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February 1–2, 1994

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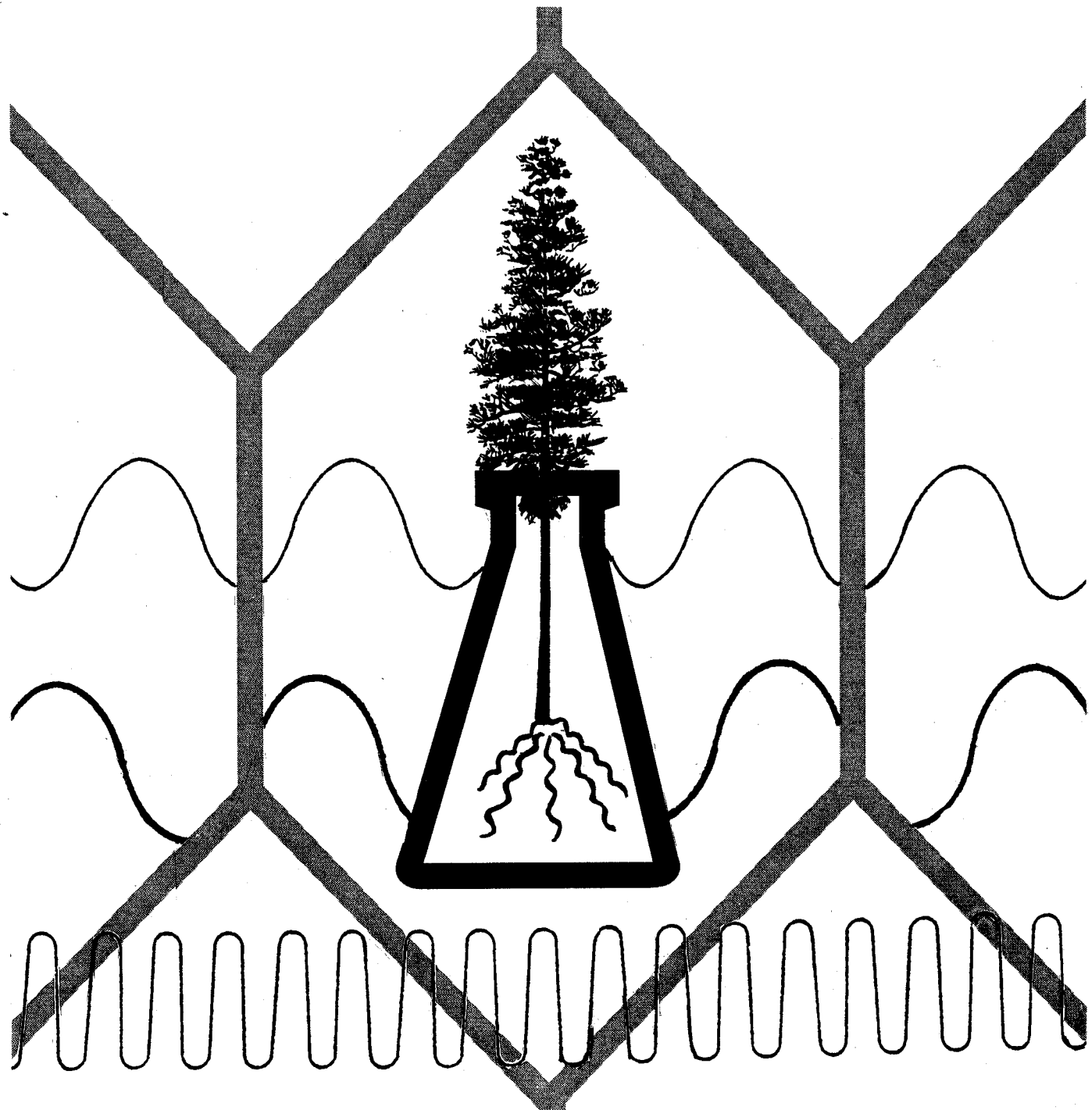

Research and Applications of Chemical Sciences in Forestry

Proceedings of the 4th Southern Station Chemical Sciences Meeting

Compiled by J. A. Vozzo

February 1–2, 1994
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PREFACE

This proceedings is the result of 65 scientists representing 34 facilities reported in 28 presentations. As titled, Research and Applications of Chemical Sciences in Forestry, the contributors represent academic, basic, and applied researchers from universities and U.S. Department of Agriculture. Their presence and experience represent a significant showing toward recognizing the importance of forestry research.

The featured speaker presented a deliberate, indepth discussion of magnetic resonance imaging in plant sciences. A second invited speaker addressed user responsibilities while applying chemical sciences in our environment. All attendees contributed to a balanced and informative program.

All contributors are responsible for content and accuracy of their contributions. Recognition goes to Frank Bonner, Project Leader of RWU-4103, for generous support of the meeting and its preparations, as well as to Jody Jones, Information and Publications Services, SOFES, for timely editorial supervision. We are all grateful for the warm welcomes from Dr. W.A. Hough, Assistant Director-Central, Southern Forest Experiment Station, USDA-FS; Dr. F.T. Bonner, Project Leader, USDA-FS; and Dr. Doug Richards, Head, Department of Forestry, Mississippi State University.

USDA Forest Service, Southern Forest Experiment Station and Department of Forestry, Mississippi State University, are proud to present these papers.

J.A. Vozzo
Compiler
Southern Forest Experiment Station

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Magnetic Resonance Imaging, a Technology for Noninvasive Plant Analysis[1]

John M. Halloin, Thomas G. Cooper, and E. James Potchen[2]

ABSTRACT

Magnetic resonance imaging (MRI), a widely used technique in radiology, provides a useful technology for noninvasive analysis of plant tissues. Most MRI procedures involve imaging of mobile protons (hydrogen nuclei), and water provides the most abundant source of these mobile protons in most plant tissues. Image intensity is influenced by proton abundance, the rates of spin-lattice and spin-spin relaxation of those protons in a magnetic field, and by imaging parameters of the instrument. Typically, images resemble tissue anatomy, but they also provide important information on physical interactions between protons and the surrounding molecular environments within tissues. Effective use of MRI takes advantage of both the physical information derived and of the noninvasive nature of the procedures. Published research employing MRI for plant investigations includes studies in the following areas: changes in abundance and distribution of water within stems and roots, water translocation, development of diseases and physiological disorders, internal structure of tissues, development of root systems within soil matrices, changes in water binding during bud vernalization, and differentiation between water and lipid distribution within imbibed seeds. Important applications of MRI in plant research will increase as the technology becomes more widely available.

INTRODUCTION

Although plant tissues were among the earliest objects imaged with MRI (Lauterbur 1974), the technology has attained extensive use as a diagnostic tool primarily in human medicine and more recently has been developed as a research tool in the plant and soil sciences. The major advantages of MRI for the study of plants are that it involves no ionizing radiation, it produces high resolution, high contrast images (Hinshaw and others 1979), and is completely noninvasive and nondestructive, allowing subsequent use of samples for additional experiments. Useful application of the technology and interpretation of results require understanding both of the physics of MRI and of the basic biology of the systems being studied.

Some of the physical principles involved in MRI and some of the technology relating to image production and resolution are briefly addressed in this paper. Additionally, the effects of plant constituents on image characteristics are discussed. Finally, demonstrated and proposed applications of MRI to the study of plants are presented. Most of the discussion concentrates on imaging of water protons (^{1}H), but the principles are applicable to several other nuclei as well as to protons of plant components other than water.

PHYSICAL PRINCIPLES AND IMAGE PRODUCTION

A thorough coverage of the physical principles involved in MRI is outside the scope of this paper. Instead, only those aspects most essential to an understanding of the subsequent material are reviewed. Readers interested in a more detailed coverage of the physical aspects of imaging are referred to general references listed at the end of this paper. The book by Bushong (1988) provides a coverage of the topic readily understood by most scientists, whereas that by Stark and Bradley (1992) provides a much more rigorous treatment.

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[1] Paper presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Physical Principles in Magnetic Resonance Imaging

For present purposes we may consider the nucleus of an atom to contain two "elementary" particles, protons and neutrons. Protons possess a positive electrical charge, while neutrons are electrically neutral. Nuclei spin, and thus may be thought of as tiny, electrically charged, spinning spheres. This spinning charge produces a magnetic moment. The magnetic moment is a vector quantity describing the magnitude and direction of the magnetic field surrounding the nucleus. This magnetic field is directionally parallel to the axis of rotation of the nucleus and is analogous to the field of a submicroscopic bar magnet (dipole). Since the axes of rotation for any group of nuclei are randomly oriented, the net macroscopic magnetic moment for a large collection of nuclei is zero.

In the presence of an externally applied static magnetic field, the randomly oriented magnetic dipoles attempt to align themselves with the direction of this field. For the most abundant isotope of hydrogen, the nucleus of which is a single proton, there are two allowable orientations or states: parallel and antiparallel. The parallel orientation is the low-energy state, whereas the antiparallel orientation corresponds to a high-energy state. Furthermore, since the nuclei are rotating, the spins do not align exactly with the external magnetic field but rather precess about the applied field at a fixed angle. This precession is analogous to the wobbling of a spinning top in the presence of the earth's gravitational field. The precessional rate of these spins (ω_o), in Hertz, may be calculated from the product of the magnetic field strength (B_o), in Tesla, and the gyromagnetic ratio (γ), in Hertz/Tesla. This is known Larmor as the relationship: $\omega_o = \gamma B_o$. For a given nuclear species, the gyromagnetic ratio is a physical constant. The proton (= hydrogen nucleus) has a gyromagnetic ratio of approximately $42.58X10^6$ Hertz/Tesla.

Precessing magnetic moments have a characteristic called phase. The instantaneous phase of the magnetic moment for a single nucleus in a static magnetic field is the location of the tip of the magnetic moment vector in the precessional orbit. It is impossible, however, to determine the action of a single nucleus. Normally, for a large collection of individual spins, the phases of the magnetic moments are randomly distributed and cannot be detected. However, if large numbers of magnetic moments are precessing in phase synchrony with one another, they produce a radio signal at the Larmor frequency (γB_o) that can be detected.

At thermal equilibrium in a static magnetic field, the number of nuclei transitioning from the low-energy (parallel) to high-energy (antiparallel) state equals the number of nuclei transitioning from the high-energy to the low-energy state. If radiofrequency energy is applied at the Larmor frequency to the ensemble of spins, the magnetic moments will be flipped from their low- to high-energy state. This is the resonance phenomenon. If the radiofrequency field is applied in a plane perpendicular to the static field, it will cause the net macroscopic magnetic moment to be tipped away from the longitudinal axis toward the perpendicular or transverse plane. The angle formed between the macroscopic magnetic moment and the longitudinal axis is called the flip angle. It is this transverse component of magnetization that induces a voltage in the receiving coil in accordance with Faraday's Law. When the radiofrequency field is removed, the net magnetization vector begins to return to its thermal equilibrium state. The voltage induced in the receiving coil decays exponentially and is called a free induction decay or FID.

Two independent characteristic time constants are associated with nuclear magnetic resonance. The first time constant is the spin-lattice (= longitudinal) relaxation time, or T1. It describes the rate at which longitudinal magnetization will return to its equilibrium magnitude once it has been perturbed. T1 is a measure of the efficiency with which nuclei transfer energy to surrounding molecules. If the efficiency of energy transfer is high, T1 will be short; conversely, low efficiency corresponds to long T1s. Distilled water has a T1 of roughly 3,000 msec, which is extremely long. The second time constant is known as the spin-spin (= transverse) relaxation time, or T2. This time constant describes the rate of loss of transverse magnetization (or loss of phase synchrony of the magnetic moments). The dominant cause of loss of transverse magnetization is the transfer of energy from one spin or nucleus to another. T2 is typically much shorter than T1 and can never exceed T1.

Spin-Echo Imaging

A magnetic resonance imaging pulse sequence is a series of radiofrequency and magnetic field gradient pulses designed to manipulate a collection of nuclei to achieve a desired contrast. The most commonly used imaging sequence employs spin-echoes. This technique uses the instrument parameters time to echo (TE) and imaging sequence repetition time (TR). When a radiofrequency pulse of appropriate intensity, and at the same frequency as nuclear precession, is applied to an ensemble of spins, the macroscopic magnetic moment will be tipped entirely into the transverse plane. This is referred to as a 90° pulse. At the instant the radiofrequency energy is removed, all spins are in phase, and the transverse

magnetization is at its maximum magnitude. However, due to localized magnetic field inhomogeneities, individual spins experience different effective magnetic fields and begin to resonate at slightly different frequencies. This causes the spins to dephase and the magnitude of the transverse magnetization to decrease. If a short time (TE/2) later a 180° radiofrequency pulse is applied, the individual spin vectors will be reflected in the transverse plane (180° from their previous orientation). This will reverse the trend of dephasing, and the transverse magnetization will begin to grow. At time (TE/2) later, the transverse magnetization is again at its maximum value (neglecting T2 effects), and a spin-echo is formed. A spin-echo consists of the mirror image of an FID, followed by an FID, and reaches its maximum intensity at the time interval TE following the initial 90° pulse. In a spin-echo imaging sequence, radiofrequency pulses are used to nutate the macroscopic magnetic moment into the transverse plane (90° pulse) and then rephase the dephasing spins (180° pulse). Magnetic field gradients are used to selectively choose a slab (or slice) of nuclei for excitation. They are also used to electronically tag or encode spins with phase and frequency information. During image processing, a Fast Fourier Transformation is used to decode this phase and frequency information into image coordinates and pixel (picture element) intensity. The time to execute a single stage of a pulse sequence is known as TR.

Image Construction and Resolution

Readers are familiar with computer displays in which the two- dimensional units of display are called pixels. With MRI, displays are two-dimensional but involve a third dimension determined by the thickness of the slice chosen for image acquisition. Thus, each unit of display represents a unit of volume in the sample rather than a unit of area. These units are termed voxels.

Most images are acquired as grids of voxels representing squared powers of 2 (e.g., 128 by 128, or 256 by 256 voxels). Typically, the image must represent a slice through the entire sample. Therefore, if the field of view is 25.6 cm across and sampling is based upon a 256 by 256 voxel grid, each voxel must represent a square 25.6 cm/256 = 0.1 cm on each side. Similarly, with a sample 2.56 cm across, each voxel could represent a square with sides 0.01 cm in length. This restraint imposed by sample size easily defines the limits of resolution. Additional limitations to resolution are imposed by the abilities of the instrument to induce detectably different frequencies of radiation from adjacent voxels. This limitation becomes increasingly important as voxel dimensions decrease due to decreased sample size and concomitant decrease in field of view.

A further constraint to resolution is imposed by slice thickness. Slice thickness determines the depth parameter of sampling, and thus the amount of tissue from which signals are produced. More tissue produces stronger, more easily detected signals. Excessively thin slices produce insufficient radiation for the instrument to differentiate it from background noise. Within an ideal subject having all structure parallel and oriented perpendicular to the plane of slicing, slice thickness would be irrelevant. However, in most tissues, nonparallelism of structure imposes a significant barrier to resolution. Selection of slice thickness therefore normally represents a compromise between the need for signal intensity and the desire to optimize resolution.

Image Intensity, Weighting, and Contrast

The relaxation parameters T1 and T2 are interrelated with the instrument parameters TR and TE and a fifth factor, spin density or proton density, in determination of spin-echo image intensity. The signal intensity (I) is related to these five parameters through the approximate expression

$$I = N_H(1-e^{-TR/T1})e^{-TE/T2}$$

where:

N_H = proton density and

e = the base of natural logarithms (Werhli and others 1983). This expression shows that an increase in the number of protons has a direct linear effect on image intensity. Thus, other factors being equal, the higher the moisture content of a system, the brighter its image will appear. Effects of other variables (T1, T2, TR, and TE) are expressed in an exponential manner as follows: assuming that all other parameters remain constant, an increase in T1 decreases signal intensity, an increase in T2 increases signal intensity, an increase in TR increases signal intensity, and an increase in TE decreases signal intensity.

Often, images are referred to as T1-, T2-, or proton density- weighted. Typically, tissues with the shortest T1 relaxation times will exhibit the greatest image intensity when short echo times are employed. Conversely, tissues with the longest T2 relaxation times will exhibit the greatest image intensity when long echo times are used. These differences in effects of echo times can produce images that differ dramatically in appearance and are referred to as T1-, or T2-weighted

images, respectively. Intermediate echo times produce images with intermediate or mixed effects that are referred to as mixed-weighted, or proton density-weighted images, as proton density is the overriding determinant of intensity in these images. Most mixed-weighted images that we have obtained from plants exhibit very little contrast between tissues, a reflection of fairly uniform distribution of proton abundance.

Image contrast derives from differences in signal intensity between neighboring voxels. When T1 and T2 relaxation times of a sample are obtained, they represent an average value for the entire sample, but provide no information about differences between cells and tissues within the sample. Because each voxel is likely to contain material with different relaxation times than neighboring voxels, each will exhibit a different signal intensity.

DEMONSTRATED APPLICATIONS OF PLANT MAGNETIC RESONANCE IMAGING

Magnetic resonance images of plants often are high in contrast between tissues and closely resemble gross anatomy. Water is the most abundant source of mobile protons within most plant tissues, and interactions of water protons with their molecular environments are responsible for differences in relaxation times. Therefore, when considered at the physiological level, most plant MRI studies, deliberately or otherwise, are studies of internal (or external in the case of some root investigations) water relations.

Plant Anatomy and Morphology

Magnetic resonance images of plant tissues often are relatively easy to obtain and, like many medical images, may resemble more conventional anatomical preparations. Proper interpretation of these images is dependent upon knowledge of proton abundance and relaxation behavior as discussed previously. Proton relaxation times within human and animal tissues have been reviewed by Bottomley and others (1984) and Mathur-DeVre (1984). Differences among these tissues in proton relaxation times commonly are attributed to binding of water protons to macromolecular proteins and nucleic acids. Few plant tissues, however, contain quantities of proteins and nucleic acids comparable to those found in animal tissues (some seeds may present an exception). Plant tissues, however, contain soluble and insoluble carbohydrates in quantities suitable to cause proton relaxation behavior similar to that associated with proteins and nucleic acids in animal tissues.

Among published studies employing MRI to examine plant structure, attributions of variations in image intensities to differences in water binding are common, but few have attempted to ascribe such binding to specific components, nor have they employed actual determinations of proton relaxation times. The role of some plant constituents affecting the relaxation behavior of water protons was studied by Potchen and others (1994). Powdered aqueous suspensions of carbohydrates (cellulose and starch) exhibited short T1 and T2 relaxation times, as did carbohydrate gels (starch and pectin). Solutions of mono- and disaccharides exhibited similarly short T2 relaxation times, but much longer T1 relaxation times than comparable concentrations of polysaccharides. Within a variety of plant tissues, T1 relaxation times were inversely related to total dry matter.

Use of MRI to nondestructively study plant tissue structure represents one of the most common applications of the technology to date. Hinshaw and others (1979) used images of apples, oranges, and plums in an early demonstration of the resolving capabilities of MRI, and Connelly and others (1987) and Chavagnat and others (1992) demonstrated high resolution in studies on germinating mung bean and pepper seeds, respectively. Veres and others (1991) used images of stems and fruits of squash to demonstrate the manner in which interactions between instrument parameters and tissue relaxation times influence the appearance of images. Similar manipulation of these interactions were employed to image stem tissues of geraniums (Brown and others 1988), and vascular bundles within stems of asparagus (Halloin and others 1994).

Several studies (Hall and others 1986, Halloin and others 1992b, Wang and Chang 1986) have demonstrated the utility of MRI for non-destructive imaging of the internal structure of wood. Structures such as annual rings, knots, and worm holes are easily distinguished, and live knots can be distinguished from dead ones (Halloin and others 1992b). Figure 1, a transverse MRI section through a willow stem, demonstrates the differentiation of annual rings, live, and dead knots that is achieved with MRI. Dried wood is unsuitable for imaging, as it lacks sufficient water protons, but rehydration of previously dried wood makes it suitable for imaging (Hall and others 1986).

Some plant components other than water are sufficiently abundant and have proton NMR spectra sufficiently different from water that their localizations within plant tissues can be imaged selectively with a technique called chemical shift imaging. With this technique, imaging principles are the same as described previously, but data are collected at the resonance frequency of the desired chemical, rather than at that of water protons. This technique has been used to selectively image the distribution of aromatic compounds in orange peel, grape berries, and fennel fruits (Pope and others 1991). The same methods were used to demonstrate differential localizations of water and lipid in imbibed pecan embryos (Halloin and

others 1993) and olive fruits. (Gussoni and others 1993). Differences in the relaxation times of water and lipid protons in the pecan embryos enabled differentiation of their localizations through conventional spin-echo methods as well, merely by altering instrument parameters (TR and TE). Several atomic nuclei other than ^{1}H are magnetically susceptible, and thus suitable for imaging when sufficiently abundant. Rollins and others (1989) applied high concentrations of a fluorinated herbicide containing ^{19}F to tomato plants and imaged localization of ^{19}F within stems.

Many of the described applications of MRI to the study of plant anatomy and morphology can be accomplished with more conventional histological methods, albeit destructively and/or with greater difficulty. The results of one application, however, can be achieved only with MRI. That application is the imaging of root systems within their soil matrices. At normal water potentials (less than field capacity), the T1 relaxation times of soil water protons are sufficiently short that they do not produce significant levels of signal with conventional imaging sequences (MacFall and others 1990). Because water protons within roots have longer relaxation times than those in soil, images of roots within soil appear as bright roots within a dark background. Rogers and Bottomley (1987) studied the suitability of a large array of soils for imaging of root systems and found that those with contents of ferric materials less than 4 percent (w/w) were suitable for imaging. Higher contents of ferric materials had distorted images that were unsuitable for most imaging applications. Sand is suitable for many applications (MacFall and others 1990), and Brown and others (1991) developed a synthetic soil mix composed of sand, peat moss, and Kaolinite clay that contained no ferric materials and yielded high quality images of root systems. Magnetic resonance imaging of root systems in soil is likely to prove valuable in future studies such as those on root development, turnover, disease, and other root-micro-organism interactions.

Physiology: Water Dynamics

Because intensities within MRI images are dependent largely upon abundance and binding interactions of water protons, water dynamics of plants is an ideal topic for exploration with MRI. This application of the technology has been well exploited by pioneering researchers through a variety of techniques.

Differences in water contents among tissues of geranium roots (Brown and others 1986) and stems (Johnson and others 1987), and changes in these distributions as a result of transpiration were among the early observations. Water movement within tissues of rapidly transpiring plants was observed by use of paramagnetic contrast agents: typically, solutions of paramagnetic ferric or cupric salts (Bottomley and others 1986, Johnson and others 1987). A recent extension of these lines of investigation (Bottomley and others 1993) employed MRI to demonstrate stem shrinkage in vetch seedlings as a result of transpiration stress, and short-term suppression of transpiration and shrinkage, but not of water transport, as a result of elevated atmospheric CO_2 concentration. Depletion of water from soil immediately surrounding roots of transpiring loblolly pine seedlings was imaged by MacFall and others (1990). They further demonstrated that this imaging was made possible by a reduction in the T1 relaxation rate of water within the soil as a result of water uptake by the plants.

Through a modification of conventional imaging techniques known as diffusion imaging, Eccles and others (1988) were able to demonstrate differences among tissues of imbibed wheat grains in rates of water diffusion. Diffusion imaging of water within a stem of box elder (*Acer negundo* L.) is shown in figure 2. Because this method differentiates between moving and relatively stationary water, it is likely to have wide applicability in studies of plant water relations. Extensions of the methods enable determinations of diffusion coefficients, flow velocities, and flow directionality.

Several plant physiology studies using MRI have been done in areas not commonly associated with water dynamics. However, as will be discussed, the successful use of MRI in these studies was dependent upon changes in associated water relations. Changes in water binding have been associated with both development of winter hardiness of plants and vernalization of plants as a result of cold treatments. Faust and others (1991) were unable to image dormant apple buds prior to vernalization, apparently due to extremely short relaxation times. They were successful in imaging buds following prolonged cold treatment, apparently due to decreased water binding and associated increases in relaxation times of the water protons. The effects of Na^+ on development of sugarbeet roots was investigated with MRI in conjunction with other methods (Kano and others 1993). Image intensity in epidermal and vascular tissues was reduced by the presence of Na^+ in the medium, apparently due to water stress. Similarly, turgor and osmotic pressure determinations in roots of a halophytic aster were related to MRI image intensities (Zimmerman and others 1992). MacFall and others (1992) imaged the effects of perfusion of nitrogen fixing nodules of soybeans with different gases. Perfusion of nodules with N_2 caused an increase in image intensity and in the T1 of both cortical tissues and inner nodule tissues as compared with tissues perfused with air or O_2. The increase in T1 was greater in the cortical than in the inner tissues, indicating a barrier to gas exchange between these

tissues. The observed increases in image intensity and T1 relaxation times likely were due to changes in the water-binding capacity and/or water contents of the tissues.

Diseases and Disorders

Research employing MRI in the study of plant diseases and disorders is merely an extension of studies on normal anatomy and/or water relations, making use of the basic advantages of MRI discussed previously. Chen and Kauten (1988) demonstrated the utility of MRI for nondestructive imaging of internal quality factors such as bruises, worm damage, voids, and dry areas in a variety of fruits and vegetables. Development of physiological core breakdown of pears was followed nondestructively by Wang and Wang (1989).

