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Induction of Root Nodule Senescence by Combined Nitrogen in *Pisum sativum* L.[1]

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ABSTRACT

Root nodule senescence induced by nitrate and ammonium in *Pisum sativum* L. was defined by determining nitrogenase activity and leghemoglobin content with the acetylene reduction and pyridine hemochrome assays. Root systems supplied with 100 mM KNO_3 or 100 mM NH_4Cl exhibited a decrease in nitrogenase activity followed by a decline in leghemoglobin content. Increasing the CO_2 concentration from 0.000320 atm to 0.00120 atm had no effect on the time course of root nodule senescence when 20 mM KNO_3 was supplied to the roots; *in vitro* nitrate reductase activity was detected in leaves and roots, but not bacteroids. Nitrate appeared in leaves, roots, and the nodule cytosol fraction but not bacteroids when 20 mM KNO_3 was supplied to roots. When nitrate entered through the shoots, however, no root nodule senescence was observed, and no nitrate was detected in root or nodule cytosol fractions although nitrate and nitrate reductase were found in leaves. The results suggest that nitrate does not induce root nodule senescence through competition between nitrate reductase and nitrogenase for products of photosynthesis.

Field-grown soybeans reportedly obtain only 25% of their nitrogen from N_2 (6). The remaining nitrogen is drawn from soil reserves of this element. Understanding the manner in which nitrate and ammonium decrease biological N_2 reduction in legume root nodules is important for devising strategies for maximizing N_2 fixation.

One method for studying interactions between combined nitrogen and N_2 fixation is to supply excess nitrate or ammonium to legumes and examine the effect on root nodule functioning. Morphological effects of supplying NH_4NO_3 to root nodules have been described (2), but the physiological basis of this response has not been elucidated. Small and Leonard (12) reported data which suggested that ^{14}C-labeled photosynthate was retranslocated out of pea root nodules on *Pisum sativum* L. plants treated with $NaNO_3$. Such a movement of photosynthate could have a considerable effect on the energy available for N_2 fixation (10).

Oghoghorie and Pate (8) explored the concept that N_2-fixing nodules and NO_3^--assimilating centers compete for supplies of reductant and carbon skeletons derived from photosynthate. If such competition results in root nodule senescence, it might be overcome by increasing the photosynthate available to root nodules. A recent report established that long term CO_2 enrichment (0.00120 atm) of the air surrounding pea plants resulted in starch accumulation in root nodules without affecting either root nodule development or functioning (9). The present study was begun in an effort to define the temporal sequence of nitrate-induced root nodule senescence in terms of nitrogenase activity and leghemoglobin content and to discover whether CO_2 enrichment could alter that time course.

MATERIALS AND METHODS

Pisum sativum L., variety Alaska, nodulated with *Rhizobium leguminosarum* 128C53 (obtained originally from J. C. Burton, The Nitragin Co., Milwaukee, Wis.) was maintained in growth chambers in the absence of combined nitrogen as previously reported (9). Carbon dioxide concentrations were maintained at either 0.00032 atm or 0.00120 atm from the time of planting. Carbon dioxide concentration was monitored continuously by a Beckman model 864 IR analyzer connected to a Beckman model 8710 strip-chart recorder with a single alarm set to activate a Skinner V52DB2052 solenoid. When activated, the solenoid allowed 99.5% pure CO_2 to enter the plant growth chamber until the alarm was deactuated by the recorder. Chamber air was sampled by a Teflon-coated pump (Thomas Industries model 106CA050-TFE, Sheboygan, Wis.) connected to a drying column filled with $CaSO_4$. This system permitted CO_2 levels to be controlled to ±2.0% of the desired setting at 0.00120 atm.

Nitrogen treatments were started 28 days after planting the peas. Each 15-cm diameter pot containing five plants was treated with 500 ml of the appropriate nitrogen-containing solution in experiments utilizing root-supplied nitrogen. When nitrate was supplied to the shoot, the apical meristem was removed, and the entire shoot was dipped into a 1-liter solution containing 3 g KNO_3 and 0.5 ml Triton X-100 for 4 hr. Control plants were treated with a solution containing only Triton X-100.

