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Ferric Leghemoglobin in Plant-Attached Leguminous Nodules[1]

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Leghemoglobin (Lb) is essential for nitrogen fixation by intact leguminous nodules. To determine whether ferric Lb (Lb^{3+}) was detectable in nodules under normal or stressed conditions, we monitored the status of Lb in intact nodules attached to sweet clover (*Melilotus officinalis*) and soybean (*Glycine max* [L.] Merr.) roots exposed to various conditions. The effects of N_2 and O_2 streams and elevated nicotinate levels on root-attached nodules were tested to determine whether the spectrophotometric technique was showing the predicted responses of Lb. The soybean and sweet clover nodules' Lb spectra indicated predominantly ferrous Lb and LbO_2 in young (34 d) plants. As the nodule aged beyond 45 d, it was possible to induce Lb^{3+} with a 100% O_2 stream (15 min). At 65 d without inducement, the nodule Lb status indicated the presence of some Lb^{3+} along with ferrous Lb and oxyferrous Lb. Nicotinate and fluoride were used as ligands to identify Lb^{3+}. Computer-calculated difference spectra were used to demonstrate the changes in Lb spectra under different conditions. Some conditions that increased absorbance in the 626 nm region (indicating Lb^{3+} accumulation) were root-fed ascorbate and dehydroascorbate, plant exposure to darkness, and nodule water immersion.

Lb, a myoglobin-like hemoprotein, is found in nitrogen-fixing leguminous root nodules, where Lb^{2+} binds with O_2 to facilitate the transfer of O_2 through the cytoplasm of the infected cell and acts as a compartmentalized buffer (Appleby, 1984). Because of its unique characteristics, Lb^{2+} supplies O_2 to the bacteroids for ATP generation while protecting nitrogenase in the bacteroids from O_2 damage. Although free and oxygenated forms of Lb^{2+} are thought to be predominant in vivo (Appleby, 1969; Layzell et al., 1990; Denison et al., 1991), other forms including Lb^{3+} and ferryl Lb and complexes with various ligands such as NO, nicotinate, and CO may exist in nodules. Lb^{3+} can be produced by an electron oxidation of Lb^{2+} or autoxidation of oxygenated Lb^{2+}. To maintain the functional state of Lb, enzymatic systems should exist in nodules to reduce Lb^{3+} to Lb^{2+}. Several research groups (Puppo et al., 1980; Kretovich et al., 1982; Saari and Klucas, 1984; Borodenko et al., 1990) have partially purified proteins that reduce Lb^{3+} to Lb^{2+}. We have isolated, purified, and sequenced FLbR from soybean (*Glycine max* [L.] Merr.) nodules (Saari and Klucas, 1984; Ji et al., 1991, 1992, 1994a, 1994b). FLbR reduces Lb^{3+} to Lb^{2+} using NADH as the reductant and is postulated to perform this reduction in nodules.

To our knowledge, Lb^{3+}, the substrate for FLbR, has not been demonstrated in intact nodules. Previously, we used spectrophotometric methods to show that Lb^{3+} can be induced and subsequently reduced in soybean nodule slices by treatment with hydroxylamine (Lee and Klucas, 1984). The objective of this study was to search for Lb^{3+} in intact nodules attached to roots using a technique that was described earlier (Klucas et al., 1985). We report the evidence that supports the existence of Lb^{3+} in aging intact sweet clover (*Melilotus officinalis*) and soybean nodules.

MATERIALS AND METHODS

Plant Culture

Yellow blossom sweet clover (*Melilotus officinalis*) seeds were surface sterilized, inoculated with *Rhizobium meliloti* (American Type Culture Collection 10312), and planted in pots containing Metromix (Scotts Co., Hope, AR) or washed sand. Soybean (*Glycine max* [L.] Merr. cv Woodworth) seeds were surface sterilized and inoculated with *Bradyrhizobium japonicum* SR123. To obtain larger numbers of somewhat flattened, translucent nodules, soybean seeds were planted in Metromix or washed sand that was placed in the outer perimeter of an 8-inch pot with a 5-inch pot concentrically positioned on the inside. Plants were grown in a plant growth chamber (24°C, 14-h photoperiod with a PPFD of 500 μmol m^{-2} s^{-1}) and supplied with a nitrogen-free nutrient solution (Evans et al., 1972). Plants at 20 to 40 d after sowing (sweet clover) or 30 to 70 d after sowing (soybean) were used for measuring Lb in nodules attached to roots.