Application of MRI to the simultaneous study of internal anatomy and water relations was employed in a study of fusiform rust galls of pine (MacFall and others 1994). Magnetic resonance imaging revealed continuity of phloem and cambial tissues, but not of xylem and water transport, between healthy and galled tissues. Several other studies have used MRI to investigate aspects of plant diseases. Goodman and others (1992) followed development of rot in raspberry fruits over a 4-day period, and found that MRI provided good differentiation between diseased and healthy tissue. Similarly, ready differentiation of healthy and diseased tissues was observed in poplar wood (Halloin and others 1992b) and sugar beets (Halloin and others 1992a). Magnetic resonance images of rot development in sugar beet roots over a 3-week period revealed different patterns of disease development within different roots, indicative of differences in disease resistance. A study on lipid distribution in pecans discussed earlier (Halloin and others 1993) demonstrated the ability to image both quantitative and distributional alterations in lipid as a result of seed rot and insect damage.

THE FUTURE OF PLANT MAGNETIC RESONANCE IMAGING

Attempts to predict future directions and developments in any area of science may be, at best, either foolish or presumptuous. Past applications of both plant and medical MRI, together with the strengths and limitations of the technology, make some applications seem highly likely.

As in the past, physiological investigations will emphasize water relations. A change in the focus of these studies, however, is likely to emphasize techniques that enable imaging and quantitation of water movement. These techniques, now gaining widespread acceptance in human angiography (Potchen and others 1993), enable determinations of localization, velocity, and volume of fluid movements. They will prove very useful for determination of disruption and other abnormalities in fluid flow by diseases and physiological disorders, as well as for studies on normal physiology.

Magnetic resonance imaging will continue to be used for noninvasive studies of plant anatomy and development. Major applications of the technology seem possible for routine quality monitoring of produce and for determination of internal structure of logs prior to sawing. Technology comparable to that required to monitor internal structure of logs is currently employed for monitoring core samples obtained during drilling of oil wells.

Anatomical investigations are readily applicable to selection of plants with desired characteristics, such as disease resistances or modified internal structure. The destructive nature of currently used methods often makes them unsuitable for such selection. Spectral shift imaging enables localization and quantitative assessment of specific chemical components. These methods could be used in selection of plants with desired modifications in the quantity or localization of those components. Nondestructive selection of seeds unusually high or low in oil content seems especially amenable to MRI.

Future developments on MRI of plants are dependent both on availability of the technology to a broader community of scientists and the specific interests of those scientists. Good understanding of the underlying principles of the technology is essential to its effective use. Magnetic resonance imaging of plants is a technology that truly is in its infancy, and many imaginative applications of it are certain to appear.

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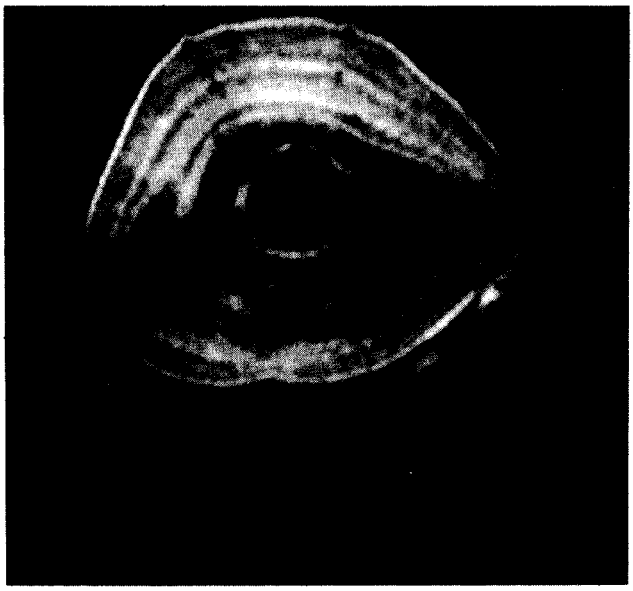

Figure 1. *Spin-echo magnetic resonance image of a transverse section through a stem of willow* (Salix *sp.*). *Annual rings are differentiated by greater image intensity in the larger vessels of spring wood. Knots (left and right) appear dark due to compression of wood, giving lower abundance of water. A live knot (right) is distinguished from a dead one by the annual ring formations around it. Maximum stem diameter = 6 cm, magnet = 4.7 Teslas, TR = 500 ms, TE = 25 ms, slice thickness = 3 mm.*

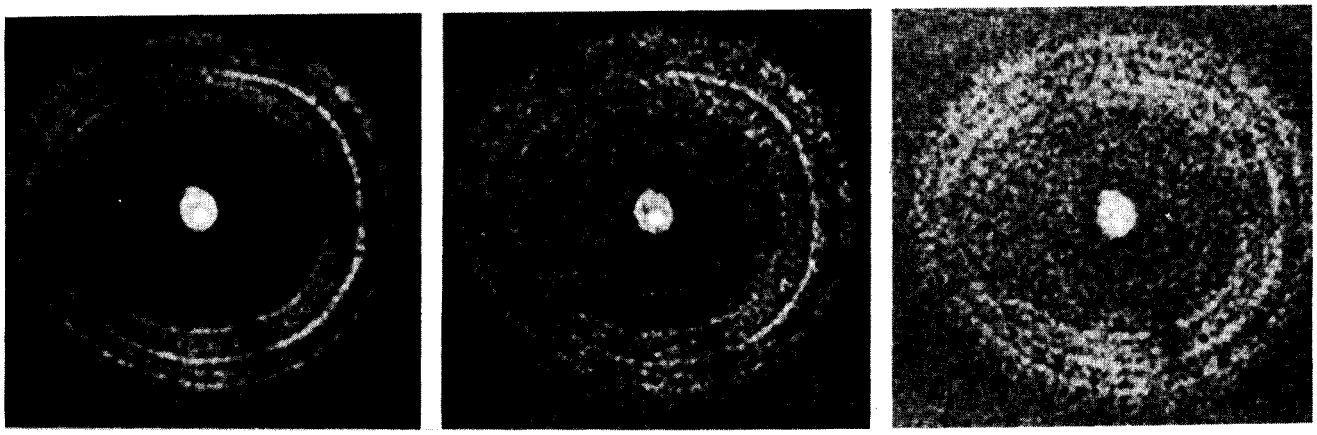

Figure 2.--*Diffusion imaging of water in a 5 cm diameter stem of box elder (*Acer negundo *L.).*
***(Left)** Transverse spin-echo image of the stem (TR = 800 ms, TE = 64 ms, slice thickness = 3 mm, maximum stem diameter = 5 cm).*
***(Center)** Image of the same slice, acquired with the same imaging parameters, but with diffusion gradients on (5 gauss/cm for 15 ms) between the initial 90° pulse and the 180° echo pulse. Use of these gradients produces a gradient in the magnetic field strength, which in turn causes dephasing of any protons that move during gradient application. This dephasing results in loss of signal from the dephased protons.*
***(Right)** Difference image obtained by subtracting voxel intensities in the center image from those in the left image. This produces an image with intensities that represent the relative abundances of protons that diffused. Pith cells and spring wood vessels, the cells with the greatest internal volumes, allow the greatest diffusion.*

Use of Magnetic Resonance Imaging for Nondestructive Assessment of Knots and Rots in Wood[1]

J.M. Halloin, J.H. Hart, T.G. Cooper, and E.J. Potchen[2]

Noninvasive visualization of internal structures of healthy logs of *Acer negundo* and *Salix* sp. and diseased logs of *Populus tremuloides* was accomplished using spin-echo magnetic resonance imaging. Image intensities and contrast are determined by the protons (H nuclei) of water in the tissues and are influenced both by the abundance of water and by physical interactions of the water protons with surrounding molecules. Observed features corresponded closely to visual appearances following sectioning of the logs. Annual ring structure was apparent, with highest image intensity in the more porous spring wood. Bark and dead knots gave low image intensity. Live knots were discernible due to changes in orientation of the annual rings. Rotted tissues produced higher image intensities than surrounding healthy tissues apparently due to less intense binding of water in the diseased tissues. Magnetic resonance imaging should prove useful for nondestructive assessment of internal structures in wood and for sequential studies of rot development.

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Magnetic Resonance Microscopy of Water Movement Through Fusiform Rust Galls of Pine[1]

Paula Spaine, Janet S. MacFall, and G.A. Johnson[2]

ABSTRACT

Galls on 10-mo slash and loblolly pine seedlings inoculated with *Cronartium quercuum* f. sp. *fusiforme* were compared with healthy stems by magnetic resonance microscopy (MRM). Stems were excised at the root collar and placed in either deionized water or water containing an MR contrasting agent. Following transpiration, high resolution images (35 to 58 um) were acquired of excised stem segments. Magnetic resonance microscopy images showed greater signal in the xylem of healthy stems than in galls, suggesting differing wood/water interactions. In 10-mo-old galled seedlings, the cambium and phloem were contiguous between healthy and galled regions. Water transport disruption occurred in the xylem at the interface between galled and healthy regions, but in the center of the galls, secondary xylem appeared waterfilled and functioning. This study shows changes in anatomy and functional physiology in vivo with respect to water relations in fusiform rust galls on pine that are detectable by MRM.

Keywords: Forestry, fusiform rust, magnetic resonance imaging, MRI, tree physiology.

INTRODUCTION

Fusiform rust is one of the most prevalent and economically damaging diseases of pines in the Southern United States. Both loblolly (*Pinus taeda* L.) and slash pines (*P. elliottii* Engelm. var. *elliottii*) are most often infected; however, longleaf pine (*P. palustris*), pond pine (*P. serotina* Michx.), and 23 other species and varieties of native and exotic pines may also become infected (Czabator 1971). Infections often result in both stem and branch galls, which lead to reduced wood marketability from growth reductions and stem and branch breakage, resulting in reduced yield and wood quality (Anderson 1986). The mortality is most often noted in nursery stock and young seedlings due to the disease. Although significant research has focused on the identification and breeding of increasingly resistant pine families, the mechanisms of resistance and tolerance have not been identified.

The pathogen, *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*, (*Cqf*), is a macrocyclic heteroecious rust fungus, alternating between oaks and pines (Czabator 1971). In active galls of pine, vegetative mycelium is restricted to the ray parenchyma and cambial layers (Jewell 1988, Jewell and Spiers 1976, Jewell and others 1962). Standard histological methods have shown seedlings of loblolly and slash pine with fusiform rust from both stem and branch galls displaying severe cellular dysplasia, hypertrophy, and alterations in cellular organization (Jackson and Parker 1958, Jewell and others 1962). Lack of cambium and xylem fibers in the most swollen regions of some stem-girdling galls has been reported, with significant necrosis observed in the constricted stem region below the gall (Walkinshaw and Roland 1990).

Although this disease has been the subject of intensive research focusing on anatomical alterations and identification of resistant germplasm (Anderson 1986, Jewell and others 1962, Rowan 1970, Walkinshaw 1978), specific mechanisms mediating gall formation and the effect of galls on tree water relations are not understood. Little is known about how trees develop strategies for rust disease tolerance and continued growth, despite the development of stem and/or branch galls.

New techniques such as high resolution magnetic resonance imaging (MRI), or magnetic resonance microscopy (MRM) offer the potential for detailed, nondestructive examination of plant tissues (Kramer and others 1990, MacFall and others

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1987). In MRM, image contrast depends not only on water distributions within the tissue, but also on physiological functions detailed by degrees of water binding. These are reflected in proton relaxation times (the rate at which ^1H nuclei return to equilibrium following excitation from an externally applied pulse). Like classical histopathology using light microscopy, strategies for image acquisition with MRM can provide detailed anatomical and functional information. Image contrast can be varied significantly, depending on the acquisition strategy used, and can be adjusted to highlight water binding, distribution, diffusion, and transport patterns (Kramer 1983, Kramer and others 1990).

The primary objective of these studies was to determine alterations in water transport processes caused by changes in stem morphology due to development of fusiform rust galls. Magnetic resonance microscopy was used to provide information on tissue organization and water transport throughout galls formed on stems of young loblolly and slash pines.

MATERIALS AND METHODS

Seeds of half-sib seedlots of slash and loblolly pine were germinated in vermiculite and transplanted after 10 days to a mix of sand/peat (1:1). Plants were maintained in the greenhouse at 20 to 30 °C, with 18 h of light. At 6 to 9 wk of age, seedlings were inoculated with *Cqf* basidiospores by the CBS (concentrated basidiospore spray) system (Matthews and Rowan 1972). Basidiospores were collected from *Quercus rubra* seedlings that had been inoculated with a mixed gall collection of aeciospores collected from Clarke County, Georgia. Individual seedlings 12 and 18 mo old that displayed a variety of rust symptoms ranging from no apparent gall formation to well-developed galls were selected for imaging.

Immediately prior to imaging, stems were excised at the root collar and placed into either tubes filled with water or tubes containing a 1:200 dilution of an MR contrast agent gadopentatae dimeglumine (Magnavist, Berlex Laboratories, Wayne, NJ). Shoots were illuminated to stimulate transpirational uptake.

Application of specific, defined dilutions of an MR contrast agent such as Magnavist causes increased signal intensity in water-filled stem regions into which Magnavist has been introduced with the transpirational stream.[3]

Following a period of active transpiration (12 to 14 h), during which at least 1 ml had been taken up, segments of stem (2 to 3 cm in length) with and without galls were excised from the shoots. Pieces of stem were wrapped in plastic foam to prevent movement and desiccation, then were placed in a custom built solenoid radio frequency (rf) coil tuned to 400 MHz. In some experiments, a healthy stem and a gall were imaged simultaneously.

Images were acquired on replicate stems. For slash pines, two healthy stems and three galls from 12-mo-old trees, and three healthy stems and five galls from 12-mo-old slash pines with Magnavist were imaged.

Images were acquired on a General Electric 9T Omega Imaging System (General Electric Medical Systems, Freemont, CA), and three-dimensional image sets were acquired. The field of view ranged from 9 to 15mm, depending on specimen size. Images were acquired with a simple spin echo pulse sequence with a repetition time of 200 ms and an echo time of 7.5 ms. The number of slices in the 3-D volume ranged from 64-256 depending on specimen size and scanner availability. Individual 2-D image slices were 256 by 256 pixels, giving a digital resolution of 35 to 58 um.

Images were reconstructed and viewed offline on a Silicon Graphics IRIX or Sun workstation. Three-dimensional rendering software was a commercial product (VoxelView ULTRA, Vital Images, Fairfield, IA). Three-dimensional image-viewing software enabled slices to be viewed both individually and as fully rendered three-dimensional datasets, allowing the 3-D volume to be "sliced" and viewed through any arbitrarily chosen slice-plane.

RESULTS

Significant differences in stem anatomy and patterns of water distribution and binding were observed between galls and healthy stems. In seedlings of both slash and loblolly pine, similar changes in anatomy and water binding/distribution were observed (fig. 1). Specific tissues could be differentiated in MRM images of both galls and healthy stems, but alterations in tissue organization could clearly be seen with gall formation.

Within the healthy stems and stems below the gall, the secondary xylem, phloem, cortical parenchyma, and epidermis were clearly distinguishable in the images (fig. 2). Concentric rings of bright and dark could be seen within the secondary xylem (fig. 2). This is a pattern that has been consistently observed in stems and taproots of pine (MacFall and others 1991)

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[3] MacFall, Janet. [n.d.] Unpublished data. On file with: In Vivo Center for Microscopy, Duke University, Durham, NC 27706.

and apparently is representative of regions of water transport up the stem (the bright zone) and regions that are not participating in longitudinal transport (the dark zone).

Identification of the regions of transpirational water transport was confirmed by the examination of stems that had Magnavist introduced into the transpirational stream. Previous studies[3], have shown that application of a 1:200 dilution of this MR contrast agent to water available for plant uptake will increase signal intensity (in images acquired with short repetition times) within regions of stems that are functioning in transpirational water transport. The observed increase in signal in both galls and healthy stems with application of Magnavist confirms that water was transported through both galls and healthy stems.

Signal increases observed in the cross-sectional views were mainly limited to the bright rings of secondary xylem, confirming that these are the regions of stems that are active in longitudinal water transport. This also confirms that the water distribution patterns within the xylem seen in the images acquired of nontranspiring stem segments are indicative of water transport patterns.

Changes in the anatomy of galled stems as seen in the MRM images were striking at the interface between healthy stem regions and galls (fig. 2). In the transition zone between healthy stem and gall, the cambium and phloem became wider, with a proliferation of cortical parenchyma and secondary xylem. Progressing further up the stem, ray parenchyma and secondary phloem had formed that were not seen in healthy stems, and that appeared to push out the cortical parenchyma. This new tissue is unique to galls, giving a striated appearance to regions outside the phloem ring.

The phloem of the galls did not appear affected by disease development. The longitudinal views through the three-dimensional MRM stem volumes of the young seedlings show that the phloem was contiguous through the gall (fig. 3). Additional changes in anatomy were observed interior to the cambium of galls and within the region of transition between healthy stem and gall (fig. 3). Disruption in the secondary xylem was observed as darkened, speckled areas in the area of transition. Travelling up the stem, this area appears as a dark speckled band sweeping inward toward the gall center. It is likely that with the rapid proliferation of tracheids formed with gall development, longitudinal connections were not fully formed, thus end-to-end transport of water was impeded.

Within the center of the well-developed gall (fig. 2), little disruption in transport was apparent in the secondary xylem. This is likely to be indicative of lateral transport within the secondary xylem, filling with water tracheids not directly contiguous with xylem tissue lower in the stem. The secondary xylem also appears more striated compared to healthy tissue, due to proliferation of ray parenchyma within the gall. Similar ray enlargement in both loblolly and slash pines has been observed with light microscopy (Jackson and Parker 1958, Jewell and others 1962).

DISCUSSION

The observations made from MRM images of fusiform rust galls reported here are important in that they clearly show changes in tree anatomy and water relations associated with *Cqf* infection and subsequent gall development. Anatomical changes included the proliferation of cortical parenchyma, that was pushed out and replaced with enlarged phloem rays. The secondary xylem was clearly seen in both galls and healthy stems, but in the galls it appeared more striated from the proliferation of ray parenchyma between the conducting tracheids. Similar changes in anatomy have been described with standard histological techniques (Jewell 1988, Jewell and Spies 1976).

A surprisingly high degree of organization was observed in both tapered and stem-girdling galls in slash and loblolly pines at 12 mo. This is in contrast to previously reported histological observations describing stem girdling galls in slash pine (Walkinshaw and Roland 1990), wherein significant cellular disorganization was observed in the cortex, cambium, and xylem. The formation of numerous wound-callus cells, thought to interfere with normal translocation and to be contributing to the mortality associated with this gall type was reported. Similar disruption in transport might have been observed in our experiments had we followed the gall formation over an extended period of time.

The imaging experiments showed differences in the patterns of water distribution/transport between healthy and galled seedlings. Disruption in anatomy and water distribution was primarily at the transition zone between healthy and gall tissue. Little disruption in anatomy was observed in the center of the gall, demonstrating compensating patterns of flow from the regional disruption in the transition area. Similar compensating paths for the ascent of sap have been observed by others following stem wounding (Kramer and others 1990).

In addition to alterations in water distribution and anatomy, the difference in signal intensity of secondary xylem between galls and healthy stem tissues suggests differences in water binding to the wood. When water without Magnavist was taken

up by seedlings, images of the excised stems from seedlings with galls showed less signal in the secondary xylem than those from healthy seedlings.

Water uptake by transpiration through stems severed at the root collar was not reduced through galled stems. Equal or greater amounts of water were taken up by stems of seedlings with galls than by healthy seedlings. This observation implies that the water content of the galls was not less than the water content of the healthy stems, as rates of transpiration were not reduced with gall formation.

Reduction in signal was, therefore, likely due to a difference in water binding between galls and healthy stems, resulting in a longer T1 for the water within the galls.

CONCLUSION

The observations made here suggest that there are alterations in the stem tissue chemistry that are reflected by changes in tissue/water interactions with fusiform rust gall development. The changes in tissue chemistry occur within the first year of growth.

Both the anatomy and water relations of the pine stem have been altered by the presence of galls, and these changes can successfully be studied with MR imaging techniques. Future studies will provide additional information regarding relationships between the wood chemistry and the plant functional physiology as seedlings age and galls develop. Potentially, plant mechanisms for reducing disruption in physiological processes such as water transport with gall development can be identified and may be associated with genetic traits for tolerance to disease.

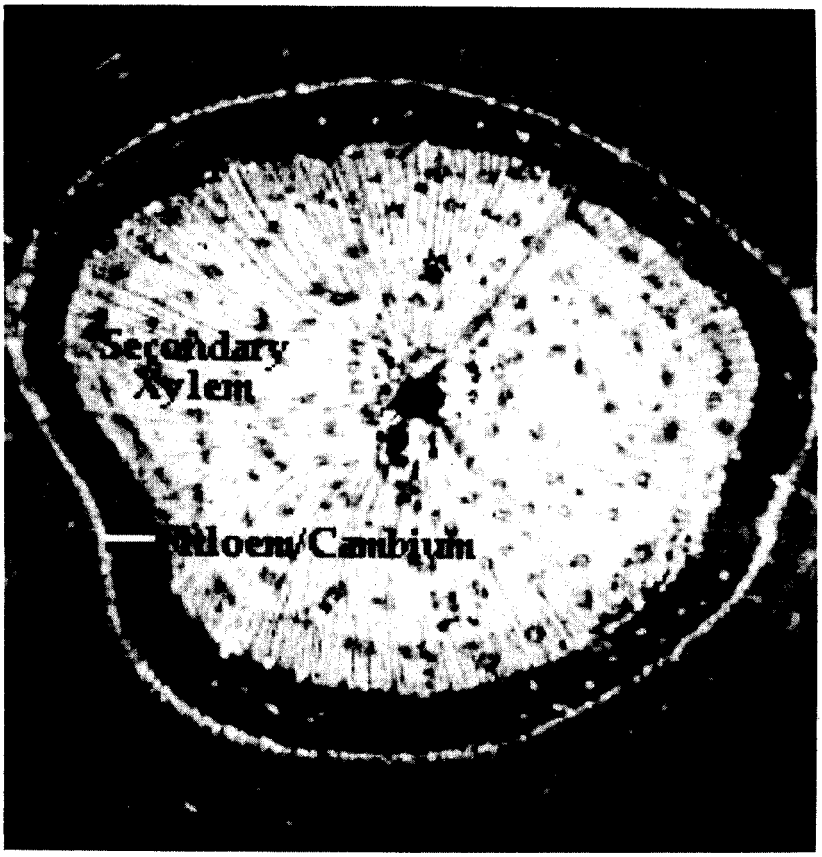

Figure 1. *High resolution image of a gall cross-section. Note the straited appearance of the tissue outside the phloem, and the rays within the secondary xylem. The alternating pattern of bright and dark concentric rings interior to the phloem is typical of both healthy and galled stems and taproots of pine. The bright inner xylem ring is functional in longitudinal water transport. The reference tube diameter was 1.12 mm.*

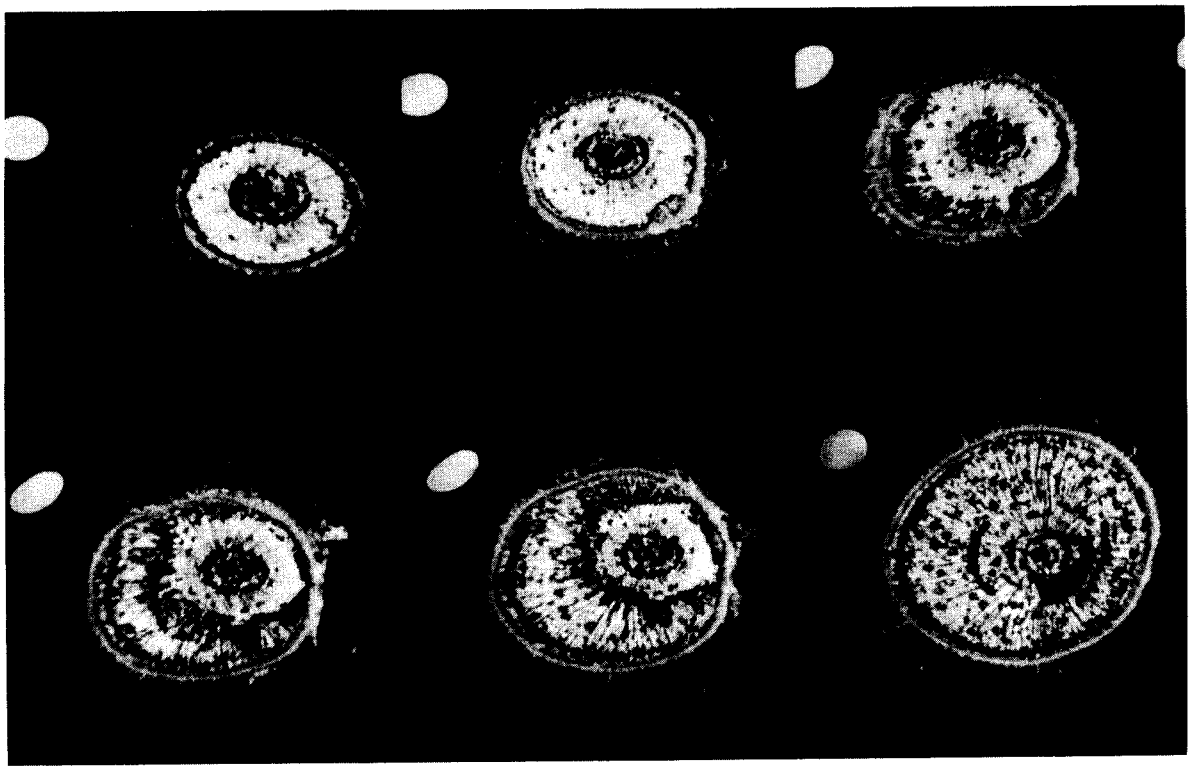

Figure 2. *Cross-sectional view at varied positions through a fusiform rust stem gall in a 9-month-old seedling. The reference tube (bright circle in upper left corner) was 1.12 mm in diameter. A. Healthy stem section below gall. B. Beginning of the transition region between the healthy and galled stem. Note the swelling of the phloem ring and proliferation of parenchyma external to the phloem. C. Lower region of the gall, with rapid proliferation of parenchyma outside the phloem, initiation of the secondary phloem, proliferation of secondary xylem, and disruption of water transport as seen by the dark regions within the secondary xylem. D. Section through gall further up the stem. Note the pushed-out appearance of the parenchyma external to the phloem and replacement by parenchyma and secondary phloem with a striated appearance. Region also shows further proliferation of the secondary xylem, with transport disruption. E. Cross section near gall center. Note the disorganized parenchyma has been nearly replaced by the striated tissue, and less disruption of water transport is seen. F. Cross section of gall center. Note change in appearance of the secondary xylem compared to the healthy stem in A, and the change in tissue organization external to the cambium and phloem. Also, note there is little disruption of longitudinal water transport in the gall center.*

Figure 3. *Longitudinal view of the same gall as was viewed in cross-section in figure 2. Three-dimensional acquisitions such as this allow repeated study of the same specimen and "slicing" through any* orientation.

