

## ***Symbiotic Nitrogen Fixation in Regenerating Black Locust (*Robinia pseudoacacia* L.) Stands***

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**ABSTRACT.** Black locust (*Robinia pseudoacacia* L.) is a dominant early successional tree in the southern Appalachians that symbiotically fixes N, grows rapidly, and has a relatively short life span. Objectives of this research were to study seasonal patterns of nodule biomass and N-fixation activity in 4-year-old black locust stands over the span of one year, and to determine the importance of symbiotic N fixation as an input to a regenerating forest at the Coweeta Hydrologic Laboratory.

Acetylene ( $C_2H_2$ ) reduction activity for nodules  $>3$  mm in length was highest during midday assays in June and September, attaining maximum values of  $1.00 \mu l C_2H_4$  mg dw  $hr^{-1}$  and  $0.94 \mu l C_2H_4$  mg dw  $hr^{-1}$ , respectively. No activity was detected in November and March. Live nodule biomass was mostly (67 percent) concentrated in the upper 15 cm of soil. Nodule biomass decreased from  $3.59 g m^{-2}$  in May to  $0.83 g m^{-2}$  in November potentially because of a summer drought during the study year.

The 4-year-old stands were estimated to have fixed  $30 kg N ha^{-1} yr^{-1}$  during the study period. Nitrogen input to the early successional forest from symbiotic fixation is large in comparison to atmospheric inputs of N and free-living N fixation reported by other investigators. FOREST SCI. 30:528-537.

**ADDITIONAL KEY WORDS:** Forest regeneration, succession, nodule biomass.

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EARLY FOREST REGENERATION on mesic sites in the southern Appalachians is patchily dominated by dense stands of black locust (*Robinia pseudoacacia* L.), a N-fixing woody legume with a relatively short life span (McGee and Hooper 1975, Boring and others 1981). Black locust comprises  $<2$  percent of the basal area in undisturbed forests, but aggressively root sprouts into disturbed sites. It may comprise up to 30 percent of the basal area on mesic sites 3 years after clearcutting, and 67 percent after 13 years of pasture abandonment (Boring and Swank, in press). The high-density stands grow rapidly, but growth rate declines by 15-20 years except on the best sites (Swank and Helvey 1970, Boring and Swank, in press).

Black locust has been used in the United States for surface mine reclamation (Jencks and others 1982), erosion control and fencepost production (Fowells 1965), and is currently being evaluated for use in short-rotation plantations as a biomass fuel source (Eigel and others 1980, Zimmerman and Carpenter 1980). The po-

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tential value of black locust for N fixation and forest products is perhaps better recognized in other parts of the world, particularly in Europe and Asia, than in North America. In these areas it is utilized for a diverse range of products, to include paneling, construction boards, fuelwood, fenceposts, livestock forage, and nectar for honey production (Keresztesi 1980).

Rates of symbiotic N-fixation in natural black locust stands are unknown, although one study in a 20-year-old black locust plantation estimated an average annual accretion of 35 kg N ha<sup>-1</sup> in the top 50 cm of soil (Ike and Stone 1958). However, this estimate did not include N accumulation in biomass, denitrification, or leaching losses of NO<sub>3</sub>, and hence represents a minimal N-fixation estimate. Given these considerations, the rates may be comparable to those reported for *Alnus rubra*, *Ceanothus velutinus*, and the lower range of rates for agricultural legumes (Zavitkowski and Newton 1968, Evans and Barber 1977, Tripp and others 1979). Acetylene reduction studies have shown that nitrogenase activity in black locust nodules approximates the activity of these other species (McNiel and Carpenter 1974, Hensley and Carpenter 1979, Moiroud and Capellano, in press). However, none of these black locust studies has combined seasonal *in situ* acetylene reduction assays with measurements of nodule biomass to estimate annual rates of N-fixation.

The long-term goal of this research is to define the role of black locust in the successional dynamics of forest structure and processes in the Coweeta Basin of North Carolina. The process studies include N-fixation, nutrient accretion in biomass, and nutrient transfers to the forest floor. Here, results are reported for 4-year-old stands which address the following objectives: (1) the determination of diurnal and seasonal patterns of activity in three size categories of black locust nodules; (2) the quantification of seasonal patterns of nodule biomass; (3) the estimation of annual rates of symbiotic N-fixation by black locust; and (4) an assessment of black locust's relative importance to ecosystem N input.

