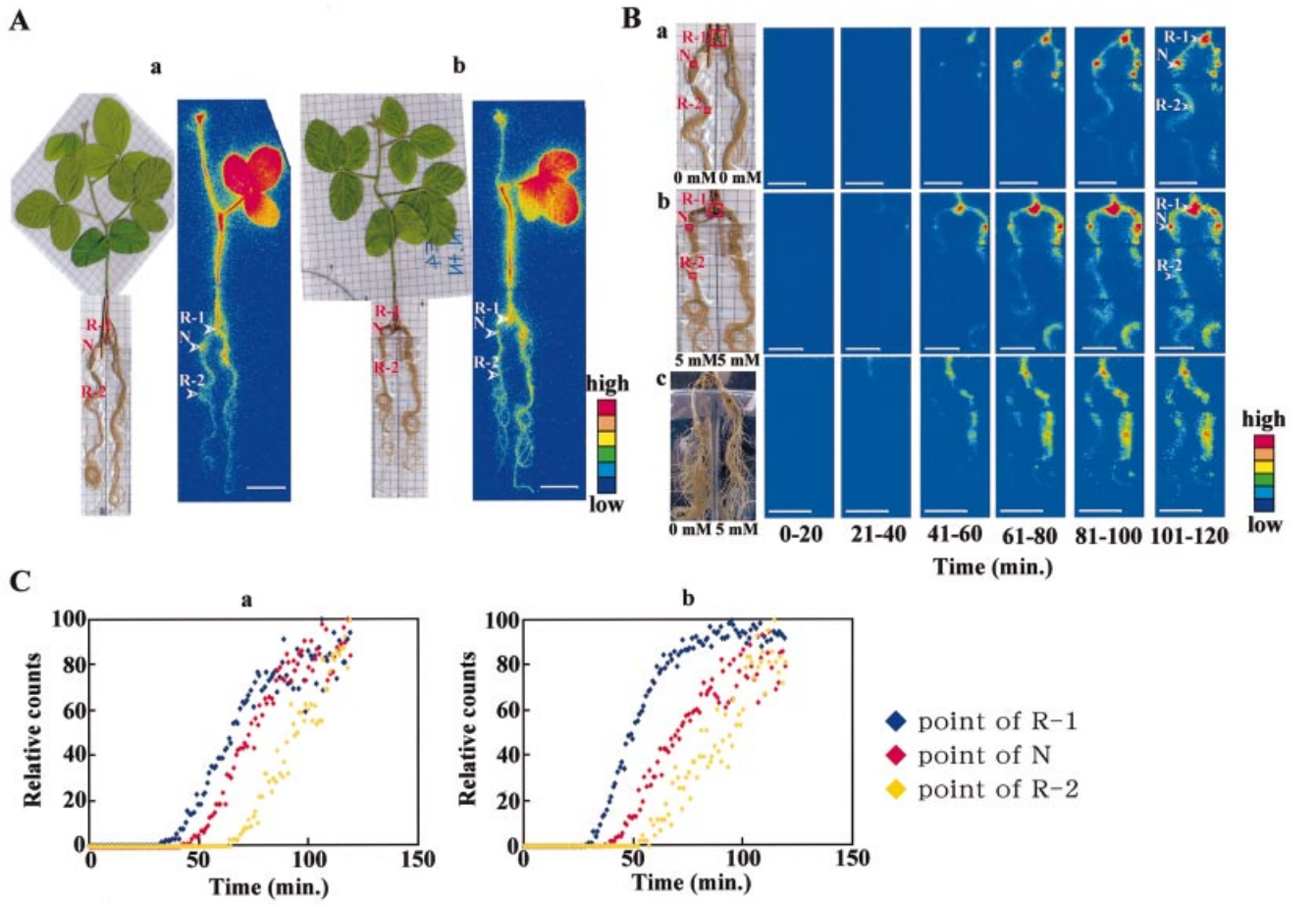

Fig. 5. ^{11}C translocation to the split root systems from first trifoliate leaves of 29-d-old soybean plants. (A) Image of the distribution of ^{11}C in soybean using BAS-1500. Bars=5 cm. (R-1) shows the point of a basal root, (N) shows a lateral root nodule, (R-2) shows lateral roots below point N. (B) The time-course for the accumulation of radioactivity as shown by PETIS. The images shown are for 20 min intervals and the data were scored every minute. Bars=2.5 cm. (C) The accumulation of radioactivity for the point of R-1 (blue), the point of N (red) and the point of R-2 (yellow). (a) Plant cultivated with nitrogen-free culture solution for 29 d. (b) Plant treated with 5 mM nitrate for 3 d after cultivated with nitrogen-free culture solution for 26 d. (c) Plant supplied with 5 mM nitrate to one side of the lateral roots for 3 d after cultivated with nitrogen-free culture solution for 26 d.

compared with the control plant with 1 mM urea (Ohyama *et al*., 1993*b*). The nitrate supply also decreased protein concentration in nodules although it increased the protein concentrations of leaves, stems and roots (Ohyama *et al*., 1993*b*). The leghaemoglobin concentrations were decreased by nitrate but the decline in protein was not specific for leghaemoglobin (Nishiwaki *et al*., 1997). By analysing protein synthesis with two-dimension gel electrophoresis, it was confirmed that the nitrogenase protein was fully retained in the