N_2 fixation associated with wood decayed by some common fungi in western Montana[1]

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Decay caused by some common wood-destroying fungi in several associated tree hosts of the Intermountain forests of western Montana was evaluated as a potential environment for the fixation of dinitrogen. Differences in the rates of fixation were demonstrated between various decay stages, tree species, decay fungi, and brown and white rots. Advanced brown-rotted wood was a more favorable system for nitrogen fixation than wood partially brown rotted. Also, brown-rotted substrates proved to be more favorable than did white rotted, particularly *Pseudotsuga menziesii* (Mirb.) Franco wood decayed by *Fomitopsis pinicola* (Swartz ex Fr.) Karst. Data demonstrate that a nitrogenase function coexists naturally with decay fungi in woody substrates and that appreciable and significant amounts of nitrogen are fixed in these substrates.

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La pourriture causée par quelques champignons communs dans des arbres cariés dans la zone Intermountain de l'ouest du Montana a été évaluée comme milieu potentiel de fixation de l'azote atmosphérique. On a observé des différences dans le taux de fixation d'azote entre divers stades de pourriture, entre les espèces d'arbres, entre les champignons responsables de la carie et entre les caries brunes et les caries blanches. Le bois qui était dans un état avancé de décomposition occasionnée par les caries brunes constituait un milieu plus propice à la fixation que le bois partiellement carié. Aussi bien, les substrats de carie brune se sont avérés plus favorables que ceux de carie blanche, en particulier le bois de *Pseudotsuga menziesii* (Mirb.) Franco carié par *Fomitopsis pinicola* (Swartz ex Fr.) Karst. Les données montrent que la nitrogénase coexiste naturellement avec les champignons de carie dans les substrats ligneux et que des quantités appréciables et significatives d'azote sont fixées dans ces substrats.

[Traduit par le journal]

The amount and the form of nutrient nitrogen greatly influence tree crops in nurseries, plantations, and naturally occurring forest stands. Nitrogen availability may not only be limited by chemical form (oxidation state) but may also be limited by the occurrence and spatial distribution of ecological niches in which nitrogen transformations occur. Since commercial nitrogen production requires enormous amounts of energy input, application in forest situations is often uneconomical. A reasonable alternative is to attempt to abet the natural processes that provide nitrogen inputs, particularly nitrogen fixation, by manipulating forest ecosystems.

Recently, both industry and government have advocated whole-tree utilization as a means of in-

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creasing the nation's fiber resource to meet existing and future needs. This practice, without due consideration to its environmental impact, could lead to deleterious side effects: disturbed nutrient-cycling regimes, site-stabilization problems may occur, and removal of residues which ultimately perform biological functions in soil systems (Harvey *et al.* 1976; Bormann *et al.* 1977).

Recently, several studies have demonstrated the association of nitrogenase activity in woody substrates in various stages of decomposition (Cornaby and Waide 1973; Sharp and Millbank 1973; Sharp 1974, 1975; Aho, Seidler, Evans, and Nelson 1974). In addition, a variety of bacterial associates have been implicated as agents of decomposition and as nitrogen fixers associated with decomposition (Ellwood and Ecklund 1959; Knuth 1964; Witz *et al.* 1967; Seidler *et al.* 1972; Knutson 1973; Aho, Seidler, Evans, and Raju 1974).

Our purpose here is to present data that demonstrate that woody residues in various states of decomposition are suitable substrates supporting appreciable amounts of dinitrogen fixation and relate this fixation to the presence and action of specific decay fungi.

Methods

A complete description of the experimental site in northwestern Montana has been given by Harvey *et al.* (1976). Decaying woody materials here selected by species and associated decay fungi: three dead. standing boles of paper birch (*Betula papyrifera Marsh.*) decayed by the white-rot fungus *Fomes fomentarius* (L. ex Fr.) Kickx; three downed logs each of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.), and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), decayed by the brown-rot fungus *Fomitopsis pinicola* (Swartz ex Fr.) Karst.; and three live. standing trees of western hemlock with a white, stringy heart rot caused by *Echinodontium tinctorium* (Ell. & Ev.) Ell. & Ev. were sampled. Each sample tree or log was dissected with a saw to locate and visually ascertain undecayed wood and decayed wood categories (incipient, advanced, or light or dark advanced for the brown cubicle decay associated with *F. pinicola*). Samples from each decay category associated with *E. tinctorium* were obtained at three different heights: 0 to 10, 20 to 30, and 40 to 50 ft (1 ft = 30.48 cm). Five replicate samples per log or tree for each decay type were placed in individual 20-ml glass Vacutainer (Becton-Dickinson Div., Becton-Dickinson) tubes. Care was exercised not to incorporate woody material from the outer exposed portions of selected substrates. No attempts were made to distinguish between heartwood and sapwood when samples were taken.