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Magnetic Resonance Microscopy (MRM) of Water Transport and Binding in Fusiform Rust Galls[1]

J.S. MacFall, P.C. Spaine, R.E. Doudrick, and G.A. Johnson[2]

Galled stems from 10-mo- to 2-yr-old seedlings of slash and loblolly pine previously inoculated with *Cronartium quercuum* f. sp. *fusiforme* were compared with healthy stems by Magnetic Resonance Microscopy MRM. Following transpirational uptake of water, high resolution images (32 to 46 um) were acquired of excised stem segments. Rapidly acquired images showed greater signal in the xylem of healthy stems than in galled stems, suggesting different wood/water interactions. In the 10-mo-old plants, the cambium was contiguous between healthy and galled regions. Water transport disruption occurred at the interface between galled and healthy regions, but in the center of the gall, the secondary xylem appeared water-filled. At 2 years of age, differences in water distribution patterns were apparent between galled and healthy stems, and between a galled stem that appeared otherwise symptomless and a galled stem from a tree showing initial symptoms of wilt. This study has demonstrated the utility of MRM in nondestructively studying changes in anatomy and functional physiology with fusiform rust gall formation in pine.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Nondestructive, Three-dimensional Study of Root Growth with Magnetic Resonance Microscopy (MRM)[1]

J.S. MacFall and G.A. Johnson[2]

ABSTRACT

Study of intact roots in soil has historically presented challenges to the researcher. MRM is a nondestructive imaging technique allowing repeated viewing of a subject over time. Three-dimensional image acquisition and rendering strategies have been developed which allow pine roots to be visually and digitally distinguished from the surrounding sand potting medium. Using this approach, the root systems of three pine seedlings have been repeatedly imaged over a 4 month period. Plants were allowed to grow undisturbed in tube containers filled with fine sand. Sequentially acquired, registered image sets of each plant showed the development of primary and secondary lateral roots, including mycorrhizal root types. Disappearance of fine roots was also observed within this period. MRM clearly has potential for the repeated nondestructive investigation of root growth over time.

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Structures and Transformations of Douglas-fir Bark Polyphenols[1]

Joe Karchesy[2]

ABSTRACT

Douglas-fir (*Pseudotsuga menziesii*) is a major timber species of western North America ranging from central British Columbia to central Mexico. The bark of this tree is an abundant source of polyphenols that have utilization potentials, possible medicinal values, and biological activities that are not completely understood. Molecular structures have been established for various procyanidin oligomers and polymers, phenolic glycosides, flavonoids, oxidatively coupled dimers of dihydroquercetin, and some lignans and related compounds. Phlobaphene polymers, which are found only in the outer bark of Douglas-fir, are significantly more complex than the procyanidins, and their structures have not been completely defined. Studies so far reveal that dihydroquercetin, polymeric procyanidin, phenolic glycoside, and lignan moieties make up polymeric phlobaphene structures. Polymer linkages are not well defined. Evidence so far suggests phenolic oxidative coupling plays a significant role in this complex chemical transformation of inner bark polyphenols to outer bark polyphenols.

INTRODUCTION

Douglas-fir (*Pseudotsuga menziesii*) is widely distributed throughout western North America where it can be found growing in a variety of habitats ranging from coastal temperate rainforests to very dry mountainous interior sites (Fowells 1965). Two varieties of this species are commonly acknowledged. The coastal variety (var. *menziesii*), which ranges from British Columbia south to California, is the larger of the two. In old-growth forests, trees in excess of 200 ft (even with tops blown out by winter storms) and 500 years of age are not uncommon. The smaller Rocky Mountain Douglas-fir variety (var. *glauca*) grows in the interior regions ranging from central British Columbia and western Alberta south to the mountains of central Mexico. While Douglas-fir is native to western North America, it has been introduced to a number of other places in the world including Europe, South America, and the South Pacific (Herman 1982, 1987).

Because of its abundance, large size, and strength properties of its wood and fiber, Douglas-fir has been the most important timber species in the Pacific Northwest, being highly valued for the manufacture of lumber, plywood, glue-laminated beams, and pulp and paper products. Such industrial operations generate a considerable amount of bark material that is presently used for its fuel value, in decorative landscaping, as a plywood adhesive extender, and for charcoal briquette production. The Muir and McDonald Company of Dallas, OR, continues to use Douglas-fir bark tannins to manufacture leather as it has since 1863.

Douglas-fir bark was recognized by earlier researchers as a rich source of phenolic extractives, and much of the natural products chemistry done on this species has been spurred on by an effort to find a better utilization of this resource (Hall 1971, Hergert 1962). Isolations and structural elucidations began in the late 1940's when the compound (+)-dihydroquercetin **(1)** was initially isolated from Douglas-fir heartwood (Pew 1948) and then bark (Hubbard and Kurth 1949). Since this compound constitutes about 5 percent of the whole bark and over 20 percent of the cork tissue of the outer bark, several generations of natural products chemists in Oregon have at one time or another investigated how this compound might be utilized. The isolation of additional flavonoid compounds from Douglas-fir occurred in following decades. Four new flavonoid glycosides were discovered in this species. Dihydroquercetin-3′-0-glucoside was found in the needles, cambium, sapwood, and bark in the late 1950's (Hergert and Goldschmid 1958). In the late 1960's, two different C-methylflavanone glucosides **(2,3)** were isolated from roots (Hillis and Ishikura 1969) and root bark (Barton 1969). The aglycone of 2, named poriol, was found to be associated with root rot (*Poria weirii*) in second-growth Douglas-fir stands (Barton 1967). Quercetin, catechin **(4)**, and epicatechin **(5)** were also found in the bark (Hillis and Ishikura 1969, Weinges 1958).

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The tannins of Douglas-fir bark also presented earlier researchers with the prospects of industrial utilization (Hall 1971, Hergert 1962). Kurth and others (1948) had shown that yields of 8 to 18 percent could be obtained by hot water extraction depending on variables such as tree age and position of bark on the tree. Younger trees showed the higher tannin contents. These results fit well with what we now know about Douglas-fir bark tannins. They are in highest concentrations in living inner bark tissues, and their concentration drops off rapidly in the outer bark as transformation to water insoluble phlobaphenes and possibly other substances takes place. Douglas-fir tannins were subsequently shown to be procyanidin oligomers and polymers with both catechin and epicatechin extending units (Hergert 1960, Karchesy and others 1976, Weinges 1958), but little else was known of the finer details of their molecular structures. Studies on the biosynthesis and location of Douglas-fir tannins (proanthocyanidins) in needles, cell suspension cultures, and bark showed that only procyanidins are found in the bark of older (80 yrs) trees, but that both procyanidins and prodelphinidins are found in the bark of very young trees (Stafford 1989, Stafford and others 1989). This has raised some interesting questions. What is the significance of this transient formation of prodelphinidins, and why and when does it stop during the trees' growth?

Another question that has been around somewhat longer is that of the nature of phlobaphene formation in the outer bark. Phlobaphenes are the reddish-colored phenolic materials from the bark that are alcohol soluble and water insoluble (Hergert 1962). Chemists have commonly believed that phlobaphenes were derived from co-occurring condensed tannins by oxidation and polymerization reactions. Kurth and others (1968) showed that dihydroquercetin was liberated from Douglas-fir phlobaphenes by an ethanolysis reaction indicating that other phenols may be involved in this complex transformation as well. During the ensuing decades, little additional information was gained about the nature of phlobaphenes because of the apparent complexity of their structures and lack of adequate purification techniques.

METHODS

By the 1980's, polyphenol natural products chemists were enjoying the use of new and far-reaching analytical techniques for structure determinations. In particular, ^{13}C NMR (Czochanska and others 1980, Porter and others 1982), 2-D NMR techniques that allowed for ^{1}H-^{13}C heteronuclear correlations (Ferreira and Brandt, 1989) and mass spectrometric techniques such as FAB and ion peak linked scanning (Karchesy and others 1986, 1989b, 1989c) gave us the unprecidented ability to determine the structures of some very complex polyphenols in their native form. The need for derivatization or degradation was significantly reduced. Of course, such compounds need to be isolated and purified, and we were fortunate to have new chromatographic gels and strategies available to help by complementing some of the older techniques that are still of great value (Karchesy and others 1989a). These analytical developments allowed us the privilege of making some significant advancements in understanding the structures and reactions of Douglas-fir bark polyphenols.

RESULTS AND DISCUSSION

Procyanidin oligomers, polymers, and associated flavonoid glycosides were isolated from the water-soluble fraction of the methanol extract of inner bark, which was partitioned between water and ethyl acetate. Study of the ethyl acetate fraction is not complete. The aqueous phase contained about 47 percent carbohydrate material by weight and the balance predominantly the polyphenols. Polyphenol-carbohydrate complexation (Haslam 1989) likely played a role in solubilizing the polyphenols into the aqueous phase in spite of repeated extractions by ethyl acetate. The finding of only C4→C8-linked procyanidin dimers in the aqueous phase and both C4→C8- and C4→C6-linked dimers in the ethyl acetate phase suggested some steric effects may be involved in such complexations as well.

Table 1. *Phenolic glycosides of Douglas-fir inner bark*

Epicatechin-7-0-ß-D-glucopyranoside
Catechin-7-0-ß-D-glucopyranoside
Catechin-4′-0-ß-D-glucopyranoside
3′-0-Methylepicatechin-7-0-ß-D-glucopyranoside
Dihydroquercetin-3′-0-ß-D-glucopyranoside
Dihydroquercetin-7-0-ß-D-glucopyranoside
Dihydrokaempferol-7-0-ß-D-glucopyranoside
Phloroglucinol-1-0-ß-D-glucopyranoside

The phenolic glycosides isolated are noted in table 1 (Foo and Karchesy 1989a). All were ß-D-glucopyranosides with glycosidic linkages through either the A-ring C-7 oxygen atom or the B-ring C-3′ or C-4′ oxygen atoms in the aglycones. Not surprisingly, most of the aglycones corresponded to either catechin, epicatechin, or dihydroquercetin. Individual oligomeric procyanidins through pentamers were isolated and their structures established as noted in table 2 (Foo and Karchesy 1989b, 1991). Structures were established primarily by use of ^{13}C NMR, IR, FABMS, and partial acid-catalyzed degradation reactions with benzylthiol. The dimers B-1 (6) and B-2 (7) were found in a significantly higher amount than B-3 and B-4 (combined wt. ratio of B1 and B2 to B3 and B4 = 6:1). Considering that both catechin and epicatechin monomers exist in abundance in the bark, it is thus not surprising to see that the rest of the oligomers, trimers through pentamers, are dominated (but not exclusively) by epicatechin extending units with C4→C8 linkages and that terminal units are mixed epicatechin and catechin. This agrees with proposals that procyanidin biosynthesis occurs by condensation of a free flavan-3-ol with a flavan cabonium ion or quinone methide to give dimers and higher oligomers (Haslam 1989). Of the trimers, tetramers, and pentamers, the most abundant compound of each group was the all epicatechin oligomer with all C4→C8 linkages **(8-10)**.

Table 2. *Procyanidin oligomers isolated from Douglas-fir inner bark.*

Epicatechin-(4ß→8)-catechin or B1
Epicatechin-(4ß→8)-epicatechin or B2
Catechin-(4α→8)-catechin or B3
Catechin-(4α→8)-epicatechin or B4
Epicatechin-(4ß→8)-epicatechin-(4ß→8)-catechin
Epicatechin-(4ß→8)-epicatechin-(4ß→8)-epicatechin
Epicatechin-(4ß→8)-catechin-(4α→8)-catechin
Epicatechin-(4ß→6)-epicatechin-(4ß→8)-epicatechin
Epicatechin-(4ß→8)-[epicatechin-(4ß→8)-]$_2$-epicatechin
Epicatechin-(4ß→8)-[epicatechin-(4ß→8)-]$_2$-catechin
Epicatechin-(4ß→8)-[epicatechin-(4ß→8)-]$_3$-epicatechin
Epicatechin-(4ß→8)-[epicatechin-(4ß→8)-]$_3$-catechin

The polymeric procyanidin fraction amounted to about 25 percent by weight of the aqueous soluble material (Foo and Karchesy 1989c). This optically active polymeric material had a number average chain length corresponding to about seven flavanoid units and was exclusively procyanidin in nature. No prodelphinidin units were detected. The extending units were composed almost exclusively of epicatechin moieties, and the terminal units were mixed catechin and epicatechin, as they were in the case of the oligomers. The C4→C8 interflavanoid bonds occurred more frequently than the corresponding C4→C6 bonds by a ratio of 4:1. This structural assessment was made by use of ^{13}C NMR and IR spectroscopy and partial degradation of the polymers with phloroglucinol in ethanol with 1 percent hydrochloric acid at room temperature and under nitrogen.

The polyphenols of Douglas-fir outer bark present a more complex picture than those encountered in the inner bark (Foo and Karchesy 1989e). The methanol soluble phlobophenes are the most abundant polyphenols. Chromatographic separation into various fractions was achieved by use of Sephadex LH-20 and methanol. Preliminary studies by use of ^{13}C NMR have indicated their structures to be composed of mixtures of polymeric procyanidin, dihydroquercetin, carbohydrate, and methoxyl moieties. The moiety composition of each major chromatographic phlobaphene fraction was distinctly different. Water insolubility appears to be in great measure due to the methoxyl groups, but other as yet undefined structural features may play a role as well. Polymer linkages remained undefined, but the ^{13}C NMR data did suggest the possibility of phenol oxidative coupling playing a role.

Confirmation of in vivo phenol oxidative coupling reactions occurring in the outer bark came with the later isolations of two dihydroquercetin dimers with B-ring biphenyl linkages. Pseudotsuganol **(11)**, a dihydroquercetin-pinoresinol coupled compound, was also the first true flavonolignan to be isolated in nature (Foo and Karchesy 1989d). [5′, 5′]-Bisdihydroquercetin **(12)** also was unique in being the first natural biflavonoid linked exclusively through the B-rings (Foo and others 1992). Both compounds further suggested that the C-5′ of the catechol B-ring may be the favored site for

oxidative coupling of dihydroquercetin in the outer bark. Additional oxidatively coupled oligomers need to be isolated and studied as models so that strategies can be developed to define such linkages in the phlobaphene polymers.

While the outer bark was investigated for other possible phlobaphene precursors (Malan and others 1992), the lignans pinoresinol **(13)**, epipinoresinol, and the furolactone **(14)** were also isolated from the methanol extract. Clearly, the isolation of pseudotsuganol indicates that lignans also can participate in phlobaphene formation and likely account for much of the methoxyl resonances observed in phlobaphene ^{13}C NMR spectra. The furolactone **(14)** gives us some additional views on polyphenol transformations in the outer bark. This relatively rare compound was previously identified by Koshino and others (1989) who isolated it as a metabolite produced from pinoresinol in cultures of *Epichole typhina*. Similar furolactones have been isolated (Castellano and others 1986, Jakupovic and others 1987); one of them, also from a tree bark extract of *Nectandra turbacensis* by De Carvalho and others (1987). Examination of the outer surface of the bark of a standing Douglas-fir tree quickly shows that a number of different fungi and lichens are living there. Are these compounds being produced by the fungi on the bark? Are fungi involved in phlobaphene formation in any way? Perhaps it is time we consider the tree, its bark, and things growing on the bark as an ecological unit.

Other potential phlobaphene precursors that have been isolated from the outer bark include several phenolic glycosides in addition to those previously reported in the inner bark. The two C-methyl flavanone glucosides **(2,3)** reported earlier in the roots by Hillis and Ishikura (1969), and root bark by Barton (1969) were among them. Also found present was 6-C-Methyl dihydrokaempferol 7-0-ß-D-glucopyransoide as well as its aglycone and 1,2-dimethoxy-4-0-ß-D-glucopuranoside benzene (Malan and others, 1992). Incorporation of such phenolic glycosides into the phlobaphene polymer structures can account for the carbohydrate (glucosyl) carbon resonances observed in the ^{13}C NMR spectra of some phlobaphene fractions.

Procyanidins such as B-1, B-2, and higher dilogomers are found in the outer bark, but in significantly lesser amounts than in the inner bark. Their oligomer and polymer profiles are not completely defined at this time. However, the proanthocyanidin dimer A-1 **(15)** was isolated, and its significance is not clear at this time since its existence in the inner bark is not resolved. It may be present in the ethyl acetate solubles of the inner bark and thus is synthesized there, or it may be formed in the outer bark as part of the array of polyphenol transformations taking place during outer bark formation. Clearly, there is a variety of phenolic compounds in Douglas-fir bark, and many remain to be identified before we can completely unravel the questions about phlobaphene formation, structure, and the nature of polyphenol transformations accompanying outer bark formation.

Oxidative coupling reactions of dihydroquercetin and pinoresinol are currently being studied in efforts to gain insight into the reaction and obtain models for phlobaphene formation [50]. In preliminary reactions so far, we have found that when dihydroquercetin is reacted with $K_3Fe(CN)_6$ under alkaline conditions, no B-ring to B-ring-linked dimers were found, but rather 3 A-ring to B-ring linked compounds were formed in low yield (12 percent or less). The C-5′ linked compound was most abundant. Dihydroquercetin was reacted with pinoresinol under similar conditions in an attempt to synthesize pseudotsuganol, but that product was not found. However, a biphenyl-linked pinoresinol dimer was obtained as one of the reaction products, again in low yield. Clearly, other reaction conditions need to be investigated to better understand this very important transformation that is going on in the outer bark.

CONCLUSIONS

Douglas-fir bark contains a wide variety of polyphenolic structures. While we have learned a great deal about these substances over the last five decades since Pew first isolated dihydroquercetin, there is still much for the natural products chemist to do. We still don't know the structures of one of the major groups of compounds--the phlobaphenes. Nor do we really understand the transformations of polyphenols that occur in the outer bark, let alone in the immediate forest ecosystem. In the past much of the polyphenol research on Douglas-fir, or any commercial timber species, has been driven by its industrial utilization. Dihydroquercetin was discovered because it interfered with pulping reactions. Bark was considered a waste product generated by production of more important lumber and plywood. The prospect of utilizing bark condensed tannins as a renewable resource to replace petrochemical-based wood adhesives is still attractive. It would be beneficial to future generations to develop new polymers and wood composites with less dependence on petroleum.

As we look to the future, one can see some new areas for polyphenol research. Increasingly polyphenols are being found to have significant medicinal values (Bisset and others 1991, Haslam and others 1989, Okuda and others 1989). Antiviral, antitumor, hypotensive, and antiulcer are just some of the demonstrated activities. Understanding the roles of molecular structures is paramount. Radical scavenging ability appears to be important, and astringency alone does not determine activity. Biologists are presently concerned with the cycling of carbon and flux of CO_2 in our forest ecosystems.

Douglas-fir is a major carbon sink in the Pacific Northwest forests. When one considers the large amounts of polyphenols produced by Douglas-fir and the other numerous woody plants in the forest, it is clear that a major flow of carbon in the environment is going through the polyphenols. Natural products chemists know that all carbon is not the same. Polyphenols can be physiologically active to a wide variety of organisms depending on various molecular structures. They also chelate and transport metals, and probably are involved in some things yet unknown.

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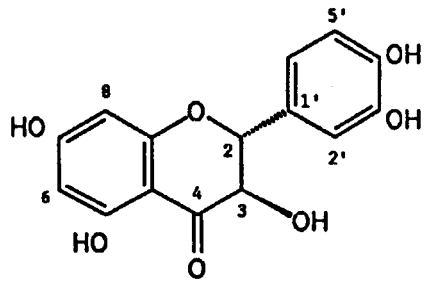

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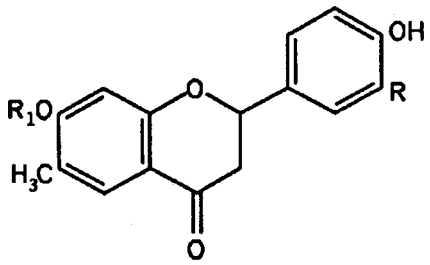

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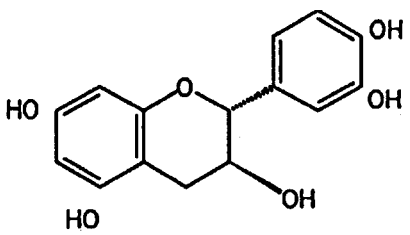
(2) R = H

(3) R = OH



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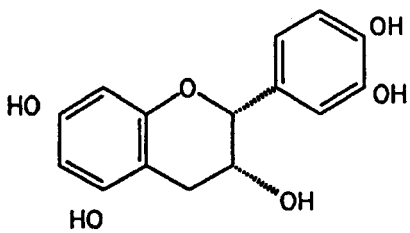

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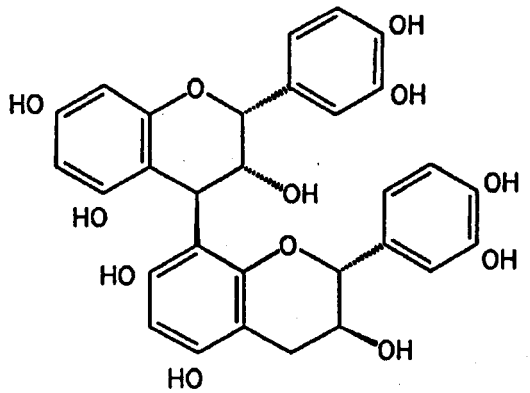

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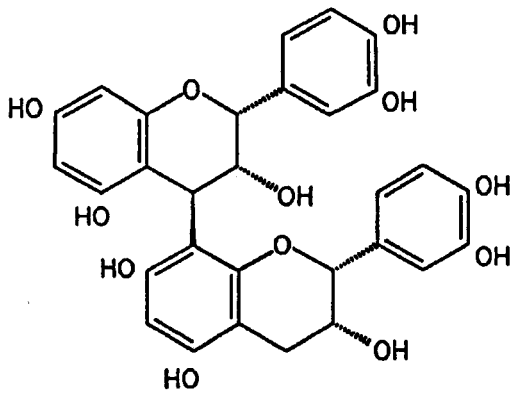

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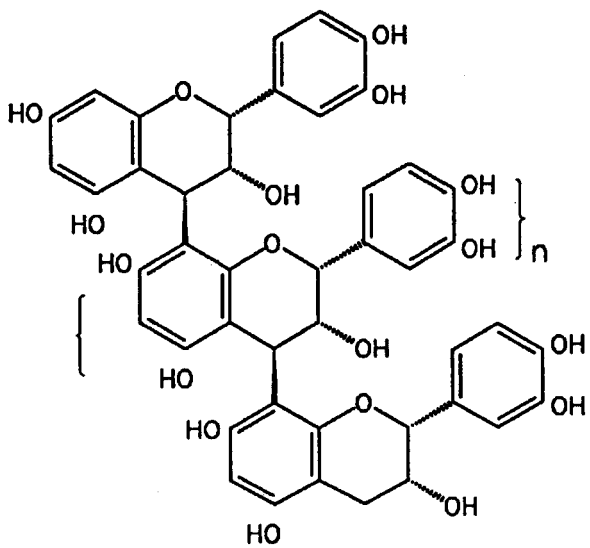
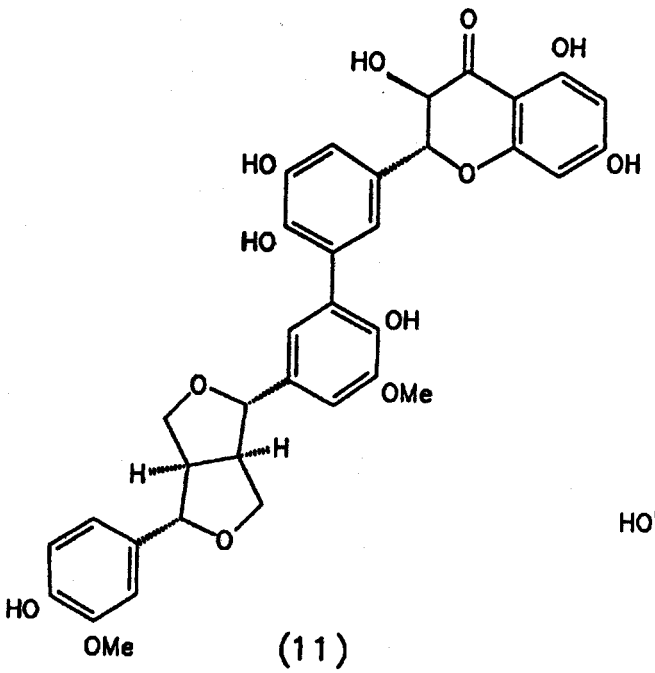