#### STUDY SITE

The black locust stands used for this study are located at the USDA Forest Service's Coweeta Hydrologic Laboratory near Franklin, North Carolina. The Coweeta Basin is in the Nantahala Mountains, part of the Blue Ridge Province in the southern Appalachians. The study sites are located within 100 m distance of each other on WS 7, a 59 ha watershed with a south aspect which was clearcut in 1977. The elevation is approximately 890 m and the slope averages 30°. The dominant soil series on these slopes is the Chandler, a member of the coarse-loamy, micaceous, mesic family of Typic Dystrochrepts. The mean annual temperature is 13°C, and the mean annual precipitation is 181 cm (Day and Monk 1974).

The study stands were dominated by vigorous, 4-year-old black locust which originated from root sprouts. There was a density of 2,760 black locust stems ha<sup>-1</sup>, and the species comprised 69 percent of the total aboveground woody and herbaceous biomass of 22,045 kg ha<sup>-1</sup>. Many of the stems exceeded basal diameters of 8 cm and heights of 8 m. Other dominant species present included *Rubus* spp., *Cornus florida*, *Liriodendron tulipifera*, and *Vitis aestivalis* var. *argentifolia*. The sites were formerly occupied by uneven-aged, mesic oak-hickory forest, dominated by *Quercus prinus*, *Quercus rubra*, *Carya* spp., *Acer rubrum*, and *Liriodendron tulipifera*. Before clearcutting, black locust comprised less than 2 percent of the total biomass, and 1 percent of total stem density (>2.5 cm dbh) on these sites (Boring and Swank, in press). The parent black locust stems probably originated from the disturbances created by the chestnut blight in 1930-1940 and selection cutting in 1927.

When the watershed was clearcut in 1977, the cutting operation consisted of

removing marketable timber by cable logging and felling of all other stems. Although several small areas were logged by a tracked skidder, the vast majority of timber removal was by cable. All logging debris was left in place with no further site preparation. Soil compaction and other disturbances of the forest floor and plant roots were minimized by this harvest technique.

#### METHODS

*Acetylene Reduction Assays.*—The acetylene ( $C_2H_2$ ) reduction assay is a sensitive yet portable, simple, and inexpensive technique for measuring  $N_2$  fixation (Hardy and others 1968, Hardy and others 1973). The techniques used were adapted from several which have received extensive use in ecological field studies of symbiotic  $N_2$  fixation (Morris and others 1974, McNabb and Geist 1979, Tripp and others 1979).

Acetylene reduction activity was determined for: (1) nodules of different sizes, to include < 3 mm, 3–10 mm, and > 10 mm in length; (2) diurnal patterns during June and September, at 0100, 0630, 0930, 1230, 1430, and 1900 hours; (3) seasonal patterns, for March, June, September, and November.

Although assays with attached nodules are preferable whenever possible, patchiness of nodule distribution on roots made this approach untenable for field studies. Instead, excised nodules were utilized. Initial time-sequence assays indicated significant decreases in activity between the 0.25 and 3 hour incubations (Fig. 1). To avoid this loss of activity, presumably due to depletion of stored carbon reserves, assays were quickly initiated within 0.25–0.50 hour following digging and removal of nodules from the root. Incubation times used were only 0.5 hour. This assay time was the minimum incubation period used, since it permitted more consistent gas diffusion and detection of activity. Although it was not possible to compare activity of excised nodules with attached nodules in the field, this technique at least minimized time-dependent decreases in nodule activity following abscission.