Spectrophotometric Technique and Conditions Surrounding the Nodule

Cuvettes with volumes of 70 or 275 mL, depending on the size of the plant and root system, were constructed

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Abbreviations: FLbR, ferric leghemoglobin reductase; Lb, leghemoglobin; Lb^{2+}, ferrous Lb, which combines reversibly with O_2 to give LbO_2; Lb^{3+}, ferric Lb; LbO_2, oxyferrous Lb.

from plastic disposable tissue culture flasks with canted necks (Corning Glass Works, Corning, NY) (Fig. 1). A plant with carefully rinsed roots was placed in the cuvette with the aerial portion of the plant projecting from the neck and with the nodulated root inside the cuvette. An exterior portion of the back of the cuvette was covered by blackened aluminum foil with a pinhole (0.4–0.5 mm in diameter). The pinhole served as a light exit when positioned behind the monitored nodule. The actual size of the pinhole could be varied to accommodate different sizes of nodules. The front of the cuvette had a 2.5-cm hole that was sealed by a rubber stopper with a glass vial passing through it. A nodule attached to the root was located between the cuvette's back wall and the bottom of the glass vial that projected from the front wall, with the pinhole on the blackened foil positioned on the back of the cuvette directly behind the nodule. The vial was pushed into the stopper until the nodule was almost in contact. The nodule was suspended from the roots of the plant and was not held in place by any other means. The stem of the plant was gently secured at the neck of the cuvette with wet sponge strips. When airtight conditions were required, modeling clay was used to seal the stem in the opening of the neck.

The cuvette was placed into the sample compartment of a Cary 219 spectrophotometer (Varian, Sugar Land, TX) with the back side adjacent to the photomultiplier tube and adjusted so that the nodule was aligned with the incident light. The cover on the sample compartment of the spectrophotometer was modified so that the aerial portion of the plant extended through it. Care was taken to keep the nodules moistened during the procedure. Direct spectra of light passing through the nodule were routinely obtained from 500 to 650 nm with a scanning Cary 219 spectrophotometer with light filters in the reference beam to balance the light passing through the nodule. For difference spectra, the data were analyzed using a computer interfaced with the spectrophotometer. Two ports on the top of the cuvette were used for changing the nutrient solution, adding chemical compounds, or flushing with gas. These manipulations could be accomplished without moving the nodule being scanned.

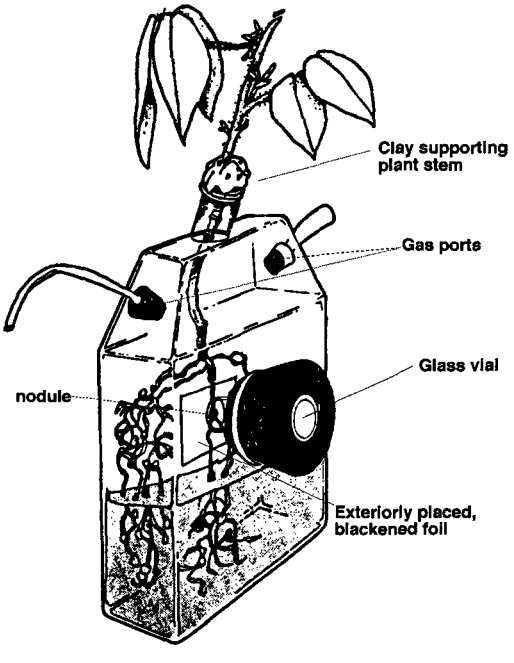

Figure 1. Diagram of the apparatus for spectrophotometric monitoring of an intact nodule attached to a whole plant. All spectra were recorded under room lighting (fluorescent) conditions, unless stated otherwise.

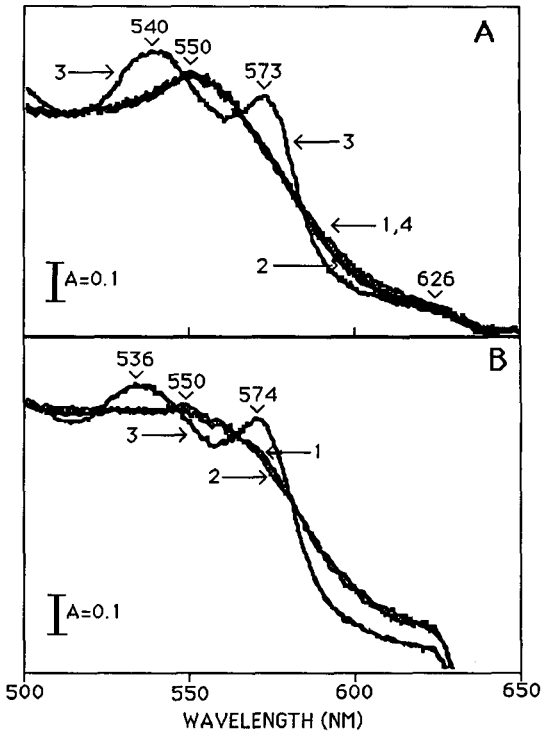

Figure 2. Spectra of Lb in intact nodules of sweet clover (A) and soybean (B), sequentially equilibrated with air for 15 min (line 1), 100% N_2 for 15 min (line 2), 100% O_2 for 30 min (line 3), and then air for 30 min (line 4).