(8) n = 1

(9) n = 2

(10) n = 3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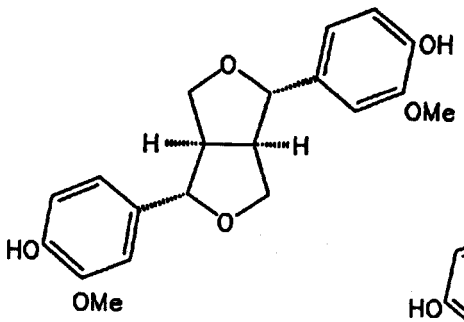
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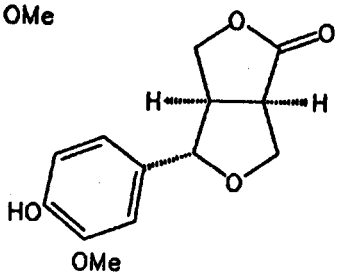
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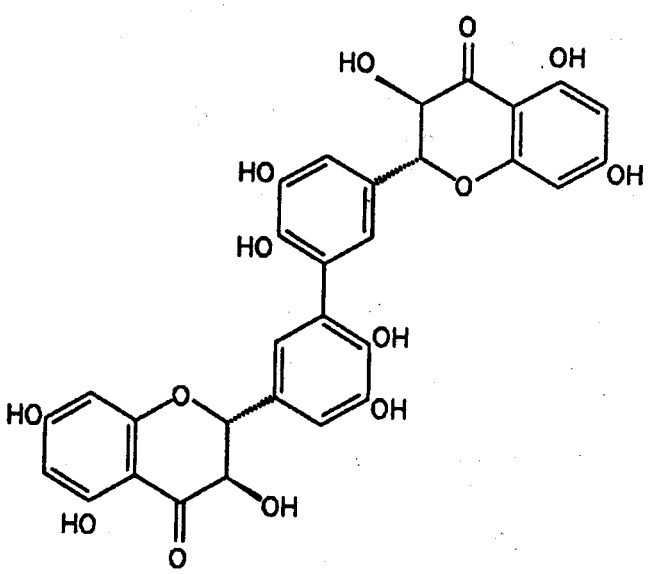
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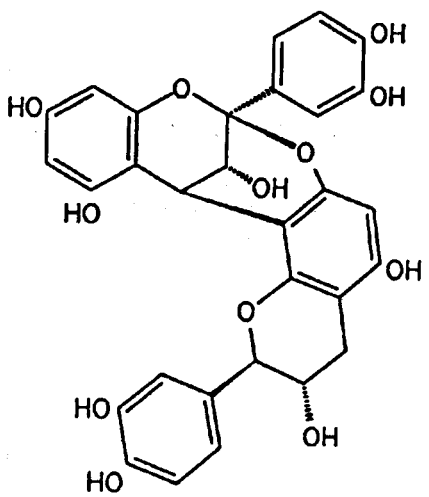
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Conformational Analysis of Polyphenols[1]

Richard W. Hemingway, Jan P. Steynberg, Wayne L. Mattice, and Fred L. Tobiason[2]

ABSTRACT

Plant polyphenols of the condensed tannin class derive most of their biological and commercial significance from their propensity to complex with proteins. In an attempt to explain specificity in the complexation of polyphenols with proteins, it was first necessary to understand the preferred shape and flexibility of these molecules. Because the condensed tannins are polymers built up of flavonoid units, both the conformation of the heterocyclic ring and rotation about the interflavonoid bond must be considered. These questions are approached by defining bond length and angles as determined in crystal structures in conjunction with fluorescence and nuclear magnetic resonance spectroscopic analyses. The conclusions reached from interpretation of physical data were compared with the results obtained from a variety molecular modeling methods. In the poster presented at this meeting, selected results of these analyses are presented to give the "flavor" of the work involved. A PC with the Alchemy II and MMX force fields is provided to permit participants to examine small model compounds of interest in their work.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Polyamines and Inorganic Ions Extracted from Woody Tissues by Freeze-thawing[1]

Rakesh Minocha and Walter C. Shortle[2]

ABSTRACT

A simple and fast method for extraction of major inorganic ions (Ca, Mg, Mn, K, and P) and cellular polyamines from small quantities of wood and woody plant tissues is described. The method involves repeated freezing and thawing of samples instead of homogenization or wet ash digestion. The efficiency of extraction of both polyamines and inorganic ions by these methods was compared for 10 different tissues. Drillbit shavings generated from wood disks or increment cores were also compared with ground wood as the starting material for ion analysis by this newly developed method of freeze-thawing. Direct use of drillbit shavings circumvents the need for making wood chips by hand and grinding in a Wiley mill. Moreover, freeze-thawing not only eliminates the need for various tissue homogenizers but is also simple enough that a large number of samples can be processed simultaneously. This method seems to be particularly useful with extremely small samples of 25 mg or less, e.g., shavings from individual growth rings of mature trees and differentiating tissues grown in vitro.

INTRODUCTION

Mobilization patterns of one or more ions within wood can be related to either wood decay processes (Safford and others 1974) or environmental stress conditions (Blanchard and others 1978; Bondietti and others 1989, 1990; Shortle and Smith 1988) in living trees. Also, the yearly variations in the inorganic ion content of a tree ring or increment core may be indicative of the composition of the nutrients taken up by the tree during that growth period (Bondietti and others 1989, Pillay 1976). Thus, changes in the ion composition of wood and woody plant tissues grown in culture may be used to evaluate the current growth potential and/or predict vulnerability of trees to environmental stress, injury, and infection.

Polyamines spermidine, spermine, and their precursor, putrescine, have been found to play a significant role in the growth and development of plant cells (Slocum and Flores 1991, Smith 1985) . The cellular polyamine content is highly regulated. A variety of stimuli including Ca and Mg deprivation (Smith 1973), pathogenesis (Greenland and Lewis 1984), ozone and acid stress (Dohmen and others 1990), aluminum stress (Minocha and others 1992), etc., all lead to an accumulation of one or more of the polyamines. Divalent cations such as Ca and Mg have been shown to substitute for polyamines in some of their metabolic activities, especially under stress conditions (Minocha and others 1992, Smith 1985). This information has prompted numerous studies on the quantitative analysis of cellular polyamines in various plant tissues (Minocha and others 1990, Smith 1991).

Most of the published work on extraction of polyamines from various tissues involved homogenization of tissue in perchloric acid (PCA) or trichloroacetic acid (TCA) using one of the following: (a) A chilled mortar and pestle with liquid nitrogen; (b) a polytron or tissumizer; or, (c) conical ground glass homogenizers (Birecka and others 1988; Faure and others 1991; Kushad and Yelenosky 1987; Maki and others 1991; Meijer and Simmonds 1988; Minocha and others 1991, 1992, 1993; Nielsen 1990; Rastogi and Davies 1989; Torrigiani and others 1987). These methods of grinding, though not very complicated, are time consuming and often noisy due to the use of polytron for long periods. Similarly, commonly used extraction procedures of dry and wet ash digestion for the determination of total inorganic ions require a rather large sample size (100 to 1,000 mg) and are time consuming, laborious, costly, and in many cases, hazardous (Anderson and Henderson 1986, Isaac and Johnson 1976, Kingston and Jassie 1986, Kuennen and others 1982, Wikoff and Moraghan 1986, Wolf 1982). In most cases, it is difficult to process a large number of samples by any of these methods. A quick and safe procedure for extraction of cellular-free polyamines and exchangeable or total inorganic ions would be useful to many laboratories.

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[1] Paper presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

[2] USDA Forest Service, Northeastern Forest Experiment Station, Concord and Mast Roads, Durham, NH 03824.

Extraction by repeated freeze-thawing as a reliable method for quantification of cellular polyamines as well as inorganic ions from woody plant tissues is presented. Homogenization of wood was also tested along with freeze-thawing to evaluate whether this procedure could also replace wet ash digestion for extraction of inorganic ions.

METHODS

Tissue Preparation

Wood

For ground wood samples, sapwood of air-dried disks taken from several mature red spruce (*Picea rubens* Sarg.) trees was chipped and ground in a Wiley mill to pass through a 420-µm sieve. This pooled ground wood was mixed thoroughly and used as an inhouse reference material since no standard reference material for nutrient content of wood was available from the National Institute of Science and Technology (NIST) for use in method development and quality control.

As an alternative to grinding, a relatively fast and effective method of drilling was developed to prepare wood samples in small quantities either from red spruce or red oak (*Quercus rubra* L.) wood disks or from tree ring cores of red spruce. Briefly, the wood surface of an increment core was cleaned by drilling a 1.0- to 3.0-mm deep hole with a 3.2-mm cobalt twist bit. A 7.9-mm titanium twist bit was used for cleaning of 5.0-cm thick air-dried disks. The shavings generated were dusted off the surface and discarded. At this point, either a 3.2-mm or 6.4-mm cobalt drill bit was used to collect either fine or coarse shavings (fig. 1). All the shavings of a certain size were pooled to provide homogenous material for method comparison (for details, see Minocha and Shortle 1993). All wood samples were ovendried at 80 °C for 16 to 24 h and cooled to room temperature over silica gel in desiccators before weighing. Five replicates were used for each treatment unless stated otherwise. Polyamine analyses were not performed for wood samples.

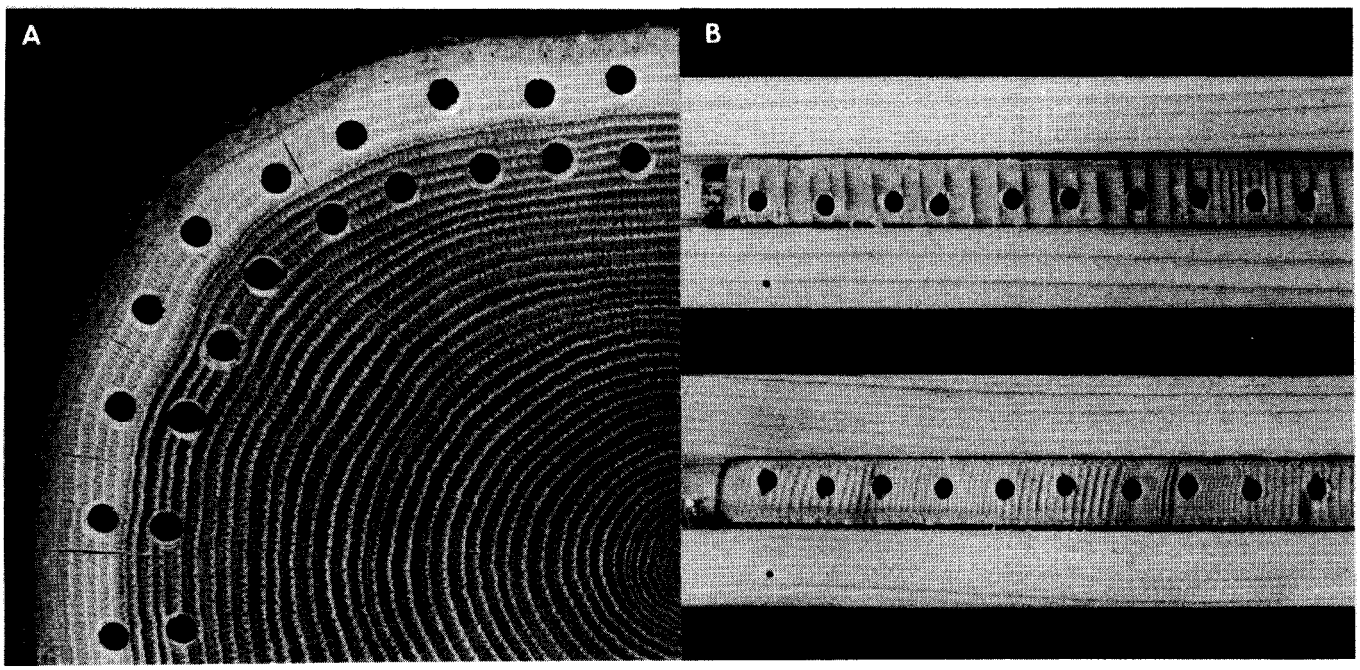

Figure 1.-(A) An oak wood disk showing the arrangement for taking sapwood and heartwood samples using 6.4-mm-diameter drill bit. (B) 12-mm-diameter increment cores from red spruce glued to grooves in wood blocks. A 3.2-mm-diameter drill bit was used to drill holes within single annual rings of a fast-growing tree (Top) or at a set distance regardless of number of rings per hole (Bottom).

Needles, Callus Tissue, and Cell Suspensions

Tissues were prepared in one of the following ways: (a) Needles from one or more red spruce seedlings were pooled, washed with distilled water, blot dried, and finely chopped; (b) callus tissue grown on agar solidified medium was pooled from several plates, blotted on filter paper to remove excess moisture and finely chopped before weighing; and (c) suspension cultures from two or more flasks were mixed, collected on Miracloth (Calbiochem-Behring Corp., La Jolla, CA), and thoroughly washed with 3 volumes of deionized distilled water. Aliquots were taken from these pooled tissues for different methods as well as for replicates within the same method.

The tissues tested included: callus of Norway spruce, *Picea abies* L. (Karst.), and aspen, *Populus tremuloides;* cell suspensions of red spruce, *Picea rubens* (Sarg.); hybrid poplar, *Populus nigra X P.maximowiczii*, and periwinkle, Catharanthus roseus; needles (1-year-old, 2-year-old, or mixed) and roots of 2-year-old red spruce seedlings. Whole dry needles from mature red spruce trees were also tested. Tissue samples were replicated four to five times unless otherwise indicated.

Wet Digestion

For wet ash digestion (W), the procedure of Isaac and Johnson (1976) as modified by Michaelson and Ping (1990) was followed. Briefly, 200 $\pm$ 0.5 mg of well-mixed sample was transferred to a 75-ml block digest tube followed by the addition of 7 ml of digestion mixture (97 g of selenous acid dissolved in 100-ml ultrapure [Millpore Corporation, Bedford, MA] water and added slowly to 4 kg bottle of concentrated sulfuric acid). Two Teflon boiling stones and 0.5 ml of 50 pecent hydrogen peroxide were added to the digestion mixture in each tube. The tube was vortexed and placed in a preheated (400 °C) block for 30 sec and then removed. The step of adding 0.5 ml hydrogen peroxide and heating for 30 sec was repeated until the solution became clear (about 4 ml). Before hydrogen peroxide was added, the tubes were removed from the heating block to allow them to cool for 1 min to avoid loss of volume due to effervescence. Once the solutions were clear, the digestion was allowed to continue for another 55 min (total digestion time 60 min). After the tubes were cooled to room temperature, ultrapure water was added to bring the volume to 75 ml, and the solution was transferred to acid-cleaned storage bottles until ready for analysis. Two blanks were digested per batch of samples.

Extraction by Homogenization

For the extraction of inorganic ions by homogenization, 5 ml of 0.01 N HCl were added to a 50-mg tissue sample in a 15-ml acid-washed glass test tube. The samples were homogenized for 90 s at 20,000 rpm using a Brinkmann Polytron homogenizer. The extracts were stored in test tubes at 4 °C until the time of analysis. Samples were filtered using a 45-µm nylon syringe filter immediately before analysis. Wood samples (25 mg) were homogenized for 90 s at 24,000 rpm with Teckmar tissumizer or Brinkmann polytron in 1 ml of 0.01 N HCl and final volume brought to 5 ml (Minocha and Shortle 1993).

For polyamine analysis, 200-mg tissue samples were transferred to 5 percent PCA (tissue:PCA ratio 1:4) in a 15-ml Corex centrifuge tube and homogenized for 90 s at 20,000 rpm using a Brinkmann Polytron homogenizer. The samples were incubated on ice for 1 h and then centrifuged at 18,000 × g for 20 min and the supernatant kept frozen at -20 °C until dansylation.

Extraction By Freeze-thawing

For freeze-thawing, the samples for both inorganic ions as well as for polyamines were frozen at -20 °C and thawed at room temperature, with the process repeated two more times. The duration of the freezing step varied from 4 h to a few days. Samples were allowed to thaw completely (not to exceed 1.5 h for tissues grown in culture and 5 h for wood samples) before refreezing. After freeze-thawing, the samples were either filtered (for inorganic ions) or centrifuged at 13,500 × g (for polyamines). Other details of sample weight and extraction volume were kept identical to the homogenization method except that the samples for polyamines were processed in 1.5 ml microfuge tubes.

Ion Analysis

The concentrations of major inorganic ions were determined by Beckman Spectra span V ARL DCP-AES (Direct Current Plasma Atomic Emission Spectrometer, Beckman Instruments Inc., Fullerton, CA) using the Environmental Protection Agency's (EPA) method number 66-AES0029 (1986).

Polyamine Analysis

Prior to dansylation, heptanediamine was added to the extracts as an internal standard for polyamine analysis. Fifty microliters of the extract were dansylated, separated by reversed phase HPLC (Perkin-Elmer Corp., Norwalk, CT) using a gradient of acetonitrile and heptanesulfonate and quantified by a fluorescence detector (Minocha and others 1990).

Statistical Analysis

Wood

Cochran's test for homogeneity of variance was performed on data sets involving comparison of more than two treatments (Cochran 1941). In cases where the null hypothesis of homogeneity was accepted, analysis of variance was carried out using SAS version 6.7 (SAS Institute Inc., Cary, NC). If the treatment differences were significant, Duncan's multiple range test was performed to separate the multiple treatment means (Duncan 1955). In cases where the null hypothesis of homogeneity was rejected, a t-test for unequal variances was performed comparing each treatment separately with the standard treatment. For data sets involving only two treatments, Satterthwaite's t-test for equal or unequal variances was performed using the same version of SAS.

Needles, Callus Tissue, and Cell Suspensions

Systat 5.02 (Systat Inc., Evanston, IL) for windows was used to perform either one way analysis of variance or a t-test for independent samples in order to evaluate if the treatment means were significantly different (two-tailed α =.05). An f-test for homogeneity of variance was performed on data to determine if a t-test for equal or unequal variances should be used (Snedecor and Cochran 1956). In cases where the null hypothesis of homogeneity was accepted, pooled variances were used for t-tests. In cases where the null hypothesis of homogeneity was rejected, a t-test for separate variances was performed.

RESULTS

Inorganic Ions

Wood

Initially, both homogenization and freeze-thawing methods were tested on the inhouse reference material (ground red spruce sapwood). The results presented in figure 2 show that there was no significant difference between amounts of Ca, Mg, and Mn extracted by any of the three methods. However, the amount of K extracted by freeze-thawing was significantly higher as compared to wet digestion and homogenization. As both fine and coarse drill shavings of red spruce and red oak, collected by drilling, were equally effective as the starting material for extraction of these ions, data are presented only for coarse shavings. The yield of Mn from red spruce sapwood and heartwood shavings was significantly higher with freeze-thawing as compared to wet digestion and homogenization (fig. 2).

In oak sapwood, Mg was fully extractable by any of the three methods. However, Mn, which was present in minute quantities, was fully extractable only by freeze-thawing and wet digestion. Calcium extraction was consistently reproducible but not complete by either homogenization or freeze-thawing (fig. 2). In oak heartwood, both Mn and Mg were present in barely detectable levels and only about 85 to 90 percent Ca was extracted by freeze-thawing or homogenization as compared to wet digestion. However, heating the tubes at 95°C for 1 h extracted all of the Ca (Minocha and others 1993). Similar to the situation with red spruce wood, K was extractable in significantly higher quantities by freeze-thawing from both sapwood and heartwood of red oak (fig. 2).

Needles, Callus Tissue, and Cell Suspensions

Whereas P could not be extracted reliably from wood, its extraction by freeze-thawing was equal to or better than that by homogenization with the exception of aspen tissue (fig. 3). Likewise, the freeze-thawing method extracted equal or significantly higher amounts of Ca, Mg, and Mn as compared to homogenization from Norway spruce callus, hybrid poplar cell suspensions, red spruce roots , and 1-year-old needles (fig. 4). Aspen callus yielded slightly lower but not significantly different amounts of all the five ions with freeze-thawing in comparison with homogenization. In the case of periwinkle and red spruce cell suspensions also, the yield of both Ca and Mn was reproducible but somewhat lower by freeze-thawing (fig. 4).

Polyamines

Freeze-thawing extracted equal or significantly higher amounts of free putrescine and spermidine from all the tissues tested (fig. 5). Spermine was present in relatively small quantities in most tissues except for red spruce cell suspensions. It was also extracted equally well with freeze-thawing as compared to homogenization (fig. 5). The HPLC profiles by either method were similar; i.e., no peaks were missing from the extracts in either case.

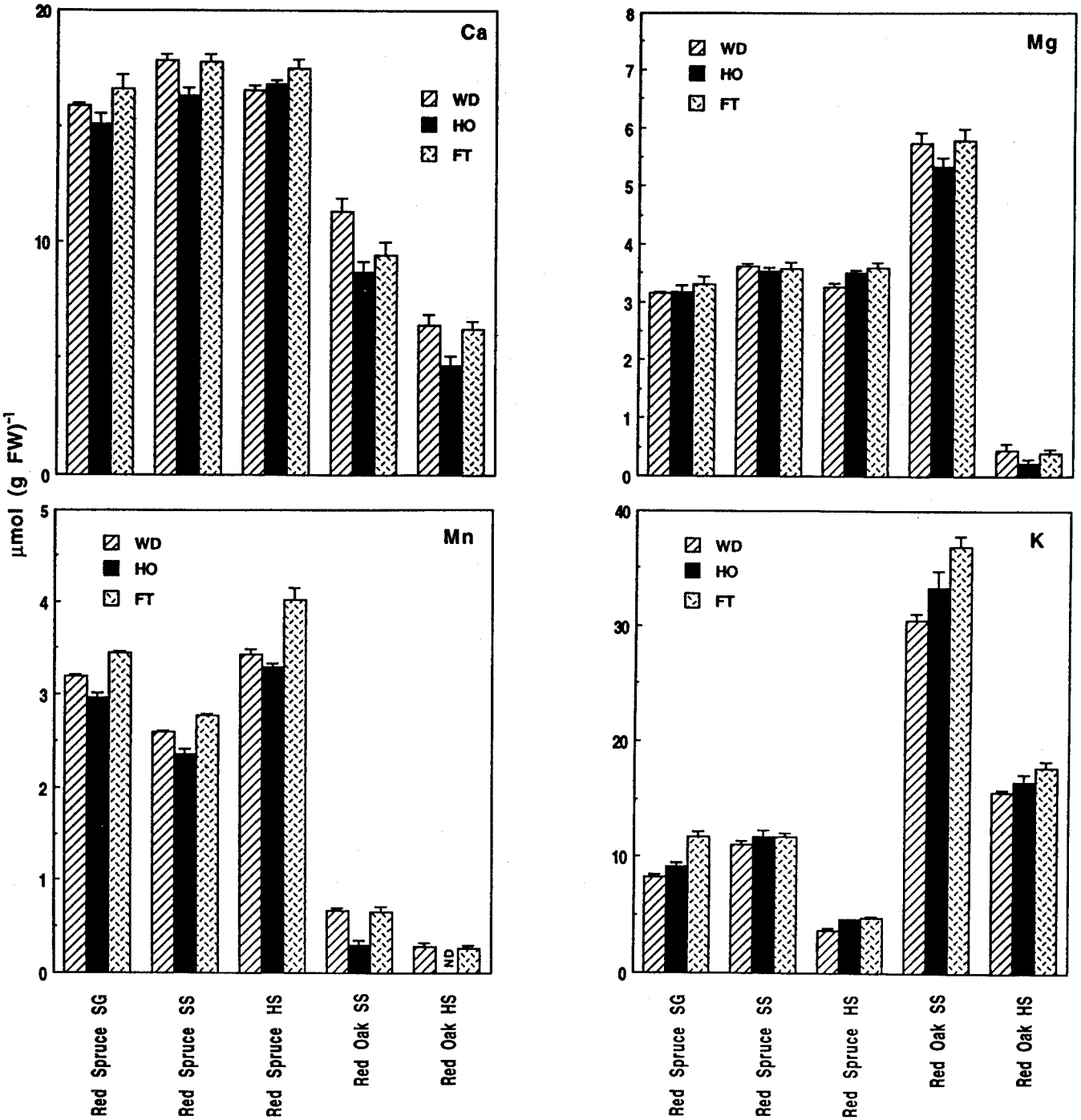

Figure 2.-Comparison of wet digestion (WD), homogenization (HO), and freeze-thawing (FT) for extraction of major inorganic cations from woody tissues. SG=sapwood ground; SS=sapwood coarse shavings; and HS=heartwood coarse shavings. Data are mean ± SE of five replicates.