nodules showing reduced acetylene reduction activity following nitrate supply (Noel *et al*., 1982). These results are in accordance with a model that a decreased availability and/or utilization of photosynthates in nodules may occur followed by nitrate exposure.

In this present study, treatment of 5 mM nitrate accelerated the transport rate of ^{11}C in the stems and roots (Fig. 5) and partitioning of ^{14}C in the roots with nitrate. However, 5 mM nitrate decreased ^{14}C partitioning in the nodules following treatment for 1 d (Fig. 6). These results were in agreement with previous studies that photosynthates partitioning to nodules is decreased following nitrate treatment (Latimore *et al*., 1977; Rabie *et al*., 1980; Kouchi *et al*., 1986; Vessey *et al*., 1988). In addition, with the split-root experiments, photosynthate translocation was especially increased in the root portions which were directly exposed to nitrate (Figs 5, 7). The combined results of a quantitative ^{14}C experiment and an ^{11}C experiment by real-time monitoring of secondary imaging with PETIS for split-root experiments support the model that a decrease in photoassimilate supply to nodules by 5 mM nitrate is involved in the reversible inhibition of nodule growth and N_2 fixation activity in soybean. The result that increased photon irradiation dramatically alleviated nitrate inhibition on acetylene reduction activity of clover nodules (Silsbury *et al*., 1986) may support the above assumption.