In the experiments in which the same attached nodule was monitored at different times, the cuvette containing the plant was returned to the plant growth chamber and aerated by an air pump until the next spectrophotometric observation.

Nicotinate effect on soybean nodules attached to the roots of 31- to 45-d-old plants was tested by the addition of 10 mM nicotinate (pH 6.5) to the nutrient solution below the nodule in the cuvette. Control plants were treated similarly but without nicotinate in the nutrient solution. The plants were treated for periods up to 24 h. When treatment ended, the nutrient solution with nicotinate was replaced by nutrient solution and monitoring continued periodically for up to 3 d.

Conditions tested for effects on nodule spectra were immersing the nodule in water and putting the plant top in darkness. The effect of root imbibition of the following chemicals on nodule spectra was tested: nicotinate (10 mM), F^- (15 mM), and ascorbate and dehydroascorbate (5 mM).

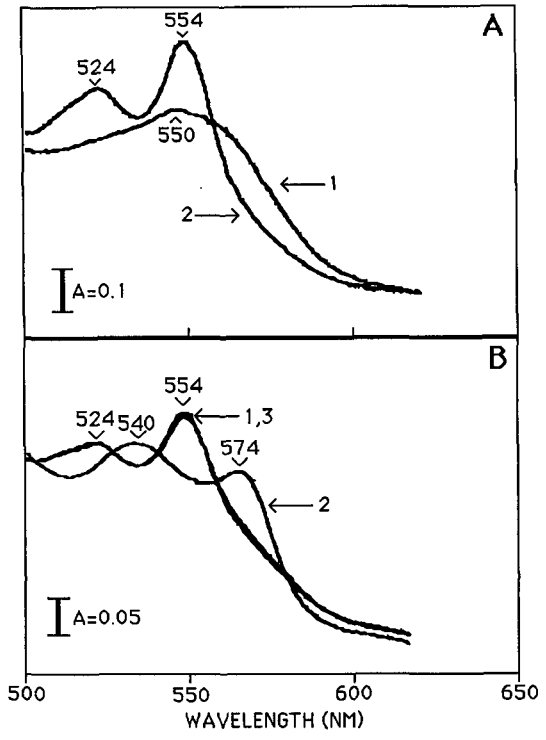

Figure 3. Spectra of Lb^{2+} in intact sweet clover nodules root-fed nicotinate (10 mM) and exposed to an O_2 stream. A, Spectra of a nodule in air (line 1) and the same after 120 min of root-fed nicotinate (line 2). B, Spectra of a nodule root-fed nicotinate for 6 h (line 1), the same nodule exposed to an O_2 stream for 10 min (line 2), and after 8 min of exposure to air (line 3).

Difference spectra were calculated to show the changes caused by the treatments.

Lb was purified from soybean root nodules using the method described by Saari and Klucas (1984). Absorption maxima of Lb isolated from soybean nodules (Dilworth and Appleby, 1979) are 555 nm for Lb^{2+}, 574 and 540 nm for $Lb^{2+}O_2$, 554 and 525 nm for Lb^{+2}-nicotinate, 626 nm for Lb^{3+}, 605 nm for Lb^{+3}-fluoride, and 557 and 527 nm for Lb^{+3}-nicotinate. Absorption maxima were not available for Lb isolated from sweet clover nodules, but spectral properties of most of the symbiotic Lbs from leguminous nodules are similar. A partially purified preparation of Lb from sweet clover nodules exhibited spectral characteristics similar to those of Lb from soybean nodules (data not shown).

RESULTS AND DISCUSSION

The absorption spectra from 500 to 650 nm of light passing through nodules were successfully obtained on intact nodules of sweet clover (Fig. 2A) and soybean (Fig. 2B). The smaller indeterminate nodules on sweet clover roots were relatively easy to position and scan because the light path through the nodule was short. Spectra of soybean nodules were more difficult to obtain because of the heavier cortex layer of these nodules; the best spectra were obtained from slightly flattened nodules that had a thin cortex layer. A series of experiments, in which predicted changes were observed when nodules were exposed to various conditions, was done to establish that Lb was the chromophore being measured in nodules.