The *in situ* field assay consisted of six steps: (1) nodules were obtained by digging up roots within a sample plot which was also sampled for nodule biomass; (2) the nodules were removed and briefly stored in a closed container, with moistened paper, on the shaded forest floor; (3) nodules were sorted by sizes and placed into each of seven replicate 50 ml incubation flasks and capped with a moistened serum stopper; (4) 10 ml of  $C_2H_2$  were injected into seven flasks using a syringe fitted with a Mininert valve to give more precision to gas injections (Supelco, Supelco Park, Bellefonte, PA); one flask with nodules but no  $C_2H_2$  was used as a gas blank, and one flask without nodules was injected with  $C_2H_2$  as a nodule blank; (5) the flasks were then buried in soil to 5 cm depth and covered with litter to maintain soil temperature for a 30 minute incubation period; (6) 10 ml of gas sample were transferred from each flask (with the syringe/valve) to Hungate tubes (Bellco, Vineland, NJ) which were used for storage and transport. Soil moisture, barometric pressure, air and soil temperatures were recorded with each assay. The nodules were later rinsed to remove soil particles and dried to constant weight at 50°C for biomass determination. Gas samples were analyzed on a Varian Aerograph 1400 gas chromatograph equipped with a flame ionization detector at 210°C, a column packed with Porapak R at 60°C, and helium carrier gas with a flow rate of 30 ml  $min^{-1}$ . Ethylene content of samples was determined by comparison with ethylene standards. Acetylene was not used as an internal standard but gas valves and Hungate tubes provided acceptable precision to gas transfers. Acetylene concentrations were also monitored closely to detect any potential leaks from the injection port on the gas chromatograph.

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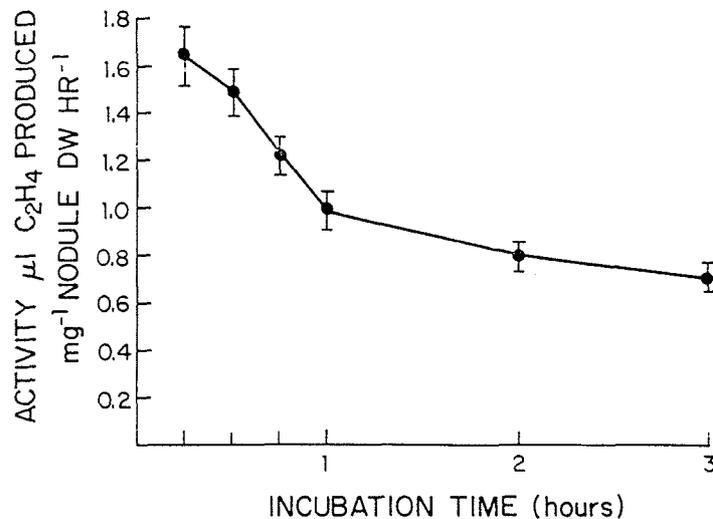


FIGURE 1. Activity for 3–10 mm nodules ( $\bar{X} \pm SE$ ) following abscission from roots in early June.

*Nodule Biomass.*—Black locust stands with similar density, biomass, soil, slope, and aspect were located close together on WS 7. Six stands were randomly chosen for sampling, and 10 × 10 m plots were marked inside each. Each of these plots was subdivided into 100 one-square-meter subplots which were used for seasonal samples of nodule biomass.

In May, July, November, and February, five subplots within each study plot were randomly chosen for nodule sampling. A 12 cm diameter steel tube was driven into the forest floor to extract soil from the middle of each subplot. Soil cores were divided into 0–15 cm and 16–30 cm depths. No samples were taken deeper than 30 cm since 80 percent of the fine root biomass is above this depth (McGinty 1976). Cores were transported to the laboratory and stored for no more than 7 days in plastic bags under refrigeration.

Nodules were separated from soil, roots, and dead organic matter by washing the samples on 1 mm mesh aluminum screen and hand sorting. Nodules were then sorted into live and dead categories with three size classes (<3, 3–10, >10 mm length), counted, then dried and weighed. The three size classes were used to separate immature (<3 mm) and potentially senescent (>10 mm) nodules from the 3–10 mm nodules, and thus reduce variability in measuring rates of activity. Length was used to divide size classes instead of diameter since the nodules were oval and elongate. Live nodules were easily distinguished from dead ones, since healthy nodules were firm-textured with variable red internal coloration from leghemoglobin. Senescent and dead nodules were hollow or very soft. Several subsamples were selected to compute fresh weight to dry weight ratios, so that C<sub>2</sub>H<sub>2</sub> reduction assays could be related to both fresh and dry weights from comparisons with other studies.