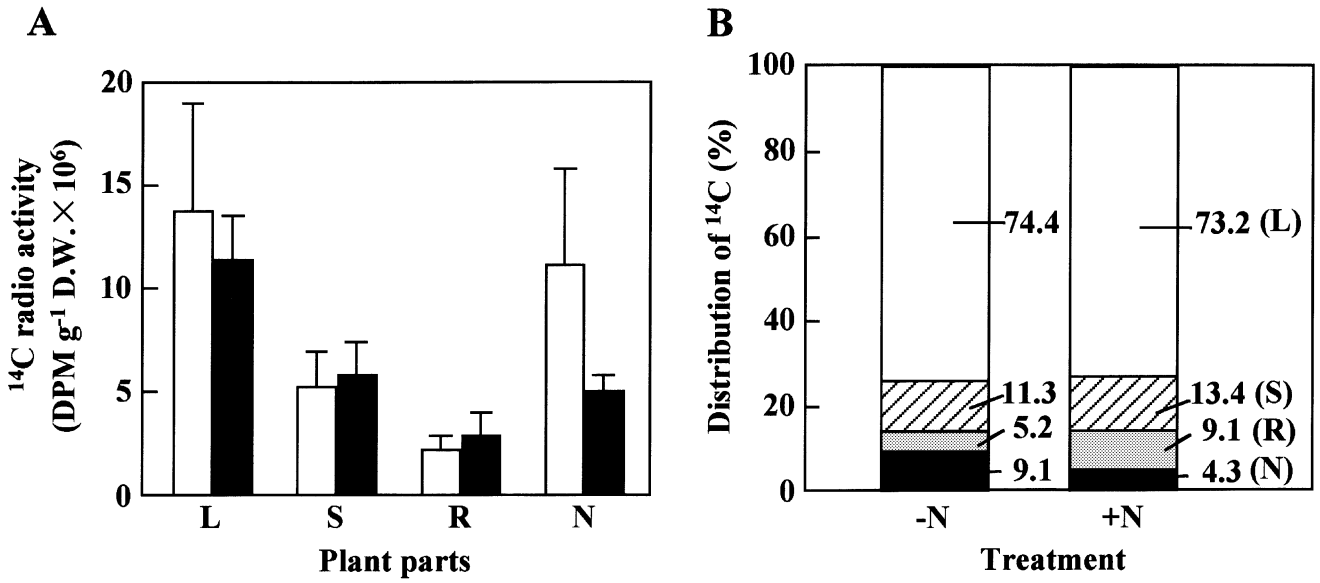

Fig. 6. Partitioning of ^{14}C labelled photoassimilates in soybean plants. (A) The radioactivity g^{-1} dry weight (DW) of each part after 1 d of 0 mM (white column) or 5 mM (black column) nitrate treatments. Bars indicate standard error of four replications in each treatment. L, leaves; S, stems; R, roots; N, nodules. (B) Proportion of ^{14}C in soybean plants cultivated with nitrogen-free culture solution for 22 d (–N) or plants treated with 5 mM nitrate for 1 d after cultivating in a nitrogen-free culture solution for 21 d (+N). The values in the figure are averages of four replications in each part as follows: leaves (white bars), stems (diagonal lines), roots (grey bars), and nodules (black bars) Samples were obtained from four independent plants.