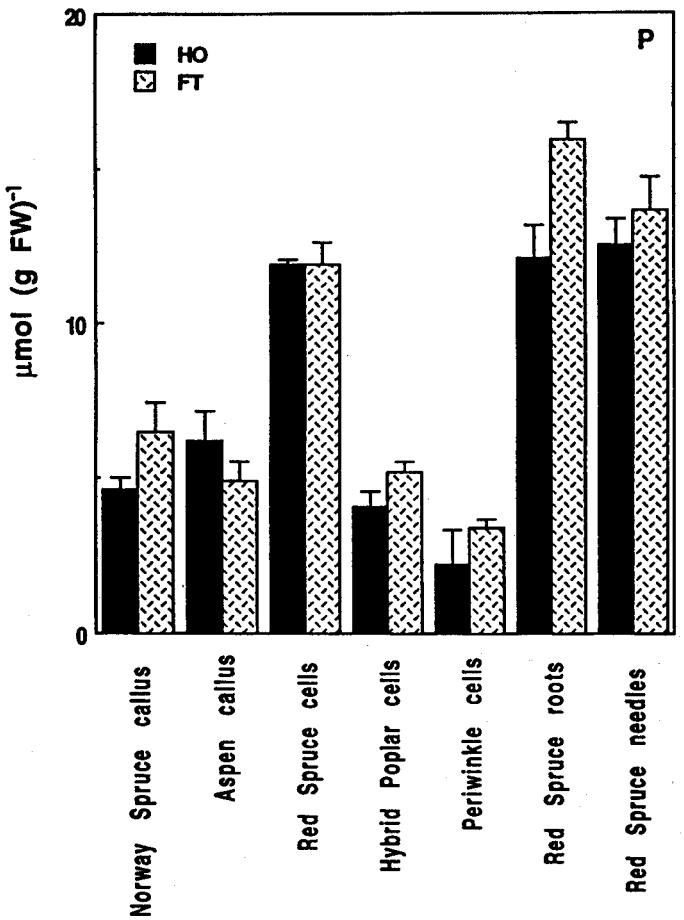

Figure 3.- Comparison of homogenization (HO) and freeze-thawing (FT) for extraction of P from various nonwoody tissues. Data are mean $\pm$ SE of four replicates for homogenization and five replicates for freeze-thawing.

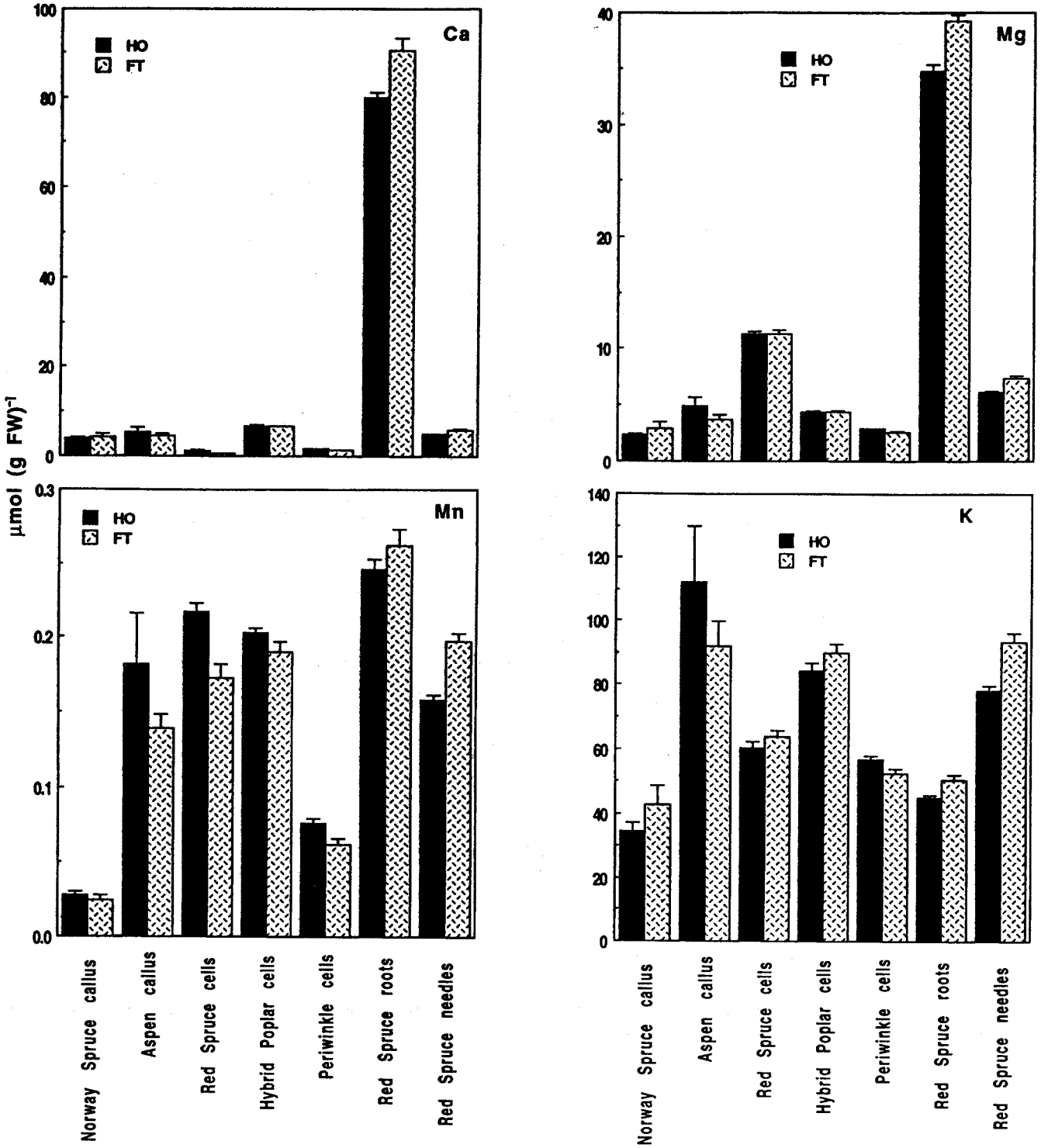

Figure 4.-Comparison of homogenization (HO) and freeze-thawing (FT) for extraction of major inorganic cations from various nonwoody tissues. Data are mean ± SE of four replicates for homogenization and five replicates for freeze-thawing.

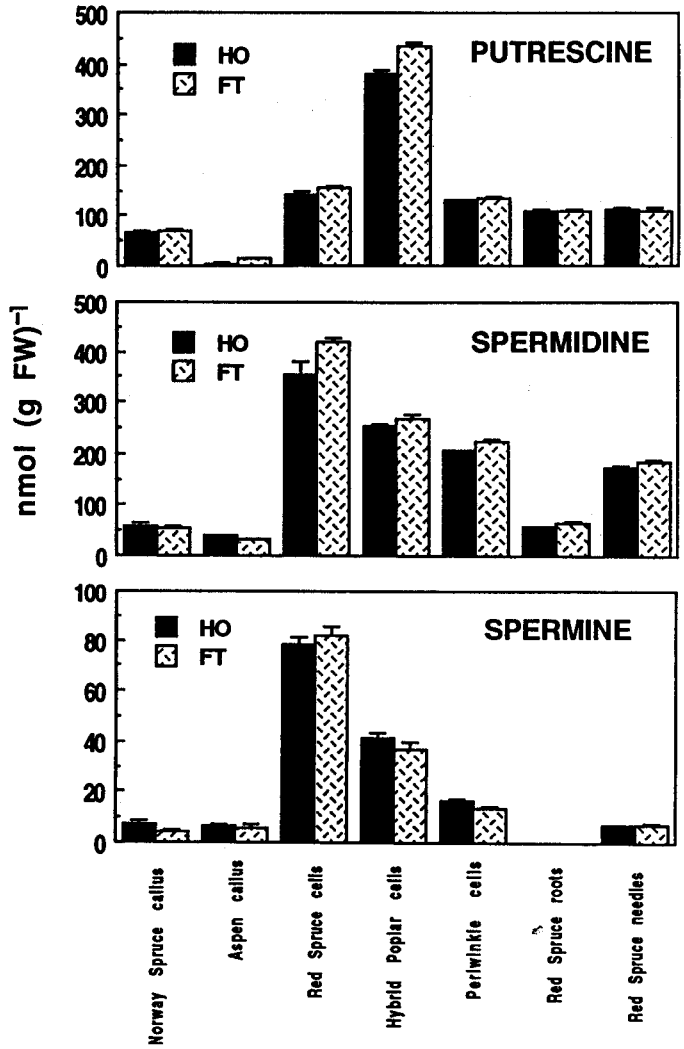

Figure 5.-Comparison of homogenization (HO) and freeze-thawing (FT) for extraction of putrescine, spermidine, and spermine from various nonwoody tissues. Data are mean ± SE of five replicates.

DISCUSSION

Wood

The use of drill-bit shavings directly for digestion of samples or for other extraction procedures has several advantages over using ground wood. It eliminates the need for chipping and milling the wood. As compared to Wiley mills, the drill-bits are easier to clean in between samplings. The samples for iron can also be analyzed by using non-ferrous drill-bits. Most importantly, they provide a fast way to study the historical record within a tree by drilling within individual tree rings of a core for fast-growing trees or within a fixed distance or decade for slow-growing trees (fig. 1).

Some of the major disadvantages of commonly used procedures of wet digestion of wood samples are: requirement for special equipment, inability to handle large numbers of samples, requirements for large dilutions that limit the analysis of micronutrient, and potential hazards of chemical explosion. In contrast, the freeze-thawing method is simple, is applicable to small amounts of tissue, and allows the processing of a large number of samples at the same time. Above all, it does not require a fume hood, complex temperature treatments, or special equipment.

Higher levels of K were consistently recovered by freeze-thawing and homogenization as compared to wet digestion. One possible explanation for this could be an earlier observation by Jackson (1962) that higher temperature during extraction by wet digestion causes volatilization of K.

Needles, Callus Tissue, and Cell Suspensions

Homogenization of tissue samples, though routinely used in most laboratories, is a relatively slow extraction procedure due to the fact that each sample must be handled separately. Freeze-thawing, on the other hand, is amenable to batch processing of a large number of samples. In our laboratory, 50 to 100 samples at a given time can be routinely processed. Moreover, no special equipment is needed. The yield of major inorganic ions by either homogenization or freeze-thawing, though repeatable, may or may not represent total ions present in the tissue. It is expected that both procedures would extract only the exchangeable or soluble ions from the tissue. Total ions from fresh or dry herbaceous tissues are usually extracted by wet or dry ash digestion. The extraction of ions from fresh needles of 1-year-old seedlings of red spruce by freeze-thawing, homogenization, and wet digestion were compared and it was found that all three methods provided complete extraction of the four ions under study. However, it was not true for dried whole needles of mature red spruce trees (Minocha and Shortle 1993). It is known that highly immobile, water-insoluble forms of major inorganic ions, such as calcium carbonate and calcium oxalate, occur in older or infected tree tissues. To mobilize ions in these forms requires increased acidity or heat, as in the case of oak heartwood (Minocha and Shortle 1993). Thus, if the goal is to study changes in exchangeable or non-covalently bound portions of ions in relation to a particular stress or a change in metabolic state of the tissue, freeze-thawing with 0.01 N HCl may be quite appropriate to use.

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In Vitro Response of *Heterobasidion annosum* to Manganese[1]

William J. Otrosina and Barbara L. Illman[2]

ABSTRACT

Manganese (Mn) is postulated to play a role in wood decay caused by certain basidiomycetes. We determined the in vitro response of *Heterobasidion annosum* (Fr.) Bref. to Mn and compared differences between three isolates each of the S and the P intersterility groups from the Western United States. On manganese-amended malt agar plates, *H. annosum* produced a brownish-black pigment that increased with increasing concentration of Mn (1, 5, and 10 mM). The amount of pigment production varied among the isolates, but isolates of the S group clearly produced more pigment than those of the P group. In pigmented areas, hyphae appeared to be packed with black granules, and brownish-black granules/crystals were present in the medium. The black pigment implicates the presence of MnO_2, potentially resulting from the activity of Mn-dependent peroxidase (MnP). An assay for MnP did not detect the enzyme in crude culture filtrates of a chemically defined, nitrogen-limited, buffered medium of the six isolates. The differential response of S and P group isolates to Mn raises questions about a role for this transition metal in *H. annosum* physiology.

Keywords: Conifers, genetic diversity, manganese dependent peroxidases, root decay pathogens.

INTRODUCTION

Transition metals such as manganese (Mn) have been reported to be involved in fungal degradation of wood and forest diseases (Blanchette 1984; Illman and others 1988, 1989; Shortle and Shigo 1973). A Mn-dependent peroxidase (MnP) secreted into culture medium by white-rot fungi (Glenn and Gold 1985; Johansson and Nyman 1987; Paszcynski and others 1985) oxidizes Mn^{2+} to Mn^{3+}, which degrades lignin-model compounds in vitro. Autoxidation of Mn^{3+} results in an accumulation of Mn dioxide. Manganese accumulated in black regions of wood decayed by several white rot fungi, including *Heterobasidion annosum* (Fr.) Bref. (Blanchette 1984).

Heterobasidion annosum is an economically important pathogen of conifers throughout the Temperate Zone forests of the world. This fungus attacks root systems and causes death of living root tissues, woody tissue decay and mortality. Three intersterility groups (ISG's) or biological species *H. annosum* are known and are associated with varying degrees of host specificity. One group, designated P, attacks mainly pine species, incense cedar, and certain hardwoods. Another group, known as the S group, attacks mainly true firs and spruce species. The third group, the F group, is specific on *Abies alba* in the Italian Alps and in the Apennines (Capretti and others 1991).

Recent population genetic studies of this fungus revealed considerable genetic diversity within and genetic divergence between ISG's from North America and Europe (Chase and Ullrich 1990; Otrosina and others 1992, 1993). In light of known host specificity differences between ISG's and genetic divergence in the fungus, as well as the postulated role of Mn in wood decay by certain basidiomycetes, *H. annosum* was employed as a model system to determine the response of *H. annosum* S and P ISG's to Mn^{2+} in pure culture and to determine if a Mn^{2+}-dependent peroxidase is excreted by *H. annosum*.

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MATERIALS AND METHODS

Culture Conditions and Enzyme Assay

Six isolates of *H. annosum* were used. Isolates 201, 214, and 247 of the S ISG were obtained from *Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr. in the central Sierra range of California. Isolates 114, 334, and 361 of the P ISG were obtained from *Pinus ponderosa* Douglas ex P. Laws. & C. Laws. hosts in Montana (114) and California (334 and 361). Isolates were grown in 125-ml Ehrlenmeyer flasks in a chemically defined, nitrogen-limited culture medium by a procedure developed for the white rot fungus *Phanaerochaete chrysosporium* Burds. (Kirk and others 1986). Each flask received a 10-mm mycelial plug from the margin of 7-day-old colonies growing on 1.25-percent malt extract agar. Flasks were kept stationary in incubation chambers for 10 days. Cultures of *P. chrysosporium* were also grown to provide a positive check on assay procedures. The culture filtrate from each isolate was assayed for the presence of Mn^{2+}-dependent peroxidase using vanillylacetone as substrate (Paszczynski and others 1985). One unit of enzymatic activity was defined as 1 µmol of substrate oxidized per minute.

Agar Culture Studies

Malt extract agar medium (1.25 percent Difco malt extract and 1.5 g agar per liter of distilled water) was amended for Mn^{2+} by adding $MnSO_4$ to yield final concentrations of 0.0, 1.0, 5.0, and 10.0 mM $MnSO_4$. $MnSO_4$ culture medium was sterilized by autoclaving for 15 minutes at 121 °C. The pH was determined potentiometrically for all media by mixing subsamples with an equal proportion of distilled 18.0 megohm water. Samples were measured after 20 minutes equilibration. The pH values for 0, 1.0, 5.0, and 10.0 mM $MnSO_4$ were 5.2., 5.1, 4.7, and 4.5, respectively.

Three replicate plates for each isolate and Mn^{2+} amendment combination were inoculated with a 4-mm diameter agar plug taken from the margins of 7-day-old malt extract agar stock culture plates. After inoculum was placed in the center of each plate, the plates were incubated on a laboratory bench at 24 °C for 8 days.

Cultures were visually and microscopically evaluated for production of a brown-black pigment in culture medium and hyphae, indicative of Mn^{4+} dioxide accumulation. A visual rating system was used to score cultures for presence, extent, and intensity of a brown-black pigment. The ratings ranged from (O) to (++++), where (O) indicates no dark pigment and (+) to (++++) indicates increasing amounts of pigment. The ratings were used to compare S and P ISG's (table 1). Samples of mycelia were transferred to glass slides, covered with a cover slip without staining, and observed at 400× for presence and location of a brown-black pigment.

Table 1.--*Relative intensity of dark precipitate formation in Mn^{2+}-amended malt extract agar for S and P intersterility groups of* Heterobasidion annosum

Mn^{2+} concentration	Relative intensity S group	P group
1.0 mM	++++	+
5.0 mM	++++	+
10.0 mM	+++	++
0.0 mM	0	0

* Plus (+) symbol represents relative precipitate extent and intensity at various Mn^{2+}concentrations. Four (+) symbols = highest extent and intensity.

RESULTS AND DISCUSSION

A brown-black pigment developed in $MnSO_4$-amended cultures of *H. annosum*. The in vitro response of *H. annosum* to Mn^{2+} is presumably due to the activity of Mn-dependent peroxidase and subsequent accumulation of Mn^{4+} dioxide.

Microscopic examination of hyphae from the dark areas of the cultures reveals granular crystals in the medium surrounding hyphae. The hyphae also appear dark and packed with this material (fig. 1). These observations are consistent

with the accumulation of manganese oxide-containing black flecks that have been reported for *H. annosum*-decayed wood in nature (Blanchette 1984).

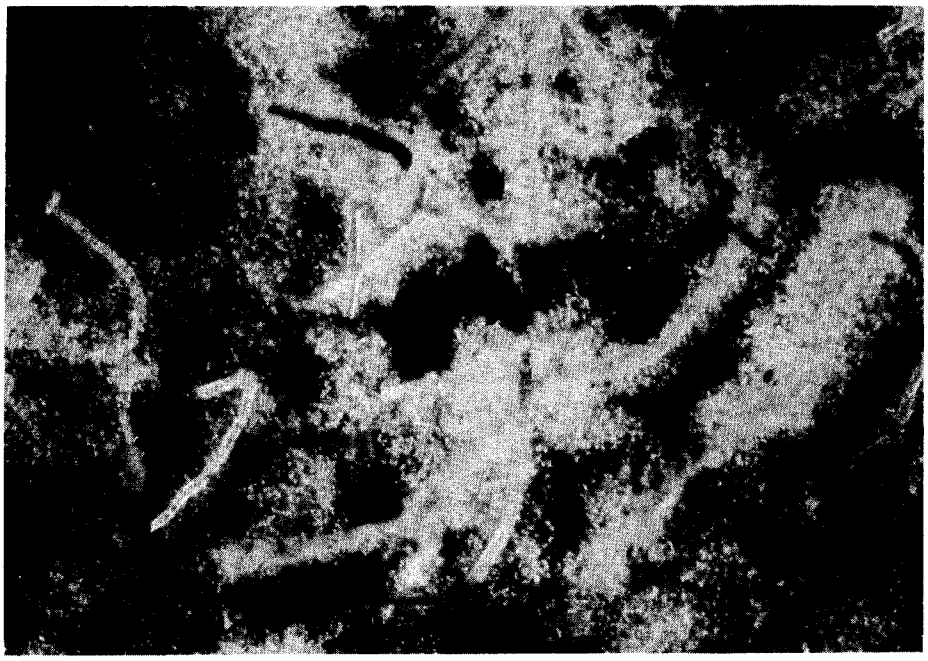

Figure 1.--*Putative oxidized manganese precipitate in* Heterobasidion annosum *hyphae. Note presence of refractile precipitate within and surrounding hyphae as it appeared under differential interference contrast. Hyphae were from 1.0 mM* Mn^{2+}*-amended 8-day-old malt extract agar culture. Magnification* × *400.*

The S and P ISG's exhibited a differential response to Mn (table 1). The S group isolates had a qualitatively more intense development of dark pigment than did the P group isolates. This was consistent with all Mn concentrations with the exception of a P ISG that produced dark pigment in 10.0 mM $MnSO_4$. The S ISG produced more pigment in 1.0 and 5.0 mM $MnSO_4$ than in 10.0 mM, reflecting a concentration optimum for Mn. Growth of S and P ISG's appeared to be depressed in the 10.0 mM concentration relative to the 1.0 and 5.0 mM concentrations. Comparisons should be made between the growth rate and Mn oxide production for a larger number of isolates within intersterility groups of this fungus.

Extracellular manganese-dependent peroxidase activity was not detected in the crude culture filtrates of the *H. annosum* isolates. Peroxidase activity was detected in filtrates of *P. chrysosporium*. The culture conditions used for *P. chrysosporium* may not be appropriate for secretion of the peroxidase by *H. annosum* in liquid culture. However, observations of the dark pigment in agar plates may be due to secretion of the enzyme under those conditions.

CONCLUSIONS

There is considerable genetic divergence between the ISGs of *H. annosum* and this genetic divergence is associated with host specificity (Otrosina and others 1992, 1993). The apparent differential response to Mn^{2+} between the S and P ISGs suggests genetic divergence may also be manifested in some unknown differences in enzymes involved in oxidative lignin degradation. Lignins differ qualitatively between hardwood and softwood species (Highley and Kirk 1979, Sjostrom 1981). Although highly speculative, differences in Mn-dependent peroxidases in *H. annosum* may be a manifestation of the same

evolutionary forces that formed the basis for host specificity in this pathogen. Further studies are planned concerning response of various geographic sources and ISGs of *H. annosum* to Mn^{2+}, isozyme analysis of Mn-dependent peroxidase of the ISGs and isolation and characterization of Mn-dependent peroxidases from this forest pathogen. This fungus can serve as a model system to study wood decay mechanisms and evolutionary processes involved in host interactions.

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Distribution of Manganese(II) in White-rot Decayed Aspen:

A New Transmission Electron Microscopy (TEM) Technique[1]

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ABSTRACT

White-rot fungi are unique in their ability to degrade lignin in wood. These fungi produce extracellular ligninolytic enzymes that can break down lignin but appear too large to penetrate the cell wall tissues. Current research suggests that manganese might facilitate the early stages of lignin biodegradation. A new transmission electron microscopy (TEM) technique to observe manganese*2 (Mn(II)) in wood has been developed. This technique utilizes a chemical spot test specific for Mn(II). A reaction product between Mn(II) and silver makes a good electron-dense marker in the TEM. Control aspen chips treated with ammoniacal silver nitrate and prepared for TEM showed Mn(II) labeling scattered throughout the cell wall tissues. Occasionally, slightly more Mn(II) appeared in the middle lamella. In aspen chips decayed for 2 weeks with *Phanerochaete chrysosporium*, Mn(II) was found in greater concentrations in cells undergoing early stages of decay; Mn(II) was found associated with fungal hyphae and the hyphae sheath in the cell lumens. In aspen chips after 10 weeks of decay, Mn(II) labeling was less in areas of extensive decay and more in areas with less extensive decay.

Keywords: Cytochemistry, manganese, transmission electron microscopy, white-rot decay.

INTRODUCTION

Some white-rot fungi decay all wood cell wall components simultaneously, totally mineralizing the wood. Other white-rot fungi can selectively degrade the wood, removing hemicelluloses and lignin, and leaving cellulose behind. Because of their ability to degrade lignin, selective white-rot fungi are of great interest with respect to their industrial uses. Applications include biopulping of wood, pulp-plant effluent cleanup, biobleaching, and bioremediation.

The mechanism of white-rot wood degradation is not fully understood, and the precise role of manganese ions during white-rot decay is unknown. Current research suggests that lignin degradation is either enzymatic or carried out by hydroxyl radicals or a combination of both. Degradative enzymes produced by *Phanerochaete chrysosporium* include lignin peroxidase (Gold and others 1983, Tien and Kirk 1983), manganese peroxidase (Huynh and Crawford 1985, Kuwahara and others 1984), and glyoxal oxidase (Kersten and Kirk 1987).

Manganese levels were shown to significantly increase during white-rot decay in soil block tests (Jellison and others 1992). Manganese is imported from the soil by the decay fungi. Bonnarme and Jeffries (1990) showed that manganese levels have a regulatory effect on enzyme production. When Mn(III) plus an organic acid is present, manganese peroxidase is predominate, whereas without the organic acid, manganese goes to manganese dioxide and lignin peroxidase is predominate (Perez and Jeffries 1992). Manganese peroxidase, codependent on Mn(II), was shown to be the predominate peroxidase detected in aspen chips decayed 3 days by *Phanerochaete chrysosporium* (Datta and others 1991). Cui and Dolphin (1990) showed that Mn(III) stabilized with an organic acid can function as a diffusible oxidant in lignin model compound oxidation. Manganese(III) is capable of oxidizing lignin substructures such as phenolic units (Bourbonnais and Paice 1989).