*Statistical Analysis.*—All biomass and acetylene reduction data were statistically analyzed using standard analysis of variance (Sokal and Rohlf 1969), SAS General Linear Model procedures, and Duncan's Multiple Range Test (Helwig and Council 1979). Mean values of nodule biomass were positively correlated with their variance, therefore biomass values were converted with a logarithmic transformation prior to analysis.

## RESULTS

*Acetylene Reduction Assays.*—In June there was a significant difference ( $P < 0.001$ ) in activity between nodules  $<3$  mm and 3–10 mm (Fig. 2). Nodules  $>10$  mm were not assayed because they were not present in sufficient numbers for replication. September assays showed no significant differences between the three size classes. The  $<3$  mm size class had lower rates in June than in September. Although  $<3$  and  $>10$  mm nodules were not always available for assays, the data are adequate to describe all patterns for the 3–10 mm nodules which comprise 60–85 percent of the live nodule biomass (Fig. 3).

Analysis of diurnal patterns of activity showed no significant differences among times of day in small nodules (Fig. 2). However, the 3–10 mm nodules revealed significant diurnal differences for both early ( $P < 0.01$ ) and late ( $P < 0.05$ ) season assays. In June, the night and evening measurements were lower than those during the day. In September, only the midday measurements were higher, as the night, morning, and evening activities were almost identical. In September, the midday activity was also significantly higher ( $P < 0.01$ ) than at night for nodules  $>10$  mm.

Trends in seasonal activity were also significant ( $P < 0.01$ ) (Fig. 2). Overall, June activity was significantly higher than September for 3–10 mm nodules. For  $<3$  mm nodules, however, activity was greatest in September. Assays on warm days in late March and early November detected no activity. Phenologically, the latter assays were conducted prior to spring budbreak and following autumn litterfall, respectively.

*Nodule Biomass.*—For all months and within both live and dead nodule categories (Fig. 3), there were significant differences ( $P < 0.05$ ) in biomass among the three size classes. For live nodules, biomass was greatest for the 3–10 mm size class, followed by the  $<3$  mm and  $>10$  mm sizes, respectively. Dead nodule biomass was greatest for the 3–10 mm size class, followed by the  $>10$  mm and  $<3$  mm sizes. In respect to nodule distribution in the soil column, pooled live and dead nodule biomass was greater ( $P < 0.05$ ) in the 0–15 cm depth ( $2.1 \text{ g m}^{-2}$ ) than in the 16–30 cm depth ( $1.2 \text{ g m}^{-2}$ ).

There was a significant difference ( $P < 0.01$ ) in monthly biomass for the 3–10 mm live nodule size class (Fig. 3). The May and July values were significantly higher than November, as 3–10 mm live nodule biomass decreased from  $3.05 \text{ g m}^{-2}$  in May to  $0.55 \text{ g m}^{-2}$  in November. Although February was not significantly different, the biomass returned to  $1.53 \text{ g m}^{-2}$ , suggesting an active winter period of root and nodule growth. There were no significant differences among monthly biomass values for  $<3$  mm and  $>10$  mm nodules.

Among the dead nodules, only the  $<3$  mm nodules showed significant differences in monthly biomass ( $P < 0.05$ ). July values were significantly higher than May or February. This indicates high mortality of immature nodules during the summer months, but persistence and presumed growth in winter and spring. There were no significant differences in monthly biomass of dead nodules 3–10 mm or  $>10$  mm although the number of individual 3–10 mm dead nodules increased from  $318 \text{ m}^{-2}$  to  $673 \text{ m}^{-2}$  between July and November.

Ratios of nodule fresh weight to dry weight gave slightly different values for each size class. From the largest to smallest size class, the means ( $\pm$ SD) were 4.82 ( $\pm 0.31$ ), 4.68 ( $\pm 0.19$ ), and 4.07 ( $\pm 0.22$ ).