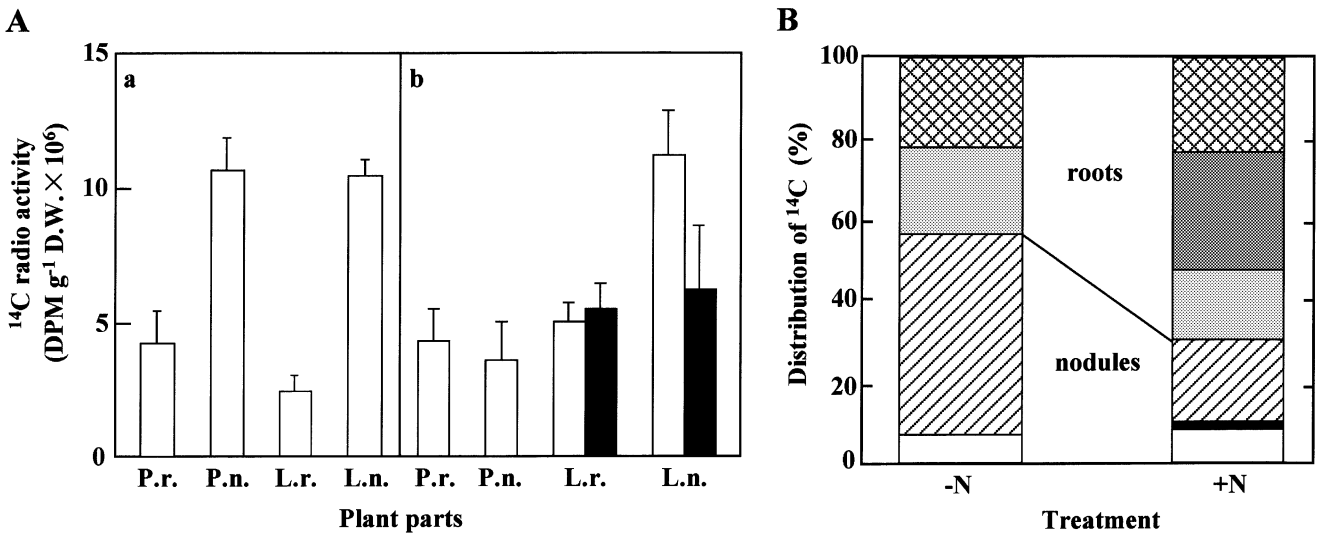

Fig. 7. (A) The ^{14}C radioactivity in each part of soybean plants grown using the split-root method. Plants were supplied with nitrogen-free culture solution to both sides of the lateral roots (a) or supplied with culture solution containing 5 mM nitrate to one side of the lateral roots (b). Bars indicate standard error of four replications in each part as follows: primary root (P.r.), primary root nodules (P.n.), lateral roots with 0 mM nitrate (L.r. white column), lateral root nodules with 0 mM nitrate (L.n. white column), lateral roots with 5 mM nitrate (L.r. black column) and lateral root nodules with 5 mM nitrate (L.n. black column). Samples were obtained from four independent plants. (B) Distribution of ^{14}C in the root system of soybean plants grown with nitrogen-free culture solution (–N) or grown in a culture solution containing 5 mM nitrate (+N). Plants were grown by the split-root method as above. Values in the figure are obtained from the average of four independent plants. Samples were obtained from each part as follows: primary root (cross hatched), lateral roots with no nitrate (light grey), lateral roots with 5 mM nitrate (dark grey), primary root nodules (diagonal lines), lateral root nodules with no nitrate (white), and lateral root nodules with 5 mM nitrate (black).

The carbohydrate deprivation hypothesis has been criticized based on the fact that nitrate sometimes did not decrease, or sometimes increased, the concentrations of sucrose in nodules (Streeter, 1988; Gordon *et al.*, 2002). However, as pointed out by Vessey and Waterer (1992) the changes in pool size may not be

indicative of changes in the flow rate of metabolite through the pool. There are many compartments in nodules, and as the concentration of metabolite in a metabolic pool is the difference between (influx+synthesized) and (efflux+metabolized), the sucrose concentration may not decrease if both supply and consumption in nodules decrease simultaneously.

Concerning the decrease in carbohydrate supply to nodules by nitrate treatments, two main causes can be assumed. One is that the decrease of photosynthate partitioning to nodules is caused by an enhancement of carbohydrate consumption for nitrate absorption and assimilation in the roots and root growth promotion. This may simply be the consequence of the increased sink strength of roots or some internal factor(s), such as plant hormones (e.g. indole acetic acid, abscisic acid), may regulate photoassimilate partitioning between nodules and roots. Another possibility is that the decrease in photosynthate partitioning is a consequence of declined sink activity of nodules, through impaired carbon metabolism and/or respiration due to decreased O_2 diffusion by accumulation of nitrate in cortex (Becana *et al*., 1989; Mizukoshi *et al*., 1995; Vessey and Waterer, 1992). It has been reported that nitrate treatment, as well as stem girdling, increased O_2 limitation of nodule respiration and nitrogenase activity, and decreased the adenylate energy charge (De Lima *et al*., 1994).

The final conclusion whether the decrease in carbohydrate supply is an initial cause by nitrate inhibition or the secondary effects from limited carbohydrate utilization in nodules has not yet been reached. Based on the result that ^{14}C labelled photoassimilate partitioning in primary nodules, which are exposed to air and not in direct contact with nitrate, also decreased following a 3 d treatment with 5 mM nitrate (Fig. 7), it is suggested that the decrease in photosynthates supply to nodules can be brought about independently of the nitrate accumulation in nodules. Of course, it is possible that both processes may occur simultaneously in nodules in direct contact with nitrate. The decrease in starch concentration in nodules (Vessey *et al*., 1988; Gordon *et al*., 2002) and the down-regulation of sucrose synthase transcript within 1 d of nitrate treatment (Gordon *et al*., 2002), may imply that nitrate reduces both photosynthate flow into the nodules and sucrose utilization in the nodules without an appreciable decrease in sucrose concentration.

In addition to sucrose supply, various internal factors may influence the suspension of cell expansion which causes the temporary interruption of nodule growth, for example, the supply of the other raw materials (amino acids and mineral nutrients), phytohormones and water. Further studies are required to clarify the primary cause of the reversible nitrate inhibition on soybean nodule growth and N_2 fixation.

Acknowledgement

We greatly appreciate Dr JG Streeter for reviewing the manuscript and for useful suggestions.

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