Spectra of Intact Nodules Equilibrated with Various Gases

The spectrum from a sweet clover nodule in air typically had an absorption peak at 550 nm (Fig. 2A, line 1). Exposure of the same nodule to 100% N_2 for 15 min changed the spectrum slightly (Fig. 2A, line 2), and subsequent exposure to 100% O_2 for 30 min changed the spectrum to show two absorption peaks, at 540 and 573 nm (Fig. 2A, line 3). This evidence is consistent with spectral characteristics of Lb^{2+} and LbO_2, respectively. Re-exposure of the nodule to air for 30 min shifted the spectrum back to an absorption peak at 550 nm (Fig. 2A, line 4). However, the shoulder at approximately 626 nm (Fig. 2A, line 3) suggests that Lb^{3+} may be present in sweet clover nodules under certain conditions. The same changes in the spectra of soybean nodules in air, 100% N_2, and then 100% O_2 were observed (Fig. 2B, lines 1, 2, and 3, respectively). No shoulder at 626 nm, which would indicate Lb^{3+}, was observed.

Spectra of Nodules Supplied with Nicotinate through the Roots

Nicotinate is a ligand that reversibly binds to Lb^{2+} and Lb^{3+} and not to any other heme proteins in the nodule (Appleby et al., 1973; Appleby, 1984). If nicotinate is imbibed by the roots and translocated to the nodule, the

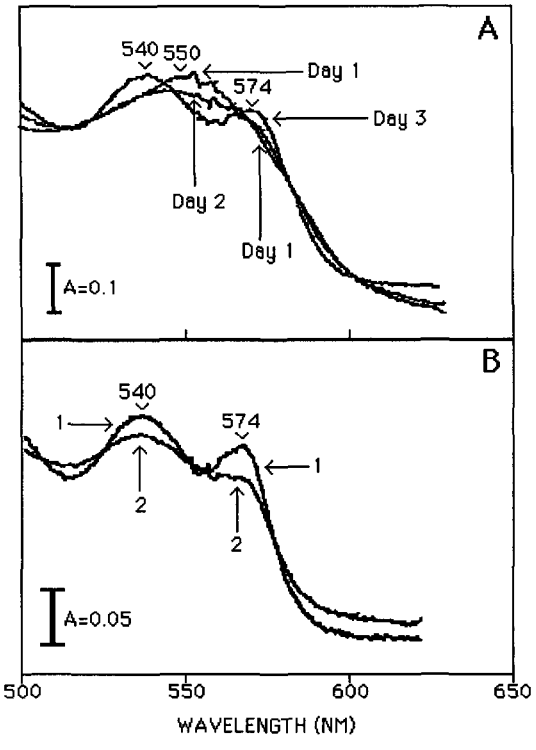

Figure 4. Spectra of an intact sweet clover nodule. A, Spectra recorded at 24-h intervals. B, Spectra of the proximal (line 1) and distal (line 2) regions of the nodule on the 3rd d.

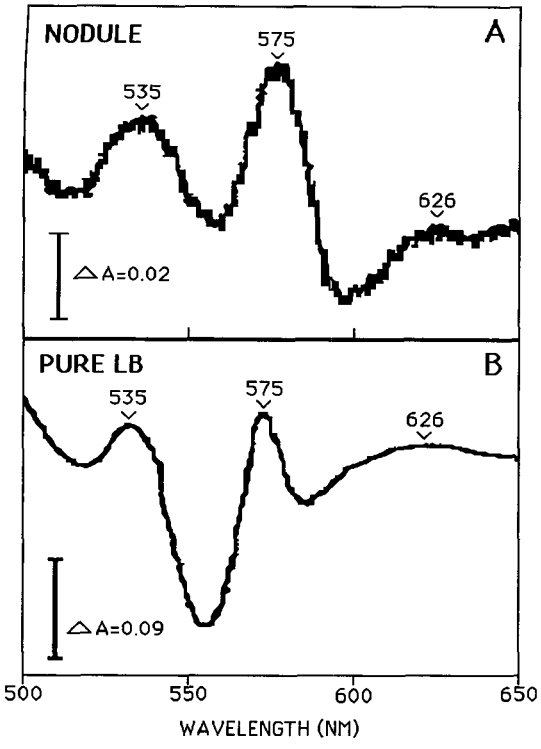

Figure 5. *A* changes of soybean Lb showing Lb^{3+} in the intact nodule and in pure preparation. A, Intact nodule (53 d): difference spectrum between the spectra after O_2 treatment (15 min) and N_2 treatment (15 min) of the same nodule. B, Difference spectrum between the spectrum of pure Lb^{2+} (37 mM) sparged with O_2 (10 min) and the spectrum of Lb^{2+} after 10 min of N_2 sparging.

formation of the Lb-nicotinate ligand would substantiate that Lb was being observed in nodules (Lee and Klucas, 1984). Spectra were taken of nodules attached to either sweet clover (Fig. 3A) or soybean roots (data not shown). The nodule being examined was positioned above the solution containing 10 mM nicotinate, in which the lower part of the root was immersed. Spectra from a sweet clover nodule exposed to nicotinate at zero time (Fig. 3A, line 1) and 2 h (Fig. 3A, line 2) are shown. The initial spectrum exhibits a typical free Lb^{2+} absorption peak at 550 nm. At 2 h, the spectrum changed to show two distinct absorption peaks, at 524 and 554 nm, which clearly indicated the formation of Lb^{2+}-nicotinate complex. This Lb^{2+}-nicotinate complex in nodules in air was still observed 24 h after the nutrient solution was replaced by nicotinate-free nutrient solution (data not shown).