In vivo nitrogenase activity of intact pea plants was measured with the acetylene reduction assay (3). Pea nodules were extracted with 0.1 M K-phosphate buffer at pH 7.5. Nodule homogenates were centrifuged at 500*g* for 5 min to remove cell debris followed by centrifugation at 10,000*g* for 15 min to sediment bacteroids and mitochondria. The supernatant, referred to as nodule cytosol, was used for both leghemoglobin and nitrate determinations. Leghemoglobin concentration was calculated by the pyridine hemochrome technique (1). Bacteroids were resuspended in 0.1 M K-phosphate buffer and broken by two passages through a French pressure cell at 20,000 p.s.i. Cell debris was removed by centrifugation at 30,000*g* for 30 min and the supernatant was saved for nitrate determinations.

Nitrate reductase was extracted from nitrate-treated pea leaves or roots with a Tekmar homogenizer using 7 ml of grinding medium/g fresh weight of materials as described by Hageman and Hucklesby (5) except that 3% (w/v) casein was added to replace the 10 mM cysteine (11), and the solution was adjusted to pH 8.8. Root extracts were passed through Sephadex G-25 to

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remove small molecules which obscured nitrate reductase activity (4). After stopping the assay reaction (5), color was allowed to develop for 10 min prior to reading A at 540 nm. A zero time reaction mixture was used as the reference solution.

Nodule bacteroids from *Glycine max* (L.) Merr. cv. Cutlass were used as a source of nitrate reductase for nitrate determinations (7). Soybean bacteroids were collected as described for pea bacteroids and used to prepare a 25% (w/w) suspension in 0.1 M K-phosphate (pH 7.5). A 1-ml reaction mixture, pH 7.5, containing 0.05 to 4.0 μg nitrate was prepared with 50 μmol phosphate, 40 μmol sodium succinate, and 0.2 ml of the bacteroid suspension. The reaction mixture was incubated for 4 hr at 27 C, and A_{540} was determined after the color development.

RESULTS

Root Applications of Combined Nitrogen. Data in Figures 1 and 2 reveal that applications of either 100 mM KNO_3 or 100 mM NH_4Cl to pea roots induced a decline in both nitrogenase activity determined by the acetylene reduction assay and leghemoglobin content detected by the pyridine hemochrome technique. Plants treated with 100 mM KCl were unaffected over the same time period. A comparison of Figures 1 and 2 shows that nitrogenase activity decreased before leghemoglobin content. Two days after the initial nitrate treatment, plants exhibited only 40% of the nitrogenase activity (μmol C_2H_4/g nodule dry weight 30 min) observed in untreated plants, but leghemoglobin levels (nmol leghemoglobin/g nodule fresh weight) were equal in treated and untreated plants. Three days after being supplied with NH_4Cl, treated plants contained 8% of the nitrogenase activity and 97% of the leghemoglobin found in untreated plants.

Increasing the CO_2 concentration from 0.00032 atm to 0.00120 atm had no significant effect on the decline of either nitrogenase activity or leghemoglobin levels in root nodules of peas treated with applications of 20 mM KNO_3 to the root substrate (Table I). The concentration of nitrate was reduced from that used for experiments reported in Figures 1 and 2 in order to provide maximum opportunity for CO_2 enrichment to overcome any competition between nitrogenase and nitrate reductase for photosynthate. Trials with levels of applied nitrate less than 20 mM did not result in reproducible root nodule senescence or nitrate reductase induction under these experimental conditions.

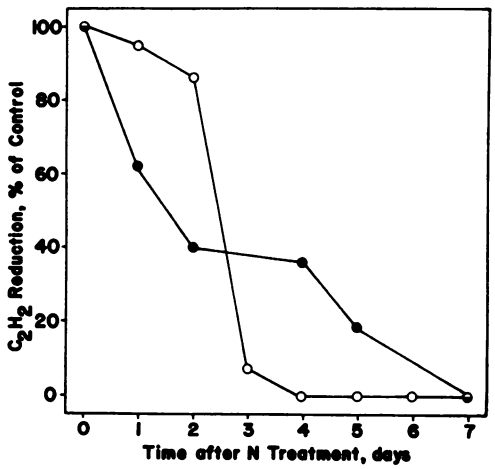

FIG. 1. Effect of root-absorbed nitrate and ammonium on nitrogenase activity in peas. Plants were grown under 0.00032 atm CO_2 and 100 mM N solutions were supplied 2 and 4 days after the initial treatment. Nitrogenase activity determined by the acetylene reduction technique was compared with control plants which received no combined N. Nitrogenase activity of control plants was 33.2 ± 1.7 μmol C_2H_4/g nodule fresh weight·hr during the experiment. (●—●): KNO_3; (○—○): NH_4Cl.