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Backa and others (1993) showed that hydroxyl radicals are present during white-rot wood decay and suggested that these radicals are responsible for the initial phase of fungal-mediated wood degradation. A transition metal ion catalyzes the reductive cleavage of hydrogen peroxide to form hydroxyl radicals. Hydroxyl radicals are small enough to diffuse into the wood tissues and cleave some covalent bonds, opening up the structure to oxidative enzymes. Research by Kirk and others (1985) showed that hydroxyl radicals are not involved in C_α—C_β bond cleavage in lignin degradation. Research suggests this is accomplished by lignin peroxidase (Kersten and others 1985, Kirk and Chang 1975, Schoemaker and others 1985).

The distribution of Mn(II) in wood at the ultrastructure level is of interest because of the uncertainty of its role during the decay process. A new technique using TEM is described, whereby Mn(II) is complexed with silver, and the complex is visible in the TEM. The technique is based on a chemical spot test developed by Feigl (1954) for the determination of Mn(II). The distribution of Mn(II) in aspen control and white-rotted chips is demonstrated.

METHODS

Aspen (*Populus tremuloides*) wood chips were inoculated with *Phanerochaete chrysosporium* BKM-F-1767 and treated in a bioreactor (Myers and others 1988) or incubated in stationary flasks. Untreated control chips, chips from the bioreactor decayed 2 weeks, and chips from the stationary flasks decayed 10 weeks were taken and processed for TEM (Kuster 1994).

Samples were cut into 1- by 1- by 2-mm segments and aspirated during treatment with ammoniacal silver nitrate (ASN) for 15 to 30 minutes. Solutions of ASN were as follows:

(1) Concentrated (28 to 30 percent) ammonium hydroxide was added to a saturated solution of silver nitrate until the silver chloride precipitate dissolved. A second volume of ammonium hydroxide equal to the first was then added.[3]
(2) As in (1), except a 4-percent solution of silver nitrate was used.

Samples were rinsed in distilled water and aspirated for 3 x 15 minutes each, dehydrated in ethanol, and embedded in Quetol 651 (Abad and others 1988). After rinsing in distilled water, some samples were additionally fixed in 1 percent osmium tetroxide for 1-1/2 hours, then processed for TEM. Thin sections were viewed and photographed on a Hitachi H-30.[4] The amount of manganese in aspen control chips was 41 ppm as determined by inductively coupled plasma emission spectrometry.

RESULTS AND DISCUSSION

A cytochemical technique for the location of Mn(II) in wood and decayed wood was developed based upon a chemical spot test for Mn(II). The spot test is a sensitive test for Mn(II) and was successfully applied to agar gels with increasing concentrations of Mn(II) and wood samples (Kuster 1994). Ferrous iron was the only other cation found that gave a positive reaction when tested with ASN. Initially, Fe(II) should not interfere because iron is present as Fe(III) in wood. It is also possible to mask Fe(II), if Fe(III) is reduced to Fe(II) during decay, using a dilute cyanide solution (Kuster 1994).

An electron-dense product forms when ASN penetrates the wood tissues at Mn(II) sites. The formation of a dark precipitate containing manganese dioxide and finely divided silver results according to the following formula:

$$Mn^{+2} + 2Ag(NH_3)_2^+ + 4OH^- \rightarrow MnO_2 + 2Ag^\circ + 4NH_3 + 2H_2O$$

The reaction product is an adsorption complex between manganese oxide and metallic silver. As with any cytochemical technique, there is concern of translocation and whether the markers reflect the in vivo sites of Mn(II) (Lewis and Knight 1992). Pulping studies found that acid conditions are needed to remove metals from fibers; washing with water alone will not remove the heavy metals. This suggests that Mn(II) is tightly bound to the cell wall under neutral or alkaline conditions. Therefore, it is probable that the Mn(II)/silver reaction products remain fixed in the wood tissues. The Mn(II)/silver reaction products have a diameter of about 30 nm in the cell wall and are somewhat larger in cell lumens. This is possibly due to limited diffusion of ASN into the cell wall versus the cell lumen or could be due to the space available between cell wall constituents for the reaction products to form.

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[3]In rare instances, an explosive substance may form if the silver ammonia solution is allowed to stand for prolonged periods (Smith 1943).

[4]The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

Ammoniacal-silver-nitrate-treated control aspen chips showed Mn(II) scattered throughout the cell walls, with occasionally more Mn(II) in the middle lamella (fig. 1). Aspen chips inoculated with *Phanerochaete chrysosporium* in a bioreactor for 2 weeks and then treated with ASN showed increased labeling in cells under fungal attack (fig. 2). Increased concentrations of Mn(II) near hyphae were also evident (fig. 2). The hyphal sheath is usually not well-preserved in conventional fixation techniques. Occasionally, membrane-like structures can be seen in what remains of the sheath. Labeling of Mn(II) in association with hyphal sheath and a hyphae is shown in figure 3.

In aspen samples decayed 10 weeks, the outer areas of the chips showed extensive decay (fig. 4). In these areas, only a small amount of Mn(II) labeling was observed after treatment with ASN. It is possible that the structure is so loosened during the decaying process that the precipitation products are washed out. Cells toward the interior of the chips, which showed less extensive decay, had more Mn(II) present in the cell walls (fig. 5).

Figure 1. *Transverse section of control aspen chip, treated with ASN at room temperature for 15 minutes. Black dots represent Mn(II)/silver distribution in the middle lamella and cell walls. G, gelatinous layer; S_2; S_1; ml, middle lamella; v, vessel cell wall; L, cell lumen. TEM, 20,000×.*

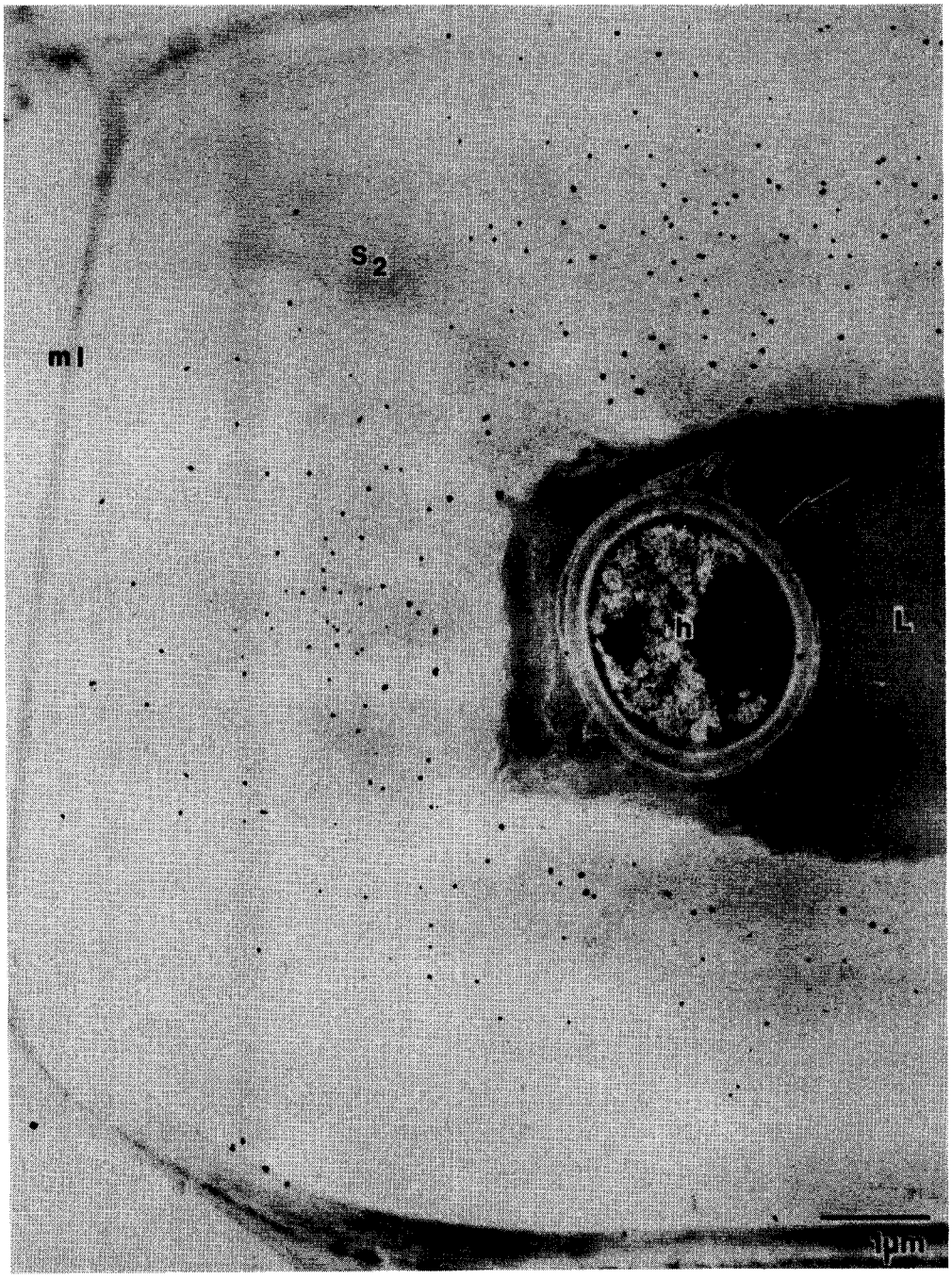

Figure 2. *Transverse section of aspen chip decayed 2 weeks, treated with ASN for 15 minutes at room temperature, rinsed, post-fixed with 1 percent osmium tetroxide showing increased Mn(II)/silver labeling in the S_2 which is undergoing decay. Mn(II) labeling (arrow) also can be seen associated with the hyphae (h) in the cell lumen (L). Middle lamella (ml). TEM, 16,500×.*

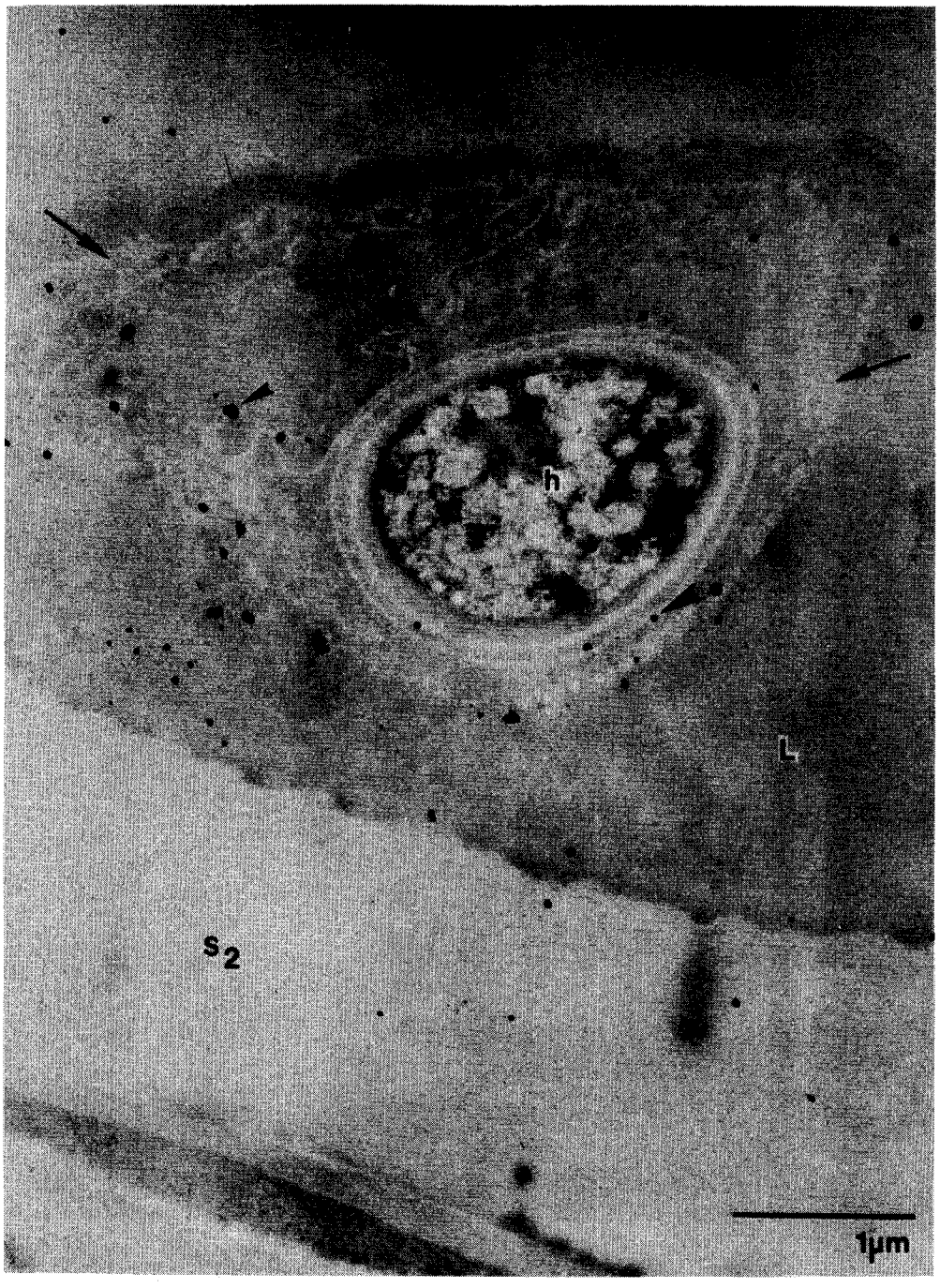

Figure 3. *Transverse section of aspen chip decayed 2 weeks, treated with ASN for 15 minutes at room temperature, rinsed, post-fixed with 1 percent osmium tetroxide showing hyphal sheath (arrows) and hyphae (h). Mn(II)/silver labeling can be seen in the sheath (small arrowheads) and near the hyphae (large arrowhead). S_2; cell lumen (L). TEM, 14,000×.*

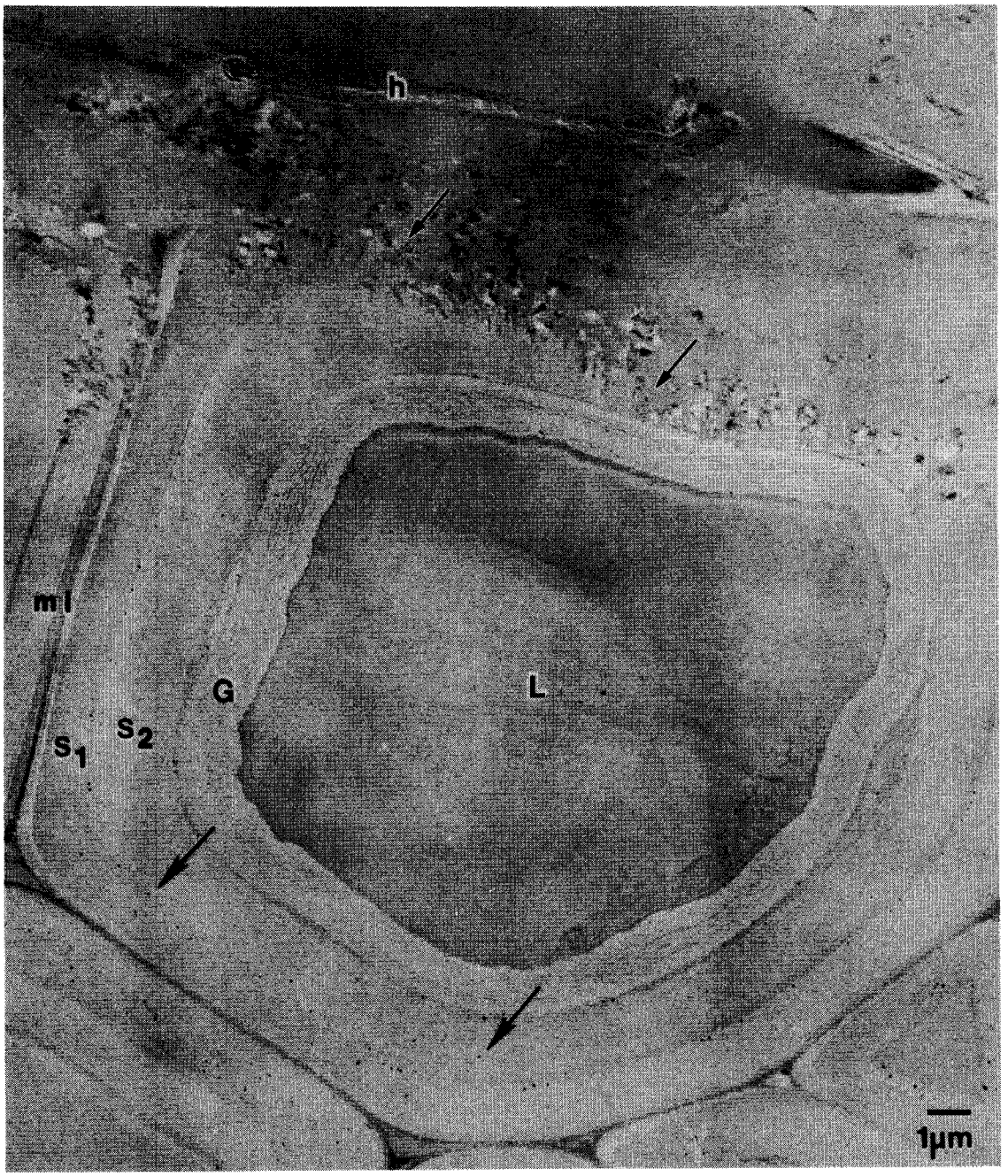

Figure 4. Transverse section of aspen chip decayed 10 weeks, treated with ASN for 15 minutes at room temperature, unfixed. Top portion of wood cell is undergoing extensive decay (small arrows) and has very little Mn(II)/silver labeling. The lower portion of the wood cell is not as extensively decayed and has considerable more labeling (large arrows). h, hyphae; G, gelatinous layer; S_2; S_1; L, lumen; ml, middle lamella. TEM, 6,000x.

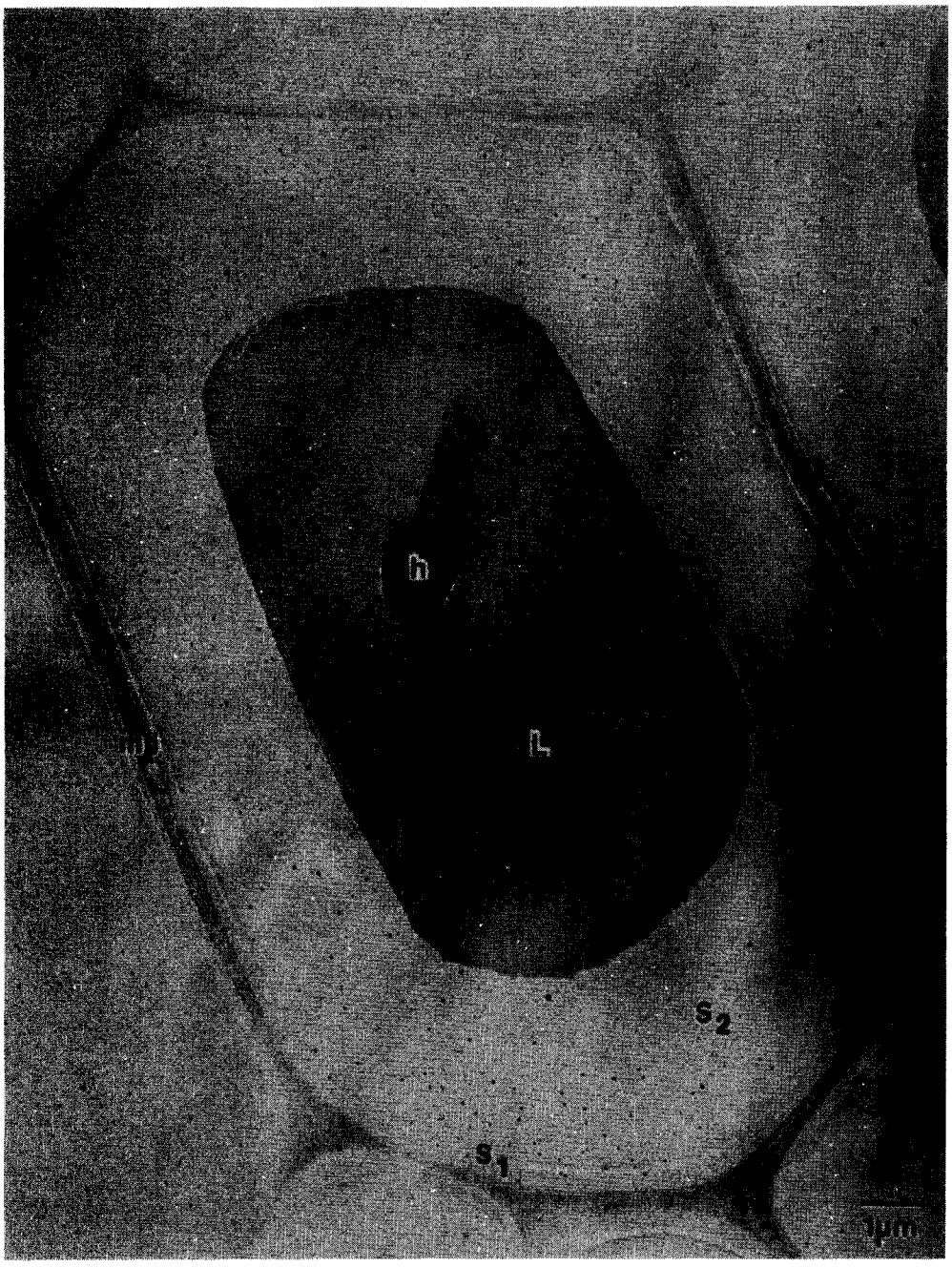

Figure 5. *Transverse section of aspen chip decayed 10 weeks, treated with ASN for 15 minutes at room temperature, unfixed. Wood cell undergoing early stages of decay showing Mn(II)/silver labeling evenly distributed throughout the cell walls. h, hyphae; L, lumen, S_2; S_1 ml, middle lamella; TEM, 9,000×.*

CONCLUSIONS

A cytochemical location technique for the detection of Mn(II) was developed and successfully applied to wood and decayed wood. A reaction product between Mn(II) and metallic silver can be observed with the TEM, thus marking the location of the Mn(II) in the wood tissues. At 41 ppm in control aspen chips as detemmined by inductively coupled plasma spectroscopy, Mn(II) was detected by this cytochemical technique.

Infommation presented in the electron micrographs on the location of manganese in the decayed wood cells suggests that manganese has a role in the early stages of decay. A greater concentration of Mn(II) was noted for cells in the early stages of decay and was found associated with the hyphal sheath. Higher manganese concentrations are associated with the production of manganese peroxidase (Bonnamme and Jeffries 1990). Datta and others (1991) showed that manganese peroxidase predominates in 3-day cultures of aspen chips being decayed by *Phanerochaete chrysosporium*. When stabilized with an organic acid, manganese peroxidase can oxidize Mn(II) to Mn(III) and cleave some lignin substructures (Bourbonnais and Paice 1989). However, it remains unknown if Mn(III) chelated with an organic acid acts as an oxidative mediator during wood decay or if Mn(II) is involved in the fommation of hydroxyl radicals.

Wood cells that showed advanced decay in the electron micrographs had considerably less Mn(II) labeling. This suggests Mn(II) is possibly (1) translocated away from this area, (2) precipitated as MnO2, (3) oxidized to Mn(III) and bound by a suitable ligand, or (4) washed out during specimen preparation. Low manganese levels promote production of lignin peroxidase (Bonnarme and Jeffries 1992). Lignin peroxidase was shown by immunocytochemistry to be involved in the later stages of decay after the cell wall is loosened (Blanchette and others 1989, Daniel and others 1990, Srebotnik and Messsner 1988). Additional Mn(II) labeling experiments with controlled culture conditions would be of interest.

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A Biochemical Assessment of the Value of Top Clipping Nursery-Grown Loblolly Pine Seedlings[1]

Shi-Jean S. Sung, Paul P. Kormanik, and Clanton C. Black[2]

ABSTRACT

Seasonal sucrose metabolism (sucrolysis) was studied in taproot cambial tissues of nursery-grown loblolly pine seedlings to assess the value of top clipping. In sucrose-importing taproots of nonclipped seedlings, sucrose synthase (SS) was the dominant enzyme for sucrose cleavage, and its activity exhibited a distinct seasonal activity. Both root SS activity and growth were most active during fall. Sucrose synthase activity decreased to the lowest level in mid-January and resumed after that. Neither root acid invertase (AI) nor neutral invertase (NI) changed activity appreciably through the seasons. Both August and September top clipping treatments decreased seedling top weight by 20 percent to 45 percent whereas total root weight was slightly decreased by August clipping only. Top clipping did not change the basic seasonal pattern of sucrolytic pathway in taproot cambial tissues. However, 2 to 3 months after top clipping, losses of root SS activity in clipped seedlings were observed. The largest decreases in SS activity occurred from November through early January followed by another decrease during active shoot elongation. Generally, August clipping decreased more root SS activity than September clipping. Neither root AI nor NI activity was affected by the clipping treatment. It was concluded that: (1) sucrose synthase was the dominant sucrolytic enzyme in cambial tissues of pine seedling taproots; (2) sucrose synthase activity can be used as an indicator for the physiological status of tissues; and (3) top clipping, especially in August, imposes stress on nursery seedlings based on biochemical analysis and growth measurements.