To determine whether the Lb^{2+}-nicotinate complex was reversible under high concentrations of O_2, a sweet clover nodule was supplied with 10 mM nicotinate through the roots for 6 h, and then nicotinate was removed. The spectrum exhibited Lb^{2+}-nicotinate absorption peaks (Fig. 3B, line 1). The nodule was then exposed to 100% O_2 for 10 min. The Lb^{2+}-nicotinate spectrum was shifted to an oxygenated-Lb^{2+} spectrum (Fig. 3B, line 2). When this nodule was returned to air or flushed with N_2, the oxygenated-Lb^{2+} spectrum shifted back to one dominated by Lb^{2+}-nicotinate (Fig. 3B, line 3). The result with soybean nodules was essentially the same. The spectra demonstrate that nicotinate was taken up by the roots, translocated to the nodules within 2 h, and ligated to the Lb^{2+} reversibly.

Long-Term Spectral Changes within a Single Nodule

To determine the effect of aging on the nodule, we examined a single sweet clover nodule during a 5-d period and differently aged segments of a single nodule. A young, elongated sweet clover nodule was placed in a monitoring cuvette. The pinhole of the aluminum foil mask that directed light to the spectrophotometer was enlarged to include most of the nodule. Initially, the spectrum showed typical Lb^{2+} (Fig. 4A, d 1). On the 2nd d, a slight shoulder at approximately 574 nm became visible (Fig. 4A, d 2), and on the 3rd d, the spectrum clearly exhibited two absorption peaks, at 540 and 574 nm, indicating gradual increases in oxygenation of Lb^{2+}. On the 3rd d, the pinhole was reduced in size and positioned so that the half of the nodule nearest the root (proximal half) and the half farthest from the root (distal half) were monitored separately. The proximal half, which is the older section of an indeterminate nodule, clearly exhibited an oxygenated-Lb^{2+} spectrum (Fig. 4B, line 1), whereas the distal half showed a less oxygenated-Lb^{2+} spectrum (Fig. 4B, line 2). On the 4th d, the whole nodule still maintained an oxygenated-Lb^{2+} spectrum, but its absorption was much lower than on the 3rd d (data not shown), and on subsequent days, no ab-

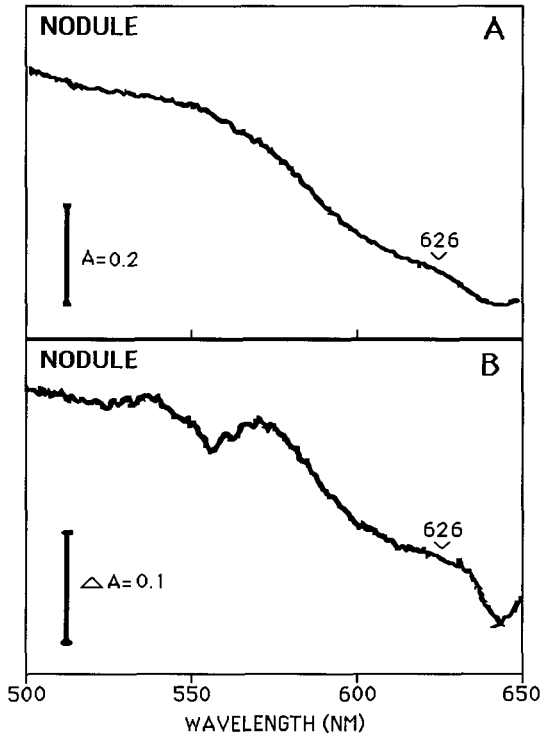

Figure 6. Intact soybean nodule (65 d) spectra. A, Direct spectrum of a nodule with no treatment. B, Difference spectrum between the above spectrum and the spectrum of the same nodule after 24 h of root-fed nicotinate.

