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\(_4\)Cl 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 contain 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\(_4\)NO\(_3\) 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 photosynthetic carbon was retranslocated out of pea root nodules on *Pisum sativum* L. plants treated with NaNO\(_3\). Such a movement of photosynthetic carbon could have a considerable effect on the energy available for N\(_2\) fixation (10).

Oghogorie 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 photosynthesis. If such competition results in root nodule senescence, it might be overcome by increasing the photosynthetic activity 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 VS2DB2052 solenoid. When activated, the solenoid allowed 99.5% pure CO\(_2\) to enter the plant growth chamber until the alarm was deactivated 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 500g for 5 min to remove cell debris followed by centrifugation at 10,000g 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 30,000g 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). However, 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...
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 KNO₃ or 100 mM NH₄Cl to pea roots induced a decline in both nitrogenase activity determined by the acetylene reduction assay and leghemoglobin content detected by the pyridine hemochromate 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 C₂H₂/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 NH₄Cl, treated plants contained 8% of the nitrogenase activity and 97% of the leghemoglobin found in untreated plants.

Increasing the CO₂ concentration from 0.00032 atm to 0.00120 atm had no significant effect on the decline of either nitrogenase activity or leghemoglobin content in root nodules of peas treated with applications of 20 mM KNO₃ 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 CO₂ 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.

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 KNO₃ 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 NH₄Cl or KNO₃ to the rooting medium of nodulated *P. sativum* L. plants
caused a decline in nitorgenase 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 CO₂ 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 CO₂ 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 CO₂ 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 photosynthesize.

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 N₂ reduction studied by other workers (8). Oghogho and Pate (8) detected movement of nitrogen supplied as 15NO₃⁻ 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 CO₂, 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 N₂ reduction by legume root nodules.

**LITERATURE CITED**