Keywords: Sucrolysis, sucrose metabolism, sucrose synthase.

INTRODUCTION

Loblolly pine (*Pinus taeda* L.) is the most commonly planted conifer throughout the United States, with 1 billion seedlings produced in the Southern United States in peak years (Johnson and others 1982). Since World War II, great progress has been made in the genetic improvement of seed source and in the nursery technology to produce this large number of seedlings. A general rule was then suggested to eliminate culling if the percentage of small, damaged, or diseased seedlings was less than 10 percent in any seedlot (May 1985b). Consequently, about 95 percent of each nursery crop was commonly reported to be "plantable seedlings" (May 1985a). However, many of these "plantable seedlings" either did not survive transplanting stress or grew poorly in the field (Weaver and others 1981, Johnson and others 1982). Nonetheless, these practices continued because the seedlings appeared fairly uniform and were readily used in artificial forest regeneration work.

Uniformity of the planting stock was achieved principally by heavy fertilization, irrigation, and by top clipping the faster growing seedlings several times during the growing season (Dierauf 1976, Mexal and Fisher 1984). But we think these procedures are flawed. For example, the more vigorous the growth of an individual seedling, the more often it was clipped. Indeed, many seedlings that were in fact poor competitors were repeatedly released from competition by these cultural practices. A close examination of these released seedlings revealed that about 20 to 30 percent of the crop still shared many morphological traits with those considered culls in Wakeley's original seedling grading system (1954). These traits include succulent stems, poor development of secondary needles, and general absence of terminal buds.

With a better understanding of loblolly seedling biology and sucrose metabolism (sucrolysis) (Kormanik and others 1990, 1991; Sung and others 1993), Kormanik and others (1992) developed a new nursery cultural technology both for growing

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and selecting loblolly pine seedlings. The technology requires as little as one-third to one-half of the top dressing nitrogen and water of those traditionally prescribed for nurseries. This technology does not employ top clipping to meet uniform target height for planting because 70 to 80 percent of the crop are plantable seedlings of uniform size.

Sucrose is the major form of translocated carbohydrate in plants (Shiroya and others 1962, Zimmermann and Brown 1971), and it initiates metabolism in recipient cells leading to growth and storage (Hubbard and others 1989; Sung and others 1989a, 1989b; Xu and others 1989). Of three enzymes cleaving sucrose, sucrose synthase (SS) is the dominant activity in actively growing and storing tissues such as loblolly pine seedling stems (Sung and others 1993) or sweetgum seedling roots (Sung and others 1989a). Sucrose synthase also has been reported to be an indicator for sucrose sink strength in growing potato tubers (Sung and others 1989b) and for the physiological status of loblolly pine tissues under cold or transplanting stress (Sung and others 1993).

It is clear that top clipping is another form of stress artificially imposed on nursery seedlings. When an actively growing loblolly pine seedling is top clipped, it loses almost half of the photosynthetically active secondary needles (Mexal and Fisher 1984). A few weeks later, numerous new shoots are initiated from cut needle fascicles (Dierauf 1976, Mexal and Fisher 1984). This observation suggests that reduced net photosynthate production and increased demand for sucrose by new shoot growth will drastically change sucrose metabolism and hence change the source-sink dynamics between seedling tops and roots. This study was a part of the nursery cultural technology trials by Kormanik and others (1992). Objectives of this study were to determine how top clipping pine seedling influenced sucrolysis and to make a reassessment of the putative value of top clipping by following the seasonal patterns of seedling sucrolysis and growth.

METHODS

Loblolly pine seeds of mixed seedlots were stratified at 4 °C for 60 days and sown in mid-April, 1988, in 18.3- by 1.2- by 1.2-m beds at the Whitehall Experimental Nursery in Athens, GA. Nursery cultural practices were as described by Kormanik and others (1990) with similar levels of nitrogen fertilizer and irrigation commonly used in most commercial nurseries. One-third of the seedlings were top clipped to 30 cm in height in mid-August, and the second one-third of seedlings were top clipped to 37 cm in mid-September. One-third of the seedlings were never clipped and served as controls. Beginning in August, 100 seedlings from each treatment were carefully harvested, and growth measurements such as top and root dry weight and root collar diameter (RCD) were made at 2-week intervals.

Biweekly sampling for enzyme analysis also began in August, and there were two replicates for each sampling. Results reported in this paper are the average of the two replications. Variations in enzyme activities between two replications were less than 15 percent at all times. Procedures for tissue sampling and biochemical analysis were the same as those described by Sung and others (1993). Root vascular cambial tissues were obtained by peeling the bark from taproots and scraping off the inner (xylem-side) cambial tissue with a razor blade. At each sampling date, 10 to 40 pine seedlings were used to obtain a composite sample of 2.5 g fresh weight root cambial tissues. Enzyme extraction and assay procedures for sucrolytic enzymes, namely sucrose synthase (SS), acid invertase (AI), and neutral invertase (NI), followed those by Sung and others (1993). The protein content of each extract was determined with Bradford reagent (Bradford 1976) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Seasonal growth measurements for all treatments are presented in figure 1. From August through October, when top growth was most active (fig. 1A), periodicity of root growth was observed (fig. 1B). In November, between the two most active periods of root growth, there was a period of slow growth. Both seedling top and root weights decreased in December and January (figs. 1A and 1B). This was also reported by DeWald and Feret (1988). It was suggested that this winter weight loss was due to a maintenance type respiration (Kuhns and Gjerstad 1991).

In addition to removal of part of the seedling top at clipping, August clipping treatment decreased the rate of top weight accumulation during the following active growing months (fig. 1A). September clipping treatment did not change top weight accumulation rate, which had almost plateaued at clipping (fig. 1A). Similarly, Dierauf (1976) reported that top clipping decreased stem diameter but not root system size. In this study, there were no significant differences in seedling root weight (fig. 1B) or root collar diameter (RCD) (data not shown) between nonclipped and September clipped seedlings. Up to 20 percent decreases in root weight (fig. 1B) and RCD (data not shown) were found in August clipped seedlings. There were also losses in both top and root weights during winter in clipped seedlings as in nonclipped controls. However, it is not known why the winter weight losses by clipped seedlings were not so obvious as those by the nonclipped controls.

One of the justifications for top clipping is to grow uniform size seedlings (Dierauf 1976, May 1985a, Mexal and Fisher 1984). Indeed, in this study, the standard deviations for nonclipped seedling top and root weights were greater than those of clipped seedlings (figs. 1A, 1B). Another reason to top clip is to improve seedling top:root ratio to 1.5:1 to 3:1 (May

1985a). In this study, top:root ratio was decreased from 6:1 in the nonclipped seedlings to 4.5:1 in the clipped seedlings (fig. 1C). This improvement of top:root ratio, however, was achieved because of decreased top weight rather than increased root biomass as reported by Mexal and Fisher (1984). These oversized seedlings, with a RCD of 5.0 to 5.5 mm when stem growth was finished for the year, were the results of heavy nitrogen fertilization and irrigation as used by most nurseries. It became clear why nurserymen top clipped the same seedlings several times in the year when weather condition was most suitable for growth. In this study, one-time top clipping in September did not affect much of the seedling top and root growth, whereas August clipping had small negative effects on top growth.

A pine seedling can be looked at in a classical manner as composed of sucrose source and sucrose sink tissues or organs. The photosynthetic needles that produce and export photosynthate in the form of sucrose are sources. Tissues such as roots that import sucrose for growth are sinks. The seasonal patterns for sucrolytic enzyme activity in nonclipped taproot cambial tissues are shown in figure 2. Similar to the findings by Sung and others (1993), SS was the dominant sucrose-cleaving enzyme activity that adapted to seasonal and developmental changes. There was a three-fold difference in SS activity between the lowest and the highest levels with the former occurring in the coldest month and the latter in November (fig. 2). A close relationship between the periodicity growth of root weight and taproot SS activity was observed throughout the study (fig. 1B vs. fig. 2). Neither invertases played a significant sucrolytic role in pine roots, and the invertase activity did not vary either seasonally or developmentally (fig. 2). It is quite clear from the results presented in figure 2 that SS can be used as a sucrose sink strength indicator with loblolly pine root.

In this study, 3 to 4 weeks after August clipping, new pine shoot tips emerged from cut fascicles. It took the September clipped seedlings 4 to 6 weeks to develop new shoots. Most of these new shoots, especially on the September clipped seedlings, did not form terminal buds in the nursery bed. Top clipping not only decreases seedling sucrose source size by removing photosynthetic needles, but also increases sucrose sink size by initiating growth of new shoot tips. This contrasting response to top clipping provided a chance to assess sucrolysis pathways in seedlings under a nonenvironmental, human-made stress.

The effects of August clipping on seedling sucrolysis are presented in figure 3. Seasonal sucrolysis pathway in August clipped taproot cambial tissues (fig. 3) was similar to that of the nonclipped controls (fig. 2) with SS being the dominant and adaptive activity. Sucrose synthase activity was 20 to 60 percent less in the August clipped seedling roots than that of nonclipped seedlings (fig. 2 vs. fig. 3). The largest SS activity losses occurred in November, December, and March. Neither AI nor NI activity was noticeably affected by clipping (fig. 3). This clipping study establishes that loblolly pine seedling taproot SS activity changes can be mediated by either seasonal or human-made stress as suggested earlier by Sung and others (1993).

All three sucrose cleaving enzyme activities in September clipped seedling taproots (data not shown) did not differ in the seasonal patterns from those of the nonclipped and August clipped seedlings. There is a similarly close relationship between root growth and SS activity for September clipped seedlings to that of nonclipped seedlings. Furthermore, SS loss by September clipping was less when compared with that by August clipping.

Both August and September clipping affected SS activity more than root weight. For example, average seasonal losses in taproot SS activity and total root weight were 35 percent and 5 percent for the August clipped seedlings and 26 percent and 0.5 percent for the September clipped seedlings. This suggests that the physiological status of pine seedlings at lifting is more critical for seedling's surviving transplanting stress than total root weight. Dierauf (1976) reported a higher field survival percentage from September clipped seedlings than from August clipped seedlings although both types of seedlings had similar top:root ratio. A general term like "physiological response" was used by Dierauf to reason this discrepancy. Results of this study, however, suggested that SS activity can be used as the qualitative and quantitative indicator for seedlinq physiological status.

CONCLUSIONS

Sucrose synthase was the dominant sucrose metabolizing activity in taproot cambial tissues during active root growth in fall and early winter with both invertase activities constant through development. The negative effects of top clipping, especially the August clipping, on loblolly pine seedlings were at least two-fold: losses of taproot cambial tissue SS activity through the seasons and decreases in top weight accumulation rate. These results support earlier conclusions about the detrimental effects of top clipping on loblolly pine seedlings.

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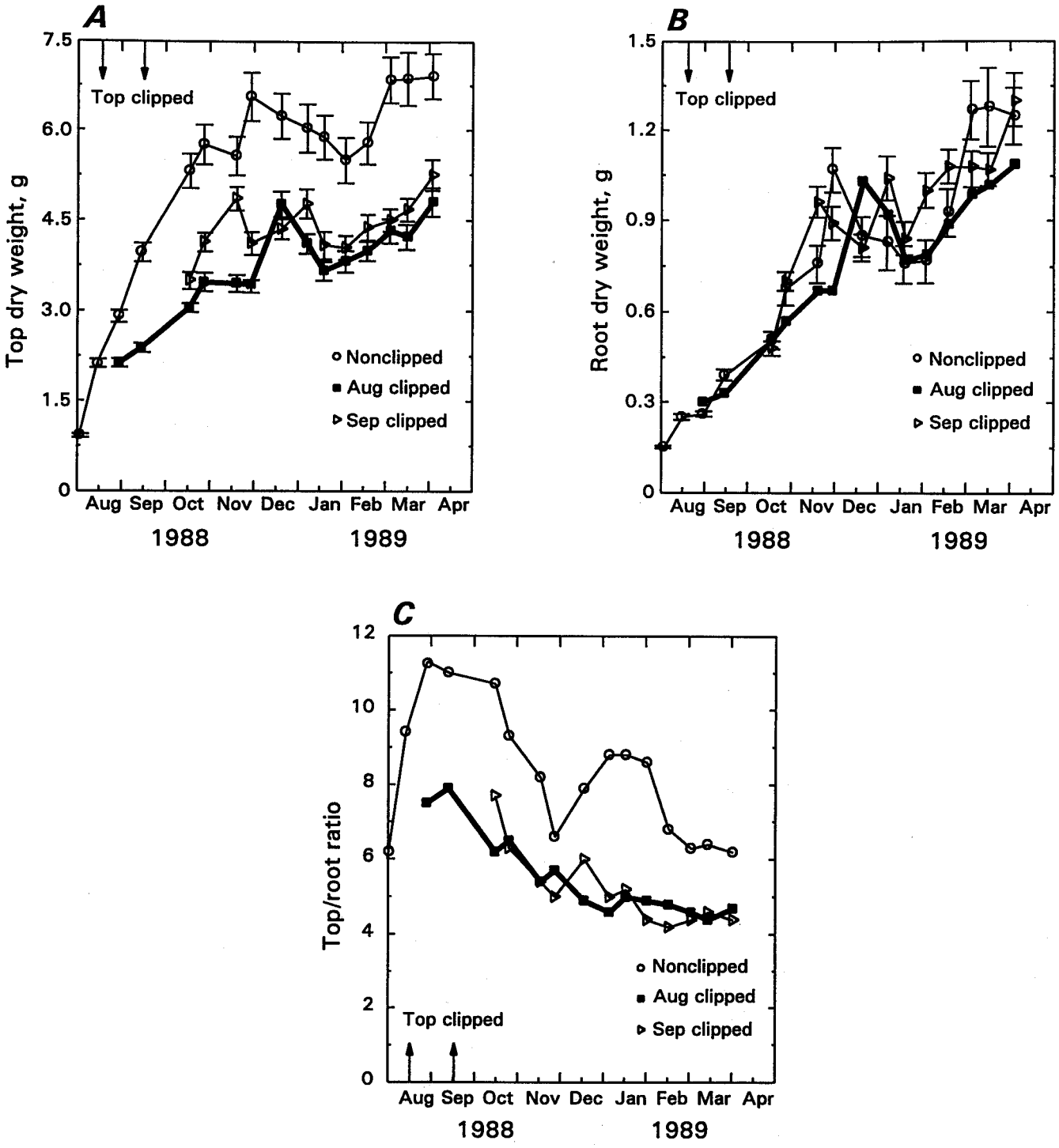

Figure 1. *Growth measurements of loblolly pine seedlings. Seeds were sown in mid-April. Data are means of 100 seedlings. Bar represents ± 1 SE. (A) Top dry weight. (B) Total root dry weight. Standard error (SE) for August clipping treatment were similar to those of September clipping and were not shown here. (C) Ratio between seedling top and total root dry weight.*

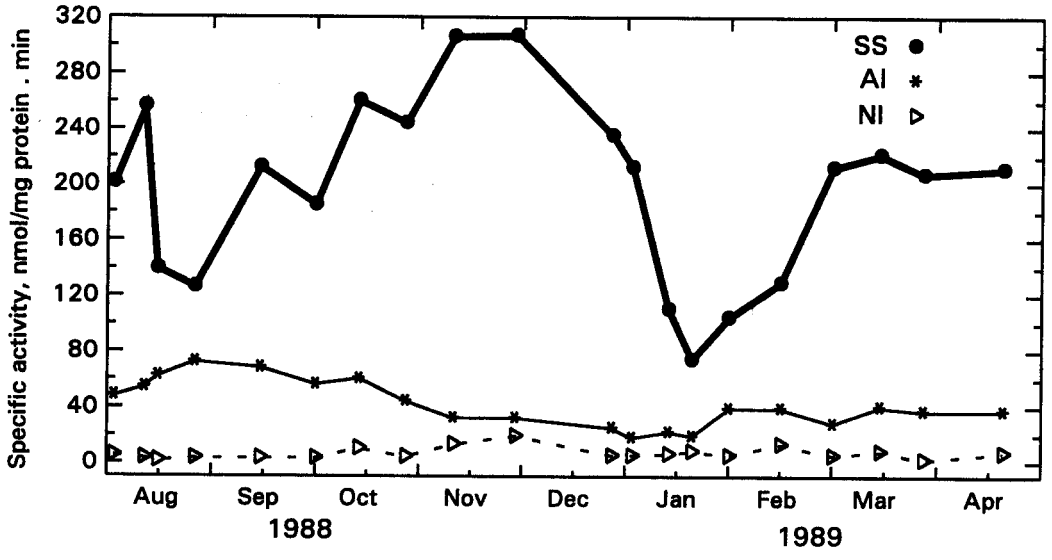

Figure 2. *Seasonal specific activity patterns of three sucrose metabolizing enzymes, sucrose syhtnase (SS), acid invertase, (Al), and neutral invertase (NI), in taproots of nonclipped loblolly pine seedlings. Soluble extracts from taproot cambial tissues were used in all assays. Each value is the average of two replications.*

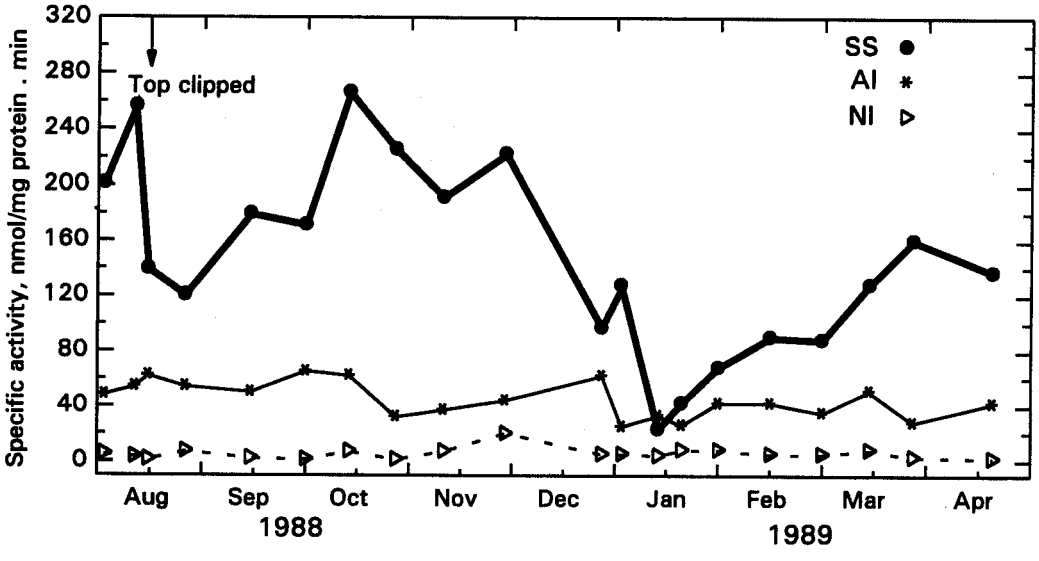

Figure 3. *Seasonal specificty activity patterns of three sucrose metabolizing enzymes in taproots of August clipped loblolly pine seedlings. Soluble extracts from taproot cambial tissues were used in all assays. Each value is the average of two replications.*

Ellagic Acid Effects on Benzyladenine-induced Adventitious Organogenesis in Longleaf Pine[1]

A.M. Diner and M. Eliasson[2]

ABSTRACT

The objective of this investigation was to identify any effects of the antimutagen ellagic acid upon cytokinin-induced adventitious shoot organogenesis in longleaf pine (*Pinus palustris* L.). Hypocotyl apices and cotyledons of S-day-germinated seedlings of longleaf pine were aseptically placed in petri dishes containing an agar-solidified Brown and Lawrence nutrient medium that was supplemented with 44 micromolar ben~yladenine (BA) or ellagic acid (EA) or 44 micromolar of each (BA+EA). After 2 weeks at 20 C under 80 micromoles-m^{-2}-s^{-1} cool-white fluorescent illumination, the tissues were transferred to the basal medium for 4 weeks.

The BA treatment induced adventitious bud primordia on all tissues. By 4 weeks posttreatment, buds were typically very small and poorly developed. Effects of the BA+EA treatment were similar to those of BA alone.

However, there were two grossly visible effects of the EA treatment. First, cotyledons were shriveled, thin, and frequently necrotic. Second, and more interesting, hypocotyl apices showed a precocious and pronounced flushing of what appeared to be the lateral bud meristems. Needles were as long as 2.5 cm and in clusters. There appeared to be no adventitious development from the apex.

Work is currently underway to do a histological examination of these tissues and to determine any physiological effect of ellagic acid on meristem dormancy as well as that imposed on seeds.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Quantitative Structure-activity Relationship Studies Of Stilbenols Against Wood-Inhabiting Fungi[1]

Tor P. Schultz, Thomas H. Fisher and Darrel D. Nicholas[2]

ABSTRACT

The agar plate fungicidal activity of a number of synthesized and natural stilbenols was measured against the white-rot fungus *Trametes* (formerly *Coriolus*) *versicolor*, the brown-rot fungi *Gloeophyllum trabaeum* and *Postia* (formerly *Poria*) *placenta*, and the blue-stain fungi *Ceratocystis minor* and *C. clavigera*. The bioactivity of a commercial biocide, 3-iodo-2-propynyl butyl carbamate (IPBC), against the wood-destroying fungi was also measured for comparison purposes. Only some reduced stilbenols (bibenzyls), 3-stilbenols and resveratrol had white-rot fungal activity. Stilbenols required a 3'-substituent for brown-rot activity. A quantitative structure-activity relationship (QSAR) study of (E)-4-stilbenols showed a parabolic hydrophobic relationship for brown-rot activity. The substitution pattern on the "A" ring did not appear to affect brown-rot activity. All bibenzyls examined had moderate brown-rot activity. No dehydrogenative dimer had activity against brown- or white-rot fungi. Only the *cis* (Z) stilbenols had significant activity against blue-stain fungi. None of the stilbenols produced by pine trees had significant blue-stain fungi activity. These QSAR and structure-activity relationship (SAR) studies were used to predict the activity of two natural stilbenols (resveratrol and chlorophorin) against white- and brown-rot fungi with mixed results. Prior literature and this study suggest that most stilbenols and wood extractives in general have low fungal activity compared to commercial biocides.

Keywords: Wood-decaying (brown- and white-rotters) and blue-stain fungi; wood durability.

INTRODUCTION

Woody plants produce several classes of secondary metabolites as defensive agents against various fungi and other organisms. Among these are hydroxylated stilbenes, which are also called stilbenols. Stilbenols and the other types of secondary metabolites can be formed in woody tissues just prior to cell death to give constitutive (passive or preformed) defense agents found in the dead tissue (heartwood and outer bark). Alternatively, secondary metabolites can be produced by living cells in response to plant stress of either a biological (micro-organism attack) or nonbiological (mechanical damage, UV radiation, desiccation, etc.) nature. These induced (active defense) antimicrobial metabolites are called phytoalexins. Constitutive or phytoalexin metabolites produced by the same tree often have the same or similar structure, but the relative concentration may vary.

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As mentioned earlier, stilbenols are one type of bioactive compounds formed by woody plants. Presented here:

(1) A brief discussion on the usefulness of quantitative structure-activity relationship (QSAR) and structure-activity relationship (SAR) studies in bioactivity studies.

(2) A brief summary of the biosynthesis of and the role that stilbenols play in the natural defense of woody plants.

(3) A summary of previously published fungicidal activities of stilbenols against wood-destroying (brown- and white-rot) fungi (Schultz and others 1990, 1991, 1992) and the resulting SAR and QSAR results.

(4) Fungicidal activities of two natural stilbenols, resveratrol and chlorophorin, against wood-destroying fungi and how the measured activities compared to the SAR and QSAR predictions.

(5) A summary of the fungicidal activities of stilbenols against two blue-stain fungi (*C. minor* and *C. clavigera*).

(6) A summary discussion on the role that stilbenols are believed to play in the natural durability of wood (Schultz and others 1994).

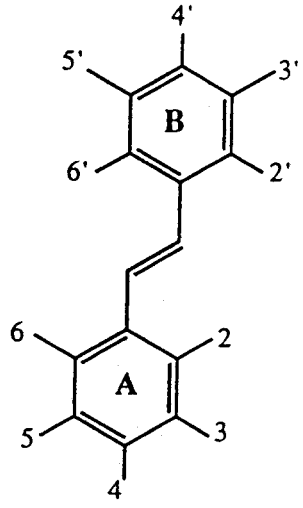
Structure-Activity Studies

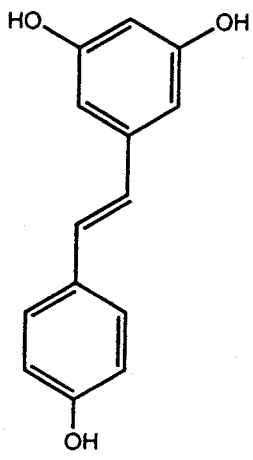
Drug design can be a tedious, trial-and-error undertaking. The ability to predict whether a compound with a specific structure will have activity (SAR), or to quantify the activity level of a series of similar compounds (QSAR), can be useful in developing new drugs (Hansch and Lien 1971, Martin 1978). Most QSAR models are based on: (1) the transportation of a bioactive agent from the application site to the micro-organism, (2) the bioactive agent fitting into the active site of a particular enzyme, and (3) the bioactive agent denaturing the enzyme site by a chemical reaction. The hydrophobicity, or lipophilicity, is used to "predict" ease of movement. Since a bioactive agent will need to move through both cell membranes (which are hydrophobic) and cell interiors (which are hydrophilic), an optimal hydrophobicity is often found with a parabolic-shaped relationship. The size (steric) factor is used to predict the ease of fitting into the enzyme site, and an electronic factor predicts the ability to denature the enzyme. Both of these factors, if significant, usually give a linear relationship with a semi-log bioactivity plot. Consequently, plotting the log of the bioactivity for a number of similar analogues versus hydrophobicity, steric and electronic factors may give a significant correlation with one of more of these factors (Hansch 1993). A parabolic hydrophobic term has been the factor most often reported in QSAR studies (Hansch 1993). Besides the three factors discussed above, other factors can also be used in QSAR studies.