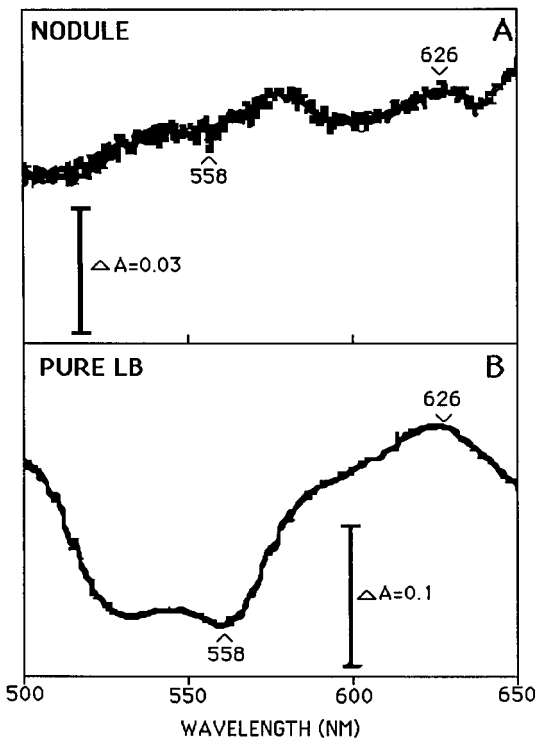

Figure 7. Difference spectra showing *A* changes of soybean Lb^{3+} in intact nodules and pure preparation. A, Spectrum of intact nodule (52 d) exposed to O_2 (15 min) preceded by N_2 (15 min) minus the spectrum of the same nodule after 3 h of root-fed nicotinate. B, Purified Lb^{3+} spectrum minus Lb^{3+}-nicotinate spectrum.

sorption peaks were observed. No absorption peak at 626 nm, which would indicate Lb^{3+}, was observed.

Observations of other sweet clover nodules invariably repeated the same pattern, although the time required for free Lb^{2+} to become oxygenated varied with different nodules. Within a single nodule, the spectra in proximal parts always showed more oxygenation than those in distal parts. As indicated by the absence of an absorption peak at 626 nm, free Lb^{3+} was not detected. If it was present, it was below the level of detection. It was observed that nodules' spectra indicated the presence of LbO_2 for 2 or 3 d preceding nodule death, as indicated by a straight line spectrum and no spectral response to N_2 or O_2 streams. An LbO_2 spectrum could be induced from control plants in air by allowing desiccation. Recovery by rehydration was possible if desiccation and nodule senescence were not too advanced.

Demonstration of the Presence of Lb^{3+} in Soybean Nodules in Vivo

Detection of low levels of Lb^{3+} in soybean nodules by spectrophotometric methods is complicated by the low extinction coefficient at 626 nm, the most prominent feature of the Lb^{3+} spectrum. Soybean nodules were used in this study because they were hardier over long monitoring periods than were sweet clover nodules. Two methods involving predicted spectral changes of Lb^{3+} ligands were used to identify Lb^{3+} in nodules. The first method is based on the disappearance of the 626-nm absorption peak when nicotinate binds to Lb^{3+}. Typically, an intact nodule showing possible Lb^{3+} A_{626} was root-fed nicotinate; the nodule was repeatedly scanned during the exposure period. The ensuing disappearance of A_{626} was indicative of Lb^{3+} in the nodule. The second method is based on a spectral shift when F^- ligates to Lb^{3+}. A plant with a monitored nodule showing A_{626} was root-fed F^-. A shift from 626 to 605 nm is indicative of F^- binding to Lb^{3+}. Computer-generated difference spectra were used to detect these small changes from before-treatment spectra to after-treatment spectra. The scanned nodule was not moved from the spectrophotometer during the treatment period.

Nodules from 45- to 60-d-old plants exposed to 100% O_2 (15 min) produced shoulders at 626 nm along with typical LbO_2 peaks. The difference spectrum (Fig. 5A) of a 59-d-old nodule treated as described above preceded by 100% N_2 (15 min) minus the spectrum after 100% N_2 (15 min) intensified the changes in A_{626}, indicating Lb^{3+}, as well as differences at 575 and 535 nm, indicating oxygenation of Lb^{2+}. The N_2 treatment was not required for the change in A_{626}. Figure 5B shows the comparable difference spectrum of pure Lb^{2+} (37 mM) sparged gently with 100% O_2 for 10 min minus the same after 10 min of N_2 sparging. The slight *A* peak of the pure Lb at 626 nm (Fig. 5B) resulted from a small conversion of LbO_2 to Lb^{3+} during O_2 sparging. Older plants (52–75 d) showed peaks at 626 nm without