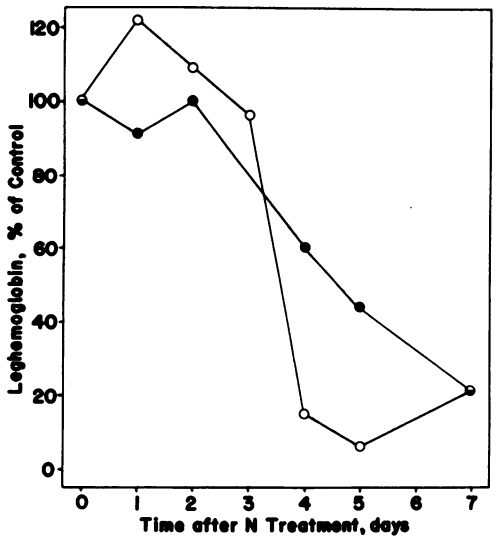

FIG. 2. Effect of root-absorbed nitrate and ammonium on root nodule leghemoglobin content in peas. Data were taken from the same plants represented in Figure 1. Leghemoglobin content of root nodules from treated plants was compared with root nodules of control plants which received no combined N. Leghemoglobin content of control plants was 69.9 ± 2.6 nmol/g nodule fresh weight during the experiment. (●—●): KNO_3; (○—○): NH_4Cl.

Table I. The effect of CO_2 enrichment on root nodule senescence of peas treated with 20 mM KNO_3 in the root substrate.

Time of nitrate applications, nitrogenase assays, and comparison with nitrate-free controls were identical to procedures used in Figs. 1 and 2. Concentrations of CO_2 were controlled from the time of planting.

Trait	Treatment	Time after initial nitrate treatment, da. 0	1	2	4	7
Nitrogenase activity/nodule mass·hr (KNO_3-treated plants as % of untreated controls)	.00032 atm CO_2	100	100	38	41	13
	.00120 atm CO_2	100	94	59	36	31
Leghemoglobin content/nodule mass (KNO_3-treated plants as % of untreated controls	.00032 atm CO_2	100	104	94	61	35
	.00120 atm CO_2	100	97	87	58	36

Efforts to detect nitrate reductase activity in extracts of leaves, roots, root tips, root nodule cytosol, and bacteroid fractions of *Pisum sativum* revealed activity in all fractions except bacteroids (Table II). Nitrate reductase activity could not be detected in roots and root tips until the extract was passed through Sephadex G-25. Bacteroids produced by 15 different strains of *R. leguminosarum* (kindly supplied by J. C. Burton, The Nitragin Co.) showed no nitrate reductase activity. Table II reveals that nitrogenase activity of treated plants declined 24 hr after moderate levels of nitrate reductase were detected in leaves and roots, and nitrate first appeared in the root nodule cytosol.

Leaf Applications of Nitrate. Data in Table III show that nitrogenase activity is not affected by the presence of nitrate and nitrate reductase activity in the leaves. Under the conditions used for these experiments, moderate levels of nitrate reductase were detected 6 hr after applying KNO_3 to the shoot and were maintained for an additional 90 hr. During this time neither nitrate nor nitrate reductase was detected in the roots or root nodules, and nitrogenase activity measured by acetylene reduction did not differ significantly between treated and untreated plants.

DISCUSSION

Our experiments indicate that addition of either NH_4Cl or KNO_3 to the rooting medium of nodulated *P. sativum* L. plants

Table II. The effect of root-absorbed nitrate on nitrogenase, nitrate reductase and nitrate levels in peas grown under 0.00032 atm CO_2.

The first treatment of 20 mM KNO_3 was supplied 28 days after planting, and a second treatment was given 48 hr later. Control plants grown without nitrate treatments contained no detectable nitrate reductase activity or nitrate.