Small pieces of woody tissue from each log were surface flame sterilized and placed on 2% malt : 2% agar (w/v) and incubated at 18°C. Fungal growth typical of *F. pinicola, F. fomentarius.* and *E. tinctorium* indicated the presence of viable and active hyphae within the substrates tested.

Nitrogen-fixation rates were estimated using an adaptation of the acetylene reduction technique discussed by Hardy *et al.* (1968). Within 2 h of sampling, the tubes were evacuated and flushed with argon five times and then filled with a mixture of 10% acetylene and 90% argon gas. Final oxygen concentrations in the tubes approximated 0.05%. Acetylene was omitted from one of the five replicates to monitor endogenous ethylene production; these values were subtracted from those obtained from samples incubated with acetylene. The sample tubes were then incubated in the dark for 24 h at ambient field temperature (12-15°C).

Gas subsamples were removed from the incubation tube by 2-ml Vacutainer tubes. These were analyzed for acetylene and ethylene levels using a Varian series 2800 gas chromatograph fitted with an 80- to 100-mesh Poropak R column. The column was run at 50°C using nitrogen as the carrier gas. After incubation the samples were dried at 105°C for determinations of dry weight and moisture content.

Results and Discussion

Our data (Tables 1–4) provide additional evidence for a nitrogenase function in decaying woody substrates. Cowling and Merrill (1966) suggested that fixed nitrogen from *in situ* N_2 fixation might supplement the existing and meager nitrogen resources in wood available to decay fungi; thus this would potentially abet the processes associated with wood decay. Recently, Sharp (1975) attempted, by an elegant procedure, to demonstrate, through the use of $^{15}N_2$, that fixed dinitrogen was taken up and utilized by wood-inhabiting fungi (principally Hyphomycetes) . Although he did demonstrate the diffusion of labeled nitrogen into woody materials and the formation of labeled nitrogen-containing compounds, his efforts to demonstrate the transference of this nitrogen to fungal mycelium were unsuccessful.

Other authors (Ellwood and Ecklund 1959; Knuth 1964; Witz et al. 1967; Knutson 1973) have reported species of bacteria associated with wood. Since the bacteria reported are known to fix nitrogen elsewhere, they may be able to perform this function in woody tissues. The presence of bacterial

TABLE 1. Mean moles per gram ovendry weight of woody tissue of net ethylene* produced in 24-h period in wood of live, standing western hemlock decayed by *Echinodontium tinctorium*

Decay stage	Moles produced, $\bar{X} \pm$ SD, $\times 10^{-9}$	Moisture content, $\bar{X} \pm$ SD, %
Undecayed	2.22 ± 0.42	144 ± 50
Incipient	2.38 ± 0.89	111 ± 27
Advanced	2.13 ± 0.71	107 ± 39

*Net ethylene is background ethylene subtracted from ethylene produced from the acetylene reduction technique.

TABLE 2. Mean moles per gram ovendry weight of woody tissue of ne! ethylene* produced in 24-h period in wood of Douglas-fir, subalpine fir, and western hemlock decayed by *Fomitopsis pinicola*