## DISCUSSION

*Nodule Biomass and Activity.*—Seasonal patterns of nodule biomass do not clearly delineate a seasonal pattern of nodulation and growth, but there appears to be

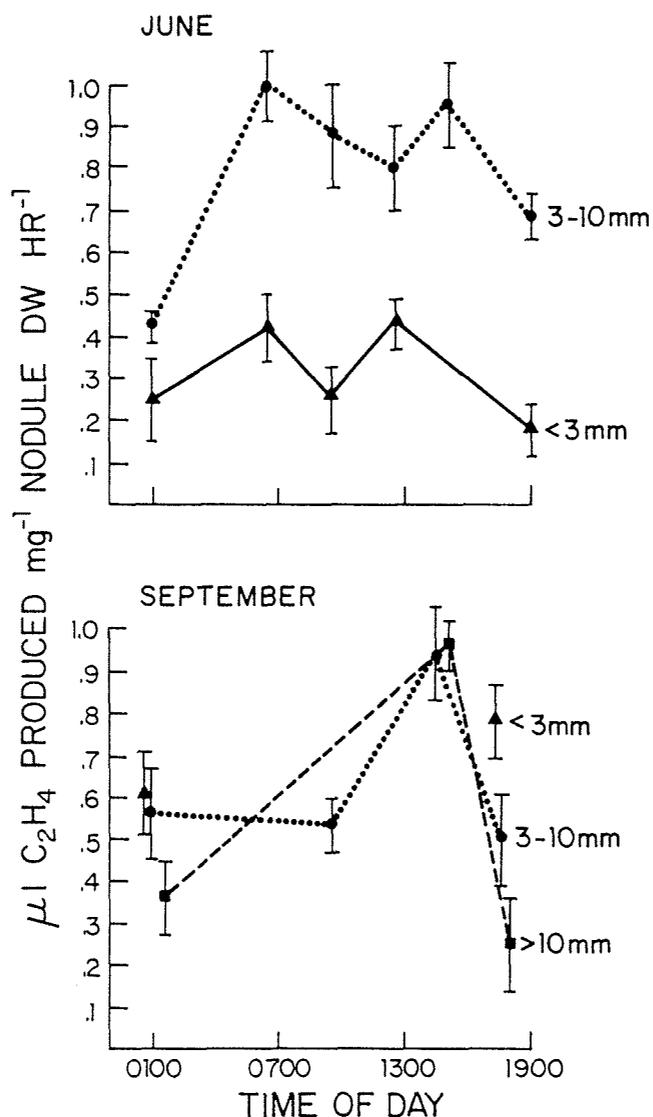


FIGURE 2. Diurnal patterns of  $C_2H_2$  reduction for June and September ( $\bar{X} \pm SE$ ), comparing the 3 size classes of nodules. Assays for March and November are not shown, as all values were 0.

high annual turnover associated with midsummer mortality. The persistence of live, large nodules over the winter suggests that at least some of these are perennial. Although black locust nodules frequently bifurcate and exceed 10 mm in length, they do not develop into large lignified structures (> 50 g each) which characterize some woody legumes, such as *Lupinus arboreus* (Pate 1977). Studies of black locust planted in France confirm the perennial nature of at least some of the nodules, but reveal no additional information on their longevity (Moiroud and Capellano, in press).

The decrease in live 3–10 mm nodule biomass through the summer may be a characteristic legume response to drought stress, as 1981 was one of the driest summers on record at Coweeta. Precipitation from May–November was 161 mm

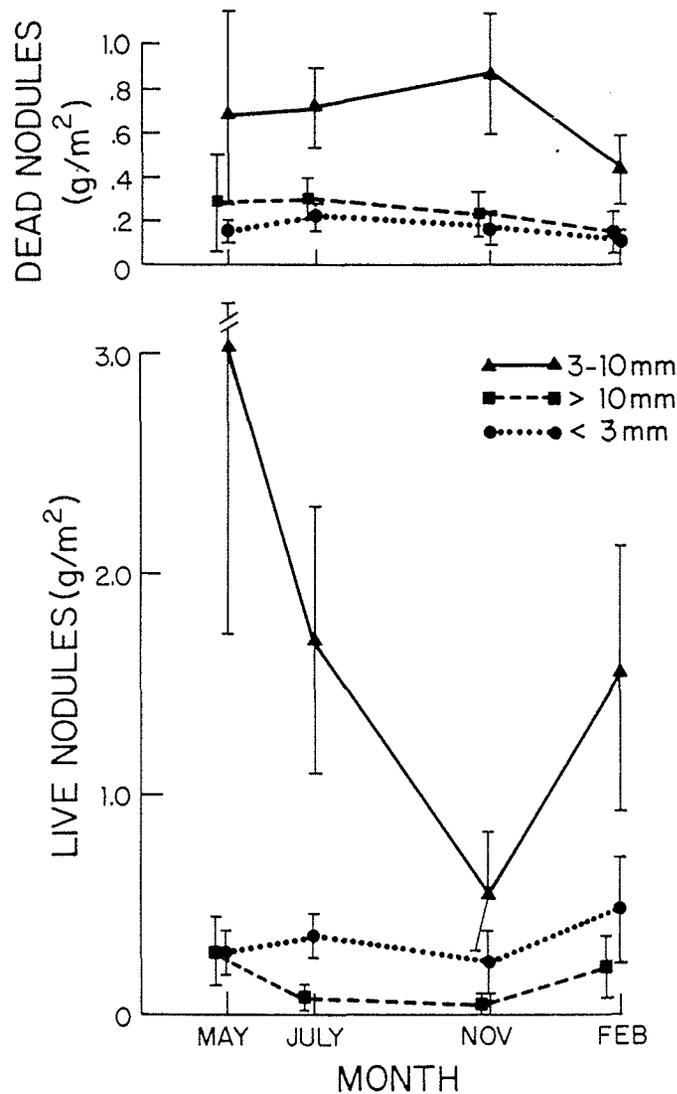


FIGURE 3. Live and dead nodule biomass by season and size class ( $\bar{X} \pm SE$ ). There were significant differences among size classes of nodules ( $P < .05$ ) and monthly biomass ( $P < .01$ ).

less than the long-term monthly means over the same period. However, hot, dry periods of varying length occur in most years, and could result in annual cycles of high summer nodule mortality with a winter period of renewal and growth. Legumes subjected to heat, drought, or other stresses have been observed to respond by sloughing nodules. In the long term, legume nodule shedding is generally followed by initiation of new nodules, with damage and recovery varying greatly among species (Pate 1977, Sprent 1979).

No  $C_2H_2$  reduction activity was detected either 30 days before budbreak or 30 days following leaf senescence. Moiroud and Capellano (in press) have found that nodules are dormant during winter months, initiate activity around spring budbreak, attain high activity following leaf expansion, and continue until leaf senescence. Seasonal nodule activity was correlated with the mobilization of carbon reserves in the spring, and the availability of photosynthate through the summer

until leaf abscission (Moiroud and Capellano, in press). These findings are consistent with whole-plant studies of *Alnus glutinosa*, which determined that actinorhizal nodule activity was significantly correlated with mean rates of net photosynthesis (Gordon and Wheeler 1978).

Diurnal variations in June and September nodule activity may be attributable to relatively low night-time soil temperatures. Although decreased photosynthate availability at night may partly explain low nodule activity, studies of *Glycine max* have shown that activity may be maintained by stored carbon reserves (Schweitzer and Harper 1980). Stored carbon may also be important in maintaining nocturnal activity for a perennial, such as black locust. As a result, relatively low soil temperatures may account for these observed nocturnal decreases (Hensley and Carpenter 1979, McNiel 1975).

*Comparisons with Other Species.*—The range of  $C_2H_2$  reduction rates in this study is similar to values from other studies of black locust (McNiel 1975, Tjepkema and Winship 1980, and Moiroud and others 1981) and provides insights in comparison to other species. Black locust generally has lower nodule activity than a wide range of agricultural legumes (Schwinghammer and others 1970, Hardy and others 1973). As expected, the black locust rates bracket and tend to exceed those of actinorhizal species such as *Alnus rubra* (Tripp and others 1979, Binkley 1981) and *Ceanothus velutinus* (McNabb and Geist 1979). Actinorhizal nodules are apparently longer lived and develop a lignified, coralloid structure several centimeters in diameter (Sprent 1979). Active tissue in actinorhizal nodules is confined to current season's growth, but most  $C_2H_2$  reduction assays usually include the entire nodule.