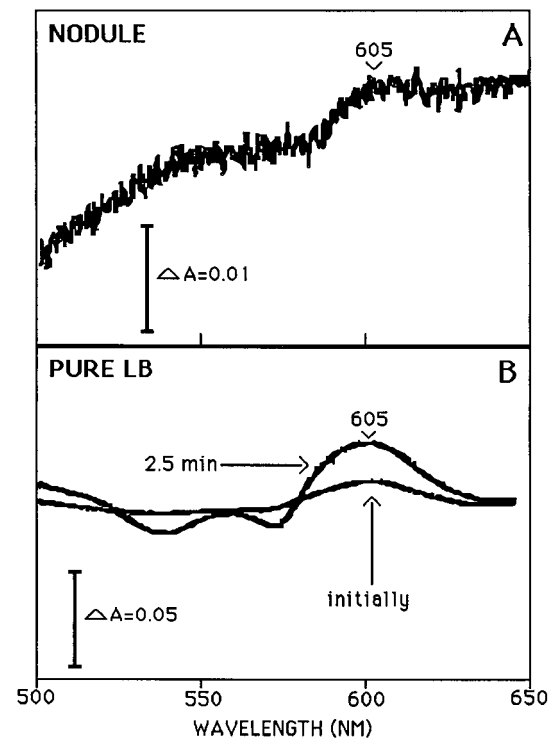

Figure 8. Difference spectra of intact soybean nodule and pure Lb. A, Spectrum of intact nodule (59 d) exposed to an O_2 stream (15 min) and root-fed F^- (90 min) minus spectrum at the initiation of F^- feeding. B, Pure Lb^{2+} sparged with O_2 in presence of F^- at initial time and after 2.5 min of sparging minus the spectrum before the F^- was added.

O_2 inducement in direct spectra (Fig. 6A, 65 d) but more distinctly in difference spectra. Figure 6B shows a nodule (65 d) in air minus the nodule at 24 h of nicotinate imbibition.

The difference spectrum of an intact soybean nodule (52 d) exposed to an O_2 stream (15 min) preceded by 15 min of N_2 exposure minus the same after 3 h of root-fed nicotinate (Fig. 7A) was compared to the difference spectrum of purified soybean Lb^{3+} minus Lb^{3+}-nicotinate (Fig. 7B). The *A* shoulder at 626 nm in these difference spectra is indicative of Lb^{3+} in both the nodule and the pure Lb.

The difference spectrum of soybean nodules (59 d) root-fed F^- for 90 min minus the initial treatment spectrum shows an increase in A_{600} to A_{610} (Fig. 8A), which indicates that Lb^{3+} is bound to F^-. The nodule difference spectrum is comparable to the spectrum of pure Lb^{2+} in the presence of F^- sparged with O_2 over time minus the spectrum of LbO_2 (Fig. 8B). The increased A_{605} is due to the presence of Lb^{3+}-fluoride.

Other treatments induced an increase in A_{626} that was detectable in the difference spectra of nodules (Fig. 9). The effects of darkness and water immersion were examined as part of a range of treatments emulating natural conditions. The reason for the nodule-spectral response is unknown. The effect of ascorbate or dehydroascorbate was tested on the spectra of intact soybean nodules because of the abundance of ascorbate in nodules (Dalton et al., 1986) and the possibility that ascorbate or dehydroascorbate might be involved in an oxidation-reduction cycle involving Lb.

Ascorbate imbibed by the roots of a 34-d-old soybean plant for 1 and 5 h caused no spectral changes in the nodule. However, 24 h of treatment caused an increase in A_{626} (Fig. 9A, line 1), which disappeared 3 h after nutrient solution plus ascorbate was replaced by nutrient solution plus nicotinate (Fig. 9A, line 2). This pattern suggests that Lb^{3+} was converted to Lb^{3+}-nicotinate with the accompanying disappearance of the peak at 626 nm.

Dehydroascorbate root-fed to a soybean nodule (62 d) caused increases in A_{626}, A_{575}, and A_{536} (Fig. 9B). The difference spectrum was obtained as the spectrum of the nodule treated for 24 h with dehydroascorbate minus the spectrum before treatment. The reason for the nodule-spectral response to ascorbate or dehydroascorbate is not known.

Subjecting the soybean plant to darkness caused rapid changes in the nodule spectra. Fifteen and 30 min after fluorescent lighting in a windowless room (PPFD of 30 μmol m^{-2} s^{-1}) was turned off, a plant-attached soybean nodule (70 d) showed an increase in A_{626} (Fig. 9C), with a concomitant reduction in peaks at 575 and 540 nm. Figure 9C shows the difference spectra between the spectrum at 15 or 30 min of darkness and a spectrum when the plant was previously exposed to room lighting. The difference spectrum between the spectrum at the initiation (2–3 min) of