	Time after initial nitrate treatment, hr			
	9	24	48	96
Nitrogenase activity/nodule mass·hr (KNO_3-treated plants as % of untreated controls)	100	100	41	37
Leaf nitrate reductase activity (μmol NO_2^- formed/g leaf fresh weight·hr)	0.68	5.42	7.33	5.85
Leaf nitrate (μg NO_3^-/g fresh weight)	6.21	38.9	55.2	51.9
Root nitrate reductase activity (μmol NO_2^- formed/g root fresh weight·hr)	0.21	1.52	2.43	2.56
Root nitrate (μg NO_3^-/g fresh weight)	56.4	78.3	123	109
Root nodule cytosol nitrate reductase activity (μmol NO_2^- formed/g nodule fresh weight·hr)	0	0.15	0.18	0.24
Root nodule cytosol nitrate (μg NO_3^-/g fresh weight)	0	15.8	17.8	22.4
Bacteroid nitrate (μg NO_3^-/g fresh weight)	0	0	0	0

Table III. The effect of foliar nitrate applications on nitrogenase, leaf nitrate reductase, and nitrate levels in peas grown under 0.00032 atm CO_2.

Nitrate was supplied every 24 hr beginning 28 days after planting by techniques specified under the methods section. Control plants grown without nitrate treatments contained no detectable leaf nitrate reductase activity or nitrate. Nitrogenase activity is reported as 100% when treated plants did not differ significantly from the control value of 33.8 ± 1.9 μmol C_2H_4/g nodule fresh weight hr.

	Time after initial nitrate treatment, hr			
	6	24	48	96
Nitrogenase activity/nodule mass·hr (KNO_3-treated plants as % of untreated controls)	100	100	100	100
Leaf nitrate reductase activity (μmol NO_2^- formed/g fresh weight·hr)	3.3	2.2	1.4	1.4
Leaf nitrate (μg NO_3^-/g fresh weight)	31.3	16.6	5.7	6.3
Root nitrate reductase activity (μmol NO_2^- formed/g fresh weight·hr)	0	0	0	0
Root nitrate (μg NO_3^-/g fresh weight)	0	0	0	0
Root nodule cytosol nitrate (μg NO_3^-/g fresh weight)	0	0	0	0

caused a decline in nitrogenase activity and leghemoglobin content determined by acetylene reduction and pyridine hemochrome assays (Figs. 1 and 2).

Two techniques were used to test the hypothesis that nitrogenase activity declined in the presence of nitrate as a result of competition with nitrate reductase for common cofactors such as ATP, reductant and C-skeletons. Increasing the concentration of CO_2 in the atmosphere surrounding the plant causes the movement of greater amounts of photosynthetic products to the root nodules in this system (9). Data in Table I reveal that raising the CO_2 concentration from 0.00032 atm to 0.00120 atm did not significantly alter the time course of nitrate-induced root nodule senescence. These results suggest that in spite of the fact that cofactors derived from photosynthetic products presumably were more available to decrease hypothetical competition between nitrate reductase and nitrogenase, nitrate still induced a rapid senescence of root nodules under the higher CO_2 concentration.

Results from a second test of the hypothesis are shown in Tables II and III. Leaf nitrate reductase, which accounts for a majority of the nitrate reductase in the present system, was induced by supplying nitrate to either the roots or the leaves. Root nodule senescence, however, was observed only when nitrate was supplied to the roots, in spite of the fact that leaf nitrate reductase induced by foliar applications of nitrate presumably utilized significant amounts of photosynthate.

Nitrogenase activity decreased before leghemoglobin content declined in all cases where root nodule senescence was observed. The possibility that nitrate exerted a direct, deleterious effect on nitrogenase probably can be eliminated. Data in Table II clearly show that nitrogenase activity declined 48 hr after the initial nitrate treatment in spite of the fact that bacteroids, where nitrogenase is located, contained no detectable nitrate. One must assume that the lack of nitrate in the bacteroids did not result from an active nitrate reductase. No bacteroids of *R. leguminosarum* exhibited nitrate reductase in the present study.

The short term induction of root nodule senescence observed in these experiments must be separated from longer term interactions between nitrate reductase and N_2 reduction studied by other workers (8). Oghoghorie and Pate (8) detected movement of nitrogen supplied as $^{15}NO_3^-$ into root nodules of *Pisum arvense* L. and discussed the concept that the root nitrate reductase previously demonstrated in their system (13), might compete with nitrogenase for products of photosynthesis. The results of the present study indicate that when nitrate reductase is present only in the shoot, sufficient photosynthate still reaches the nodules to maintain nitrogenase activity (Table III). On the other hand, under conditions where nitrate and nitrate reductase activity are present in the root system (Table II), supplying higher concentrations of CO_2, which increases net photosynthesis in this system (9), has no effect on root nodule senescence (Table I). These results strengthen the case presented by Rigaud (10) that products of nitrate reduction are responsible for depressing N_2 reduction by legume root nodules.

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