Tree species	Decay stage	Moles produced, $\bar{X} \pm$ SD, $\times 10^{-9}$	Moisture content, $\bar{X} \pm$ SD, %
Douglas-fir	Undecayed	1.83 ± 0.59	37 ± 11.6
	Incipient	1.69 ± 0.62	38 ± 4.7
	Advanced (light)	1.42 ± 0.29	72 ± 17.6
	Advanced (dark)	10.55 ± 6.08	412 ± 86.2
Subalpine fir	Undecayed	1.45 ± 0.24	53 ± 7.9
	Incipient	1.59 ± 0.41	79 ± 12.5
	Advanced (light)	1.38 ± 0.62	74 ± 23.9
	Advanced (dark)	2.99 ± 1.95	157 ± 70.9
Western hemlock	Undecayed	2.40 ± 0.47	34 ± 7.1
	Incipient	2.04 ± 0.33	75 ± 13.2
	Advanced (dark)	5.32 ± 2.57	132 ± 61.1

*Net ethylene is background ethylene subtracted from ethylene produced from the acetylene reduction technique.

species associated with decaying wood capable of fixing nitrogen has been reported by Seidler et al. (1972) and Aho, Seidler, Evans, and Raju (1974). In our experiments we have not identified the bacteria associated with the demonstrated nitrogenase function. However, several anaerobic and aerobic forms that have been isolated are likely candidates as dinitrogen fixers.

Several reports have noted the presence of nitrogenase activity in fungal-infected wood (Cornaby and Waide 1973; Sharp and Millbank 1973; Sharp 1974, 1975). Aho, Seidler, Evans, and Nelson (1974) detected nitrogenase activity in decaying sapwood from living white fir trees but concluded their results were inconclusive due to excessive ethylene production. However, they did demonstrate nitrogenase activity in decaying heartwood of fir.

The data presented here, derived from several different woody substrates decayed by various fungi, depict notable statistical differences between the various paired associates (host substrate and kind of decay) as well as differences between decay stages. Most substrates (Tables 2 and 3) demonstrate an increased capability for supporting the nitrogenase function with decay progression if moisture contents are considered. The exception is the western hemlock - E. tinctorium association (Table 1) . In this association, however, several factors may have affected the results: difficulty in obtaining sound and partially decayed wood due to extensive internal decay and variability of data indicating insufficient sample size. Furthermore, the net ethylene produced from undecayed wood samples (Table 4) suggests the samples may have been in a partially decayed condition not visibly discernible or an indigenous population of N_2-fixing bacteria may be present in the absence of decay, or both conditions. Substrate permeability in conjunction with moisture content must also be considered when examining the results. Acetylene penetration may differ radically between decayed and undecayed samples and may be compounded by variable moisture contents. Although the data have not been restructured within this context, they indicate that at extremely high moisture contents acetylene penetration may be retarded.

If kind of decay is considered (brown rot versus white rot) significant differences (p = 0.001) are observed between advanced brown and white rots, with the exception of the subalpine fir - *F. pinicola* association. The Douglas-fir - F. pinicola brown-rot system supports the highest fixation detected in these experiments, particularly in the dark-advanced decay. Data published by Harvey et al. (1976, 1978) show that this kind of substrate (brown cubical-decayed wood) is an important ecological niche for mycorrhizae formation and activity when it becomes a functional part of the soil profile. This suggests a strong nutritional relationship between mycorrhizae and nitrogen fixation, in addition to the demonstrated physical relationship of mycorrhizae and brown-rotted wood (Harvey et al. 1976).

No significant differences in N_2 fixation were detected between samples taken at various heights in western hemlock decayed by *E. tinctorium.*

The kind of decay and the associated rates of nitrogen fixation have not been previously noted. Cornaby and Waide (1973) reported fixation rates for decaying logs of American chestnut (*Castanea dentata* (Marsh.) Borkh.) but did not characterize the decay system. Baxter (1931) provided an extensive survey of the fungi causing decay of Ameri-

TABLE 3. Mean moles per grain ovendry weight of wood substrate of net ethylene* produced in 24-h period from white birch wood decayed by *Fomes fomentarius*

Decay type	Moles produced, $\bar{X} \pm$ SD, $\times 10^{-9}$	Moisture content, $\bar{X} \pm$ SD, %
Undecayed	1.45 ± 0.30	59.4 ± 15.5
Incipient	1.65 ± 0.19	115.9 ± 39.5
Advanced	2.5 ± 0.43	140.9 ± 50.5

*Net ethylene is background ethylene subtracted from ethylene produced from the acetylene reduction technique.

can chestnut. An examination of Baxter's list reveals that nearly all the fungi produced white rots. Thus it is conceivable that the experimental substrate investigated by Cornaby and Waide (1973) was decayed principally by white rotters and less so by brown rotters, as would be expected in an eastern deciduous forest ecosystem. The initial work of Seidler *et al.* (1972) and Aho, Seidler, Evans, and Nelson (1974) utilized the heartwood of living white fir (*Abies concolor* (Gord. & Glend.) Lindl.) decayed by fungi that produce white heartrots.