The difference in nodule size between black locust and actinorhizal species is shown in nodule biomass comparisons between black locust (this study) and alder stands (Binkley 1981). For black locust, nodule biomass ranged from  $3.59 \text{ g m}^{-2}$  in May to  $0.83 \text{ g m}^{-2}$  in November. In comparison, on clearcut sites in British Columbia, red alder (*Alnus rubra*) had  $39 \text{ g m}^{-2}$  and Sitka alder (*Alnus sinuata*)  $11 \text{ g m}^{-2}$  (Binkley 1981). Seasonal patterns of actinorhizal nodule biomass have not been reported, although they could also be sloughed in response to environmental conditions.

An estimate of the quantity of N fixed by black locust may be computed using the mean  $C_2H_2$  reduction values per unit nodule biomass (weighted for diurnal patterns) for each size category, the assumed conversion ratio of 3 moles  $C_2H_2$  per mole of  $N_2$  (Hardy and others 1973), and the mean nodule biomass for each size category of nodules in May, July, and September (the latter calculated as the average of July and November biomass). In spite of relatively low nodule biomass, the estimate of  $30 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  for the 4-year-old stands is generally comparable to well-documented woody actinorhizal N-fixing species (Zavitkovski and Newton 1968, Binkley 1981), but is below the range of most agricultural legumes (Evans and Barber 1977).

There is a possibility that this N-fixation estimate may be low, since there was such high nodule mortality during the dry summer. However, since this study was not replicated during a wetter year, it is impossible to interpret the impact of the 1981 drought.

Nitrogen fixation by black locust may be even greater in intermediate-aged stands. In a related study at Coweeta, there were estimated net N-accretion (biomass, litter, and soil) rates of 48, 75, and  $33 \text{ kg ha}^{-1} \text{ yr}^{-1}$ , respectively, in 4-, 17-, and 38-year-old stands (Boring and Swank, in press). Also, November live nodule biomass in the sequence of stands was 8, 106, and  $4 \text{ kg/ha}^{-1}$ . This suggests a potential peak for N fixation considerably greater than  $30 \text{ kg ha}^{-1} \text{ yr}^{-1}$  in intermediate aged stands, and a decrease as the stand declines.

*Ecological and Management Implications.*—The plots used for N-fixation studies are representative of the high density black locust stands on a regenerating clearcut at Coweeta (WS 7), so symbiotic fixation rates of 30 kg N ha<sup>-1</sup> yr<sup>-1</sup> are estimated for 25 percent of the watershed. Assuming that black locust stem density is proportional to its N fixation, a weighted estimate of fixation over the area of the whole watershed is 10 kg N ha<sup>-1</sup> yr<sup>-1</sup>.

Relative to all measured potential N inputs to an Appalachian deciduous forest, symbiotic fixation by black locust during early successional stages is the largest. In Coweeta hardwood forest, inputs from forest floor, soil, and lichen fixation, and atmospheric deposition would account for 2.3, 8.6, 1.9, and 8.8 kg N ha<sup>-1</sup> yr<sup>-1</sup>, respectively (Todd and others 1978, Swank and Waide 1979).

This regenerative pulse of N may be most important in the long-term replacement of net N losses that result from forest harvesting. Following clearcutting, hydrologic export (Swank and Waide 1979), stream sediment denitrification (Swank and Caskey 1982), soil denitrification, and sawlog removal may remove appreciable quantities of N. By assuming a 20-year life span for the subsequently established black locust, the dense stands could fix at least 600 kg N/ha<sup>-1</sup>, and the watershed as a whole could receive a weighted total exceeding 200 kg N ha<sup>-1</sup>. Cumulatively, symbiotic N fixation may more than compensate for any N losses associated with forest harvesting. Detailed patterns of N accumulation and retention are reported elsewhere (Boring and Swank, in press).

This quantity of N fixed by black locust needs to be compared with inorganic fertilization from both biological and economic considerations. Included among these are additions to fertility, soil organic matter and site productivity, effects of plant competition, costs of fertilizer production and application, and costs of silvicultural treatments. Also, many questions remain about immobilization of symbiotically fixed N in biomass and litter, and the timing of increased N mineralization and availability. Black locust's potential for a broader range of applications warrants further research and development.

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