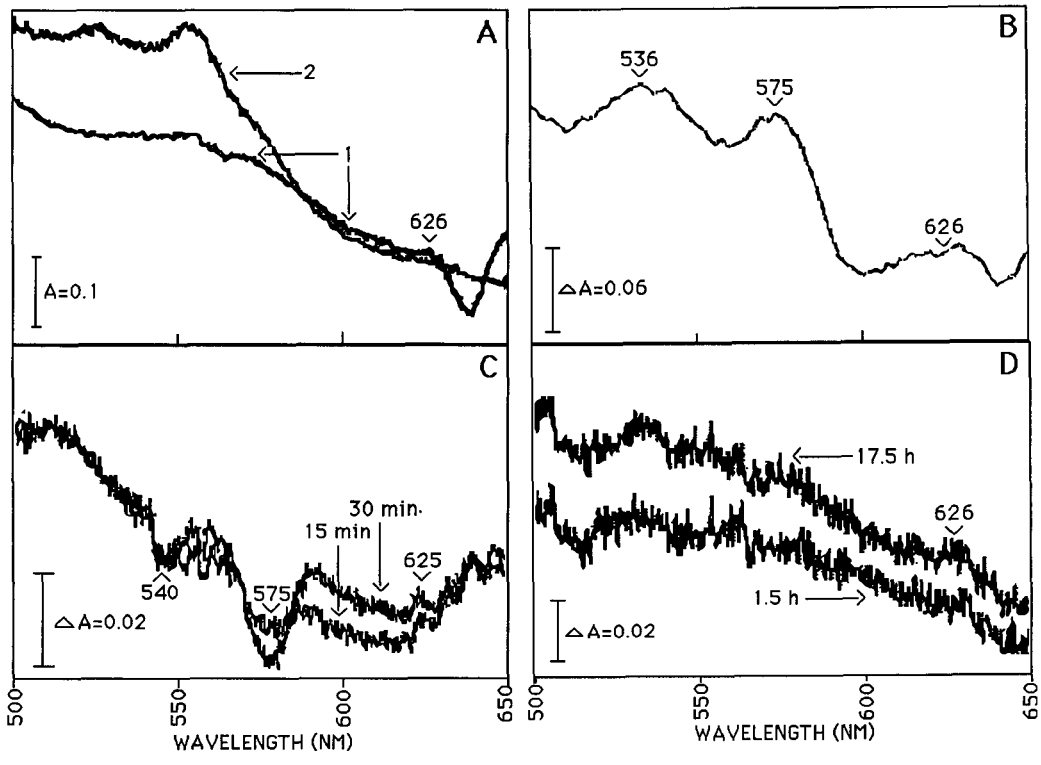

Figure 9. Spectra of intact soybean nodules under conditions that resulted in increased A_{626}. A, Direct spectra of a nodule (34 d) after 24 h of ascorbate (5 mM) (line 1) compared to the same nodule after ascorbate was replaced by nicotinate (10 mM) for 3 h (line 2). B, Difference spectrum of a nodule root-fed dehydroascorbate (5 mM) for 24 h minus the spectrum before treatment. C, Difference spectra of a root-attached nodule (70 d) at 15 and 30 min of plant top darkness minus the spectrum of the nodule with the plant top in light. D, Difference spectra of an immersed nodule (62 d) after 1.5 or 17.5 h of immersion minus the spectrum at the initiation of the treatment.

darkness and the spectrum when the plant was in light showed no change at 626 nm (data not shown), indicating that the change was not an artifact.

Immersion of several soybean (57, 62, 65, and 76 d) nodules in water (21 h) increased the A_{626}. Figure 9D shows difference spectra of a nodule (62 d) at 1.5 and 17.5 h of immersion minus the spectrum at initiation of the treatment. After the immersion treatment, root-fed F^- caused A peak shifts from 600 to 610 nm in 1.5 h. In other nodules, exposure to air for 5 to 6 h after immersion removed the peak at 626 nm (data not shown).

We have presented qualitative evidence that Lb^{3+} is detectable in soybean and sweet clover nodules using direct spectrophotometry of intact nodules attached to roots. The observed spectral changes in the nodules were confirmed to be attributable to Lb by comparisons with known characteristics of Lb's, which included predicted changes when Lb^{+3} and Lb^{+2} are exposed to O_2, nicotinate, and F^-. Lb^{3+} was shown to be present in nodules that were exposed to high levels of O_2 or to other treatments, but it was also detected in untreated nodules, especially in older nodules. Although Lb^{3+} could not be detected at atmospheric levels of oxygen in 34-d-old nodules, difference spectra showed Lb^{3+} to occur in older nodules (52 d) as well as in younger nodules (45 d) exposed to 100% O_2 (15 min), sometimes, but not necessarily, preceded by 100% N_2 (15 min). This effect was observed also in nodule slices (Lee and Klucas, 1984). The spectra of intact nodules in response to root-fed nicotinate and F^- confirmed the presence of small amounts of Lb^{3+} in the intact nodule.

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