Moisture contents of decaying residues of various tree species have also provided some insight into nitrogen-fixation regimes. With the progression of brown cubical decay, moisture contents eventually increase with concomitant increases in rates of fixation. Reductions in rates of fixation were observed, but were not significant, between incipient and light-advanced decay in the subalpine fir - *F. pinicola* association and are attributable to data variability (Table 4). A similar phenomenon was also noted for the Douglas-fir - *F. pinicola* association (Table 4); the only difference was a significant reduction between undecayed wood and light-advanced decay. These reductions do not appear to be moisture dependent and reflect either an inadequate sampling or a parameter associated with the nature of the substrate in the light-advanced stage of decay with which we are unfamiliar.

Cornaby and Waide (1973) reported that 30.54 mol ($\times 10^{-9}$) acetylene-dependent ethylene per gram of substrate (285 g ($\times 10^{-9}$) N) were produced in a 24-h period in American chestnut logs in Georgia and on a yearly basis estimated that annual input was 0.89 kg/ha per year or 4.1 % of the

TABLE 4. Comparison of mean moles ($\times 10^{-9}$) of net ethylene* produced by stage and type of host substrate, and associated fungus†

Host substrate (and associated fungus)	Undecayed	Decay: Incipient stage	Decay: White-advanced stage	Decay: Brown-advanced stages: Light	Decay: Brown-advanced stages: Dark
Brown rot					
Douglas-fir (*Fomitopsis pinicola*)	1.83	1.69		1.42	10.55
				a	
		a			
		a			
		c			
Subalpine fir (*F. pinicola*)	1.45	1.59		1.38	2.99
				c	
		c			
		b			
Western hemlock (*F. pinicola*)	2.40	2.04			5.32
		a			
		a			
White rot					
White birch (*Fomes fomentarius*)	1.45	1.65	2.5		
		a			
	c				
		a			
Western hemlock (*Echinodontium tinctorium*)	2.22	2.38	2.13		

*Net ethylene is background ethylene subtracted from ethylene produced from the acetylene reduction technique.
†Significant differences between values indicated by solid line between respective values; level of significance indicated by *a* (p = 0.001), *b* (p = 0.02). and *c* (p = 0.05).

annual input. Aho, Seidler, Evans, and Nelson (1974), using similar techniques, reported 142 mol ($\times 10^{-9}$) for a 24-h period in decaying white fir heartwood samples 20 cm in diameter and 20 cm long. Assuming that the samples were 50% decayed (specific gravity (sg) 0.18) they would weigh approximately 1200 g wet weight (or 600 g ovendry weight, assuming 100% moisture content). The fixation rate can be calculated to be approximately 0.3 mol ($\times 10^{-9}$) (2.8 g ($\times 10^{-9}$) N) per gram ovendry weight substrate, a figure which appears rather low.

Control areas on our experimental sites have a residue loading of almost 113 tonnes/ha. Assuming an average rate of 7 mol ($\times 10^{-9}$) (65 g ($\times 10^{-9}$) N) of acetylene-dependent ethylene produced per gram ovendry weight substrate per 24 h, we calculate approximately 735 g nitrogen would be fixed annually (assuming 100 days). If this annual figure is used for computation of nitrogen accrued for stand rotation at 100 years, then the amount of nitrogen fixed is considerable. In contrast, rates of symbiotic nitrogen fixation are noticeably higher, for Hardy *et al.* (1968) reported 33 kg/acre N fixed per annum (82.5 kg/ha) for nodulated soybeans.

We conclude that the rates and the quantities of dinitrogen fixed in decaying woody residues are important components of the nitrogen cycle in the Intermountain regions of western North America. Therefore, woody residues must be viewed not only as a fiber resource but as a substrate with important and unique biological functions.

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