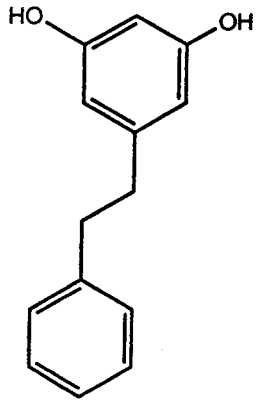
While the QSAR model for a particular biocide class against a specified micro-organism is only an empirical correlation, many benefits can be obtained from a QSAR study. First and most important, the QSAR model can predict the compound expected to have the greatest activity. Consequently, from the bioactivity data of a limited number of compounds researchers can synthesize and test what often is a previously unknown compound but which may have optimal activity against a certain pathogen. Thus, the trial-and-error approach is avoided. Secondly, the QSAR model might provide some information about the mode of action. Finally, a particular derivative that has greater activity than predicted can suggest new research directions. Disadvantages of QSAR are that many analogues must be obtained and tested, and that a QSAR model is specific for the particular compound type/micro-organism examined.

Biosynthesis and Activity of Stilbenes

The biosynthesis of stilbenols has been reviewed by a number of authors (Gorham 1989, Hart 1981, Kindl 1985); therefore, this area is only briefly covered. The general stilbene structure is shown in scheme 1. Stilbenols are biosynthesized from two pathways, the shikimic acid pathway (ring "A" on the bottom) and the acetate (or malonate) pathway (ring "B"), and initially follow the same biosynthetic pathway as the flavonoids. The "B" ring can be hydroxylated in both the 3' and 5', or sometimes only the 3', positions. These hydroxy groups may be further modified by one or both groups being methylated, or the 3' position can have an 0-glycoside. The "A" ring has the substitution pattern expected from the shikimic pathway: no substitution, 4-hydroxy, 3,4-dihydroxy, 4-hydroxy-3-methoxy, 4-hydroxy-3,5-dimethoxy, etc. The ethene bridge is typically found in the (E) (*trans*) isomer shown in scheme 1, but sometimes small amounts of the (Z) (*cis*) isomer are found along with the (E) isomer. (E/Z) isomerization may occur during isolation, and the (Z) isomer may thus be an artifact. The most common stilbenol found in woody plants is resveratrol [(E)-3',4,5'-trihydroxystilbene] (Kindl 1985), shown in scheme 1. In addition, the ethene bridge can be reduced in vivo to form a bibenzyl, such as dihydropinosylvin. Bibenzyls can act as plant growth regulators in addition to being fungicidal.







RESVERATROL DIHYDROPINOSYLVIN

SCHEME 1

As with any natural metabolite, a large variety of derivatives and exceptions to the above general rules can be found (Kindl 1985). Various biomodifications include "unusual" aromatic hydroxylation and/or carboxylic acid derivatives, the cyclization of stilbenols or bibenzyls to phenanthrenes, a 3-hydroxy-4-methoxy substitution on the "A" ring, C-alkylation with isoprenoid groups in the 4' position, oxidative polymerization to form various stilbenol oligomers such as the viniferins, etc.

Prior studies on the role which stilbenols play in wood durability have reached few definitive conclusions (Hart and Shrimpton 1979, Kindl 1985). (Indeed, the reason for the natural durability of certain hardwoods is poorly understood). In brief, it has been shown that stilbenols are bioactive against many but not all wood-inhabiting fungi. Also, it is generally assumed that the stilbenols present, in conjunction with the other extractives found in heartwood, have a synergistic effect against fungi.

There are several reasons for the confusion on the role which stilbenols play in the natural durability of wood. First, stilbenols, as with extractives in general, are difficult to extract in sufficient amount and purity for bioactivity studies. Second, only a limited number of stilbenols are present in a particular woody plant, and thus a study might involve only two or three compounds. In addition, wood biocide measurement methods are generally imprecise and very method specific; consequently, results obtained at one lab are not comparable to results from another lab. Finally, only a few researchers have also run a commercial biocide of known activity when they measured the activity of isolated extractives. All these reasons make it very difficult to draw many conclusions and comparisons on the bioactivities of different stilbenols from prior work.

In reviewing the literature on the effect of stilbenols in wood durability, we felt that additional work was worthwhile. To have sufficient amount and variety of stilbenols for SAR and QSAR studies, we synthesized stilbenols (Schultz and others 1990, 1991, 1992). The bioactivity of these synthesized stilbenes, and also of a few stilbenols isolated from woody plants, was measured using the agar plate procedure. The agar plate method is very reproducible and requires a minimal amount of biocide as compared to the agar block and soil block methods (Archer and others 1993). (A few stilbenols were also run using the soil block method to provide additional comparative data). In addition, a commercial biocide, 3-iodo-2-propynyl butyl carbamate or IPBC, also called polyphase, was run with both the agar plate and soil block methods so that the activity of a particular stilbenol could be directly compared to a "good" biocide. Though the major effort of this work concentrated on heartwood durability (Schultz and others 1990, 1991, 1992), the activity of some stilbenols and derivatives against the blue-stain fungi *C. minor* and *C. clavigera* was also examined.

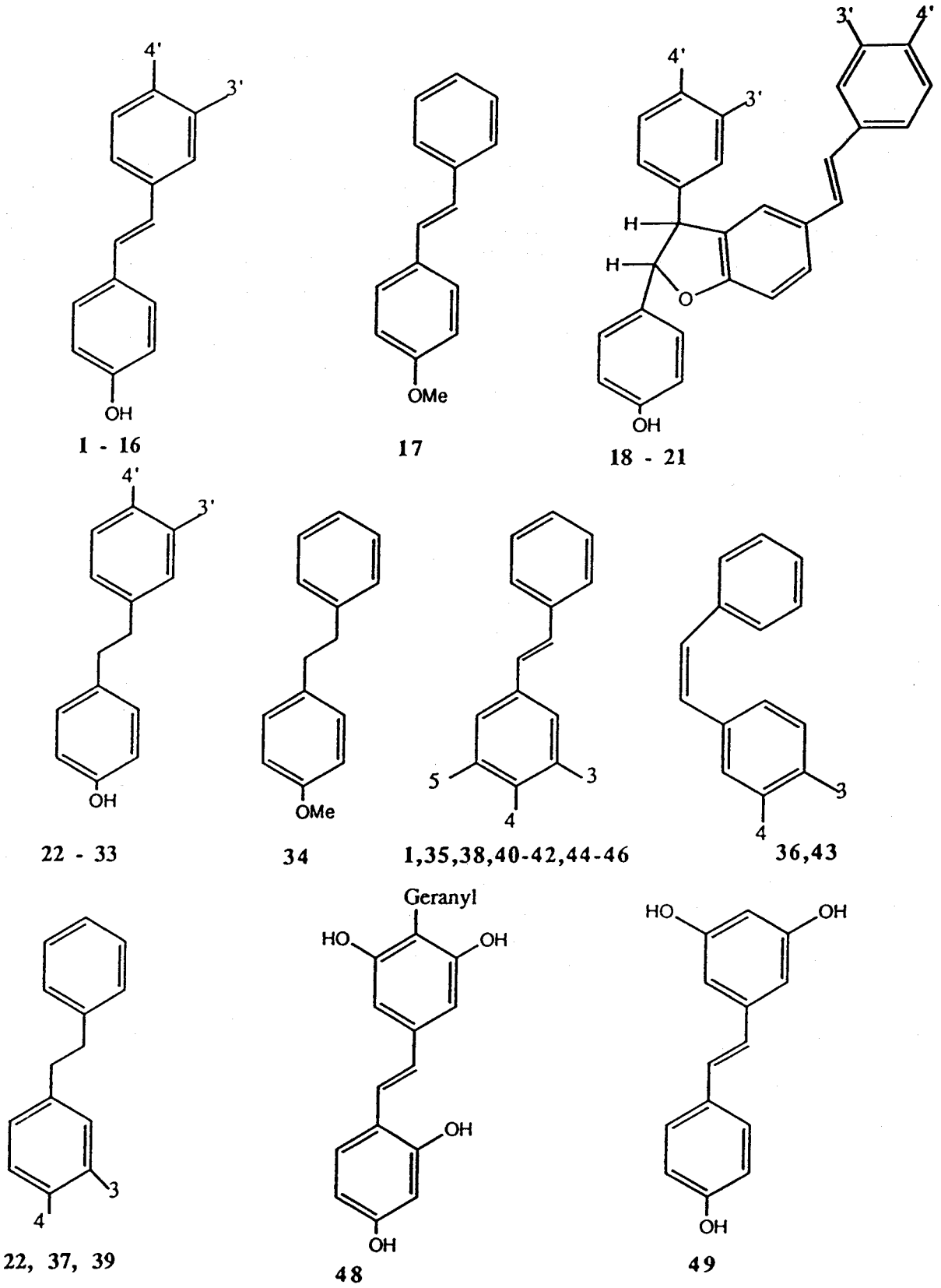


Figure 1. Stilbenols Examined.

RESULTS AND DISCUSSION

Stilbenol Bioactivity Against Wood-Destroying Fungi

The agar plate bioactivities (IC_{50}, ppm; concentration at which the relative radial fungal mycelium growth is inhibited by 50 percent) for the wood-destroying fungi are given in table 1 and the structures in figure 1. Compounds which have been found in woody plants are **1** (questionable), **13**, **26**, **30**, **44** (pinosylvin), **45** (pinosylvin monomethyl ether), and **46** (pinosylvin dimethylether) (Kindl 1985, Lorimer and others 1993, Norin 1989). The bioactivity data from these compounds were used to develop SAR and QSAR correlations, where appropriate, with the wood-destroying fungi. To verify these SAR and QSAR correlations, two natural stilbenols were used (chlorophorin, **48**; and resveratrol **49** (Schultz and others 1994)).

Table 1. *Bioactivity (IC_{50} ppm) for stilbenols and related compounds against wood-destroying fungi*

	Brown-Rotters		White-Rotter
Compound	*G. trabeum*	*P. placenta*	*T. versicolor*
(E)-4-OH Stilbenes and Controls			
1 4'-H	31/25	1	>250
2 4'-Me	>250	-	>250
3 4'-OMe	>250	-	>250
4 4'-F	>250	-	>250
5 4'-Cl	~125	-	>250
6 4'-OH	>250	-	>250
7 3'-Me	16	1	>250
8 3'-OMe	22	6	>250
9 3'-F	16	5	>250
10 3'-Cl	13	13	>250
11 3',4'-diOMe	>250	-	>250
12 3',4'-diCl	50	-	>250
13 3'-OH	53	16	>250
14 3'-Br	23	46	>250
15 3'-OPr	10	~0.5	>250
16 3'-OBu	83/82	33	>250
17 4-OMe (Non-phenolic control)	>250	-	>250
(E)-4-OH Stilbene Dehydrogenative Dimers			
18 4'-H Dimer	>250	-	>250
19 4'-Cl Dimer	>250	-	>250
20 4'-OH Dimer	>250	-	>250
21 3'-Me Dimer	>90	>90	-
4-OH Bibenzyls			
22 4'-H BB	38	20	224
23 4'-OMe BB	>90	15	>250
24 4'-Cl BB	69	17	113
25 3',4'-diCl BB	69	~0.5	43
26 3'-OMe BB	87	11	>250
27 3'-Me BB	44	13	112
28 3',4'-diOMe BB	>90	21	>250
29 3'-F BB	77	9	>250
30 3'-OH BB	>90	39	>250
31 3'-Cl BB	60	~0.5	>250

Table 1. *Bioactivity (IC_{50}, ppm) for stilbenols and related compounds against wood-destroying fungi--Continued*

32 3'-Br BB	68	27	>250
33 4'-F BB	69	~0.5	>250
34 4-OMe BB (Non-phenolic control)	>90	>90	>250
A-Ring Stilbenes and Bibenzyls			
(**1** 4-OH)	31/25	12/7	>250
(**22** 4-OH BB)	38	20	224
35 3-OH	35	54	49
36 cis-3-OH	65	25	75
37 3-OH BB	44	49	78
38 4-OH,3-OMe	40	40	>350
39 4-OH,3-OMe BB	>90	13	>350
40 4-OH,3,5-diOMe	60	49	>350
41 4-OH,3-Cl	43	40	>350
42 3,4-diOH	34	33	>350
43 cis-3-OH,4-OMe	64	8	>350
44 3,5-diOH	29	>90	140
45 3-OH,5-OMe	42	>90	163
46 3,5-diOMe	>90	>90	>350
Commercial Biocide			
47 IPBC	6	1	1
Natural Stilbenes Used to Verify SAR and QSAR Correlations			
48 Chlorophorin	45	-	511
49 Resveratrol	197	-	226

White-rot fungus

No 4-stilbenol was active against the white-rot fungus *T. versicolor* (table 1). Several researchers have previously reported that stilbenols are active against brown-rot but not the laccase-producing white-rot fungi (Hart and Shrimpton 1979, Lyr 1961). However, other studies have reported that some 4-stilbenols can be active against white-rot fungi (Hart and Hillis 1974, Woodward and Pearce 1988).

Four 3-stilbenols were active against *T. versicolor* (**35**, **36**, **44**, and **45**). One *cis* 3-stilbenol had no white-rot activity (**43**). Thus, it can be tentatively assumed that white-rot activity for stilbenols is confined to 3-stilbenols. The IC_{50} values of the four 3-stilbenols that had white-rot activity were all much higher (less active) than the commercial biocide IPBC (**47**), however.

None of the dehydrogenative dimers (**18-21**) were active. Since none of the monomeric precursors had white-rot activity, it is not unexpected that the dimers were also inactive.

Only a few of the bibenzyls had moderate to poor activity against *T. versicolor* (**22**, **24**, **25**, **27**). No consistent structure requirement could be found to differentiate between the active and inactive bibenzyls.

Brown-rot fungi

The (E)-4-stilbenols (**1-17**) showed a structure-activity relationship with *G. trabeum*. Specifically, it appears that 4-stilbenols with only 3'-substituents had activity, with the exception of the chlorosubstituted stilbenols **5** and **12**. Figure 2 gives some data for the chloro and methoxy substituents, expressed as the relative radial fungus growth at 125 ppm stilbenol concentration. As can be seen for the methoxyl derivatives (Figure 2), only the 3'-OMe stilbenol **8** had any *G. trabeum* activity. With the chloro substituents, the 3'-Cl stilbenol **10** had much higher activity than the other two chlorinated

stilbenols. No activity was observed for the 4'-Me, 4'-F and 4'-OH stilbenols **2**, **4**, and **6**. Low activity for the 4'-Cl stilbenol **5**, and moderate activity for the 3',4'-diCl stilbenol **12**, were observed.

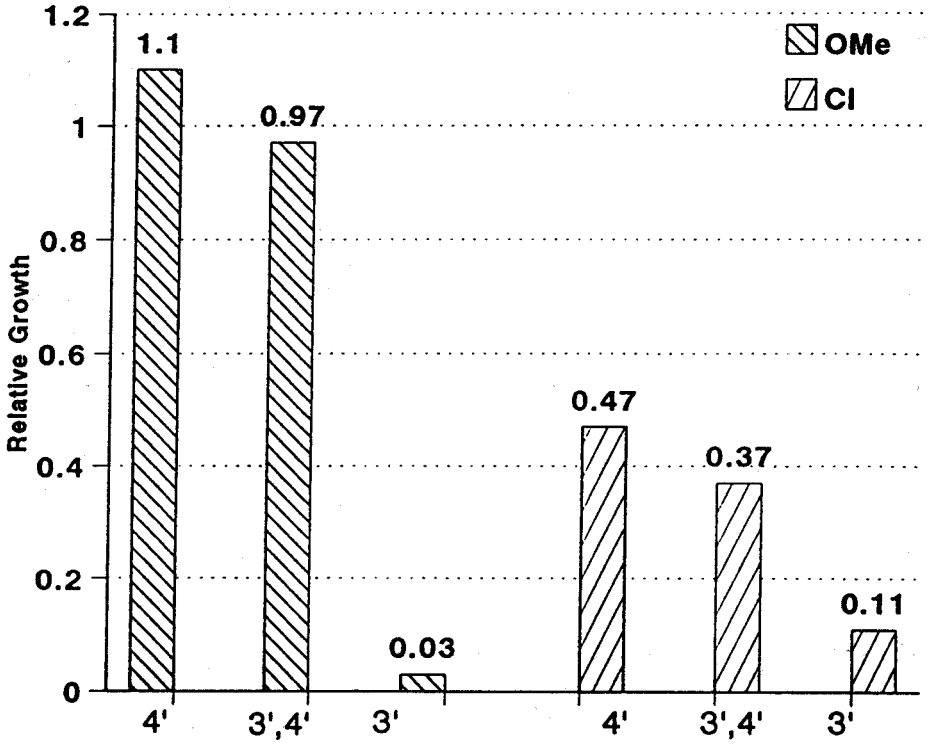


Figure 2. *Relative* G. trabeum *fungal growth at 125 ppm stilbenol concentration.*

The activity values for the (E)-3'-substituted-4-stilbenols against the brown-rot fungi *G. trabeum* and the copper-tolerant *P. placenta* were subjected to a QSAR study using various electronic, steric, and hydrophobic parameters (Schultz and others 1991). Only hydrophobicity was found to be a significant parameter. A hydrophobicity plot, in which the IC_{50} values were converted to mM concentrations, gave a roughly shaped parabolic curve for *G. trabeum* (r^2=75.2 %), and also for *P. placenta* when the 3'-Cl and 3'-Br derivatives were deleted (r^2=60.2 %). The highest activity was observed for compounds with moderate hydrophobicity, with the most hydrophilic (**13**) and hydrophobic (**16**) stilbenols having the poorest activity. Thus, partial in vivo methylation to give a more hydrophobic stilbenol, such as the biosynthesis of pterostilbene [(E)-3',5'-dimethoxy-4-hydroxystilbene] from resveratrol, would be anticipated to give a stilbenol with moderately high brown-rot activity but no white-rot activity. Indeed, pterostilbene, a heartwood constituent of *Pterocarpus* species, was found to be very toxic to a brown-rot but not a white-rot fungus (King and others 1953). Pterostilbene has also been found to be a grapevine phytoalexin with relatively high fungal activity (Langcake and others 1979).

A free phenolic group is necessary (Hart 1981), as can be seen by the inactivity of (E)-4-methoxystilbene **17**.

No dimer (**18-21**) had brown-rot activity, even though two of the monomeric precursors had good brown-rot activity. Langcake and Pryce (1977) reported that stilbenol oligomers were more fungitoxic than the monomeric precursor resveratrol. Alternatively, Dereks and Creasy (1989) found that resveratrol was about twice as fungicidal as a resveratrol dimer. The results obtained with "artificial" dimers may not necessarily be indicative of the fungicidal properties of natural stilbenol oligomers.

The bibenzyls (**22-33**) all had some brown-rot activity, but the activity was approximately only about one-half that of the equivalent stilbenol. An exception to this was that all 4'-substituted bibenzyls had at least some fungicidal activity against at least one brown-rot fungus, whereas the 4'-substituted stilbenols (**2-6**, and **11-12**) had no activity except for the chlorinated derivatives **5** and **12**. A control bibenzyl, in which the phenolic group was methylated (**34**), had no activity as expected (Lorimer and others 1993). No significant QSAR correlation was found for the bibenzyl brown-rot activities.

All the so-called "A"-ring modified stilbenols had moderate brown-rot activity against at least one fungus. However, the substitution pattern of the stilbenols appeared to have little influence on the activity. The three "A"-ring bibenzyls also had moderate brown-rot activity, but the limited number studied makes data interpretation difficult. *cis/trans* Isomers appear to have similar *G. trabeum* activity, although this is based on only one isomer pair (**35** vs. **36**). The isomer effect is less clear for *P. placenta*. As mentioned earlier, while most natural stilbenols are *trans*, a few studies have also isolated small amounts of a co-occurring *cis* isomer. The *cis* isomer may have been formed during isolation, since stilbenes are well known to undergo *trans/cis* photoisomerization. The only "A"-ring stilbenols with low activity against the copper-tolerant fungus *P. placenta* were the natural stilbenols pinosylvin and pinsoylvin monomethyl ether (**44** and **45**). (Note: The hydroxylation pattern of **44** and **45** is from the acetate-derived ring). These results were surprising, since these stilbenols were active against *G. trabeum*, and previously we found that stilbenols active against *G. trabeum* were usually even more active against *P. placenta*. However, in this study, all three (E)-3-stilbenols that had no 4-substituent (**35**, **44**, and **45**) were less active against *P. placenta* than *G. trabeum*. It is possible that whatever gives *P. placenta* copper tolerance also provides some tolerance against stilbenols with a specific structure.

Activity of Natural Stilbenols Against Wood-Destroying Fungi

The activity of two natural stilbenols, chlorophorin (**48**) and resveratrol (**49**), was measured using the white-rotter *T. versicolor* and the brown-rotter *G. trabeum* (Schultz and others 1994). These measured activities were then compared to the values predicted from the SAR and QSAR work described above.

Chlorophorin (**48**) (figure 1) has a 4-hydroxy group. Consequently, it should have very poor white-rot activity, which is what was observed (IC_{50} of 511 ppm, against *T. versicolor*) (Schultz and others 1994) (table 1). In addition, since chlorophorin has a 4'-substituent (an isoprenoid group), little brown-rot activity is predicted, which is not what was observed (IC_{50} of 45 ppm against *G. trabeum*). (The nonisoprenoid substituted analogue of chlorophorin, oxyresveratrol, had very poor brown-rot activity (Schultz and others 1994)). Since chlorophorin had much higher activity than anticipated, it appears that isoprenoid-substituted stilbenols may affect brown-rot fungi by a different mechanism than nonisoprenoid-substituted stilbenols.

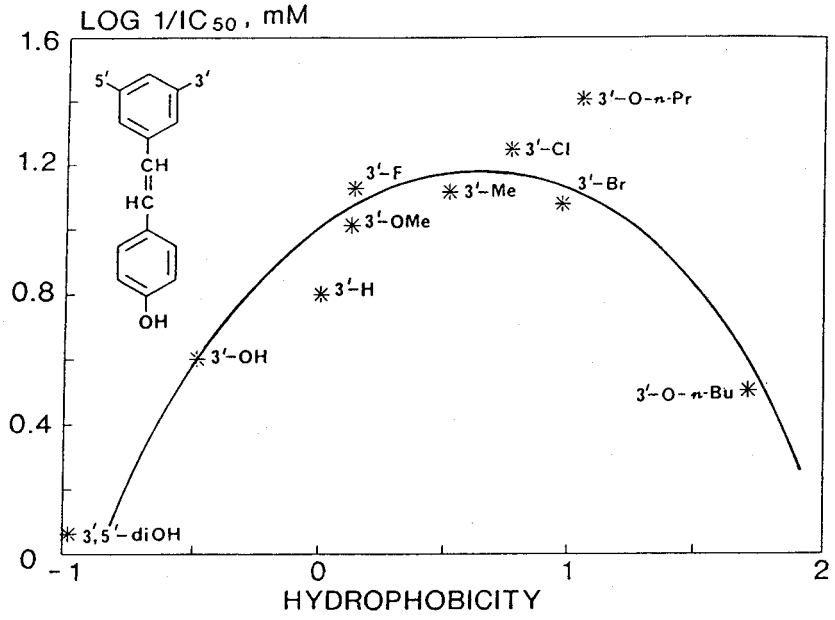


Figure 3. *Quantitative Structure-activity Relationship plot for activity of 4-stilbenols against* G. trabeum.

Resveratrol (**49**) is the most commonly-found stilbenol (Kindl 1985). Based on the additivity of substituents and a parabolic QSAR, resveratrol would be expected to have poor *G. trabeum* activity, with an IC_{50} of about 300 ppm. The measured value of 197 ppm (Schultz and others 1994) is close to the predicted activity based on a QSAR hydrophobicity plot (figure 3). Several assumptions were made, however, in using prior data to predict resveratrol's low *G. trabium* activity. For example, it was assumed that 5' substituents act in a similar manner as 3' substituents, since only the 3' and/or 4' positions were studied (Schultz and others 1990, 1991). With a 4-hydroxy, it would also be expected that resveratrol would have no or little white-rot activity. The measured value of 225 ppm, while low, is higher than we expected. Prior work (Hart and Hillis 1974) also found that resveratrol has white-rot activity, although other studies have generalized that stilbenols are not active against white-rot fungi (Hart and Shrimpton 1979, Lyr 1961). We generalized earlier that white-rot activity is limited to 3-stilbenols (Schultz and others 1992).

Based on the above, it appears that the SAR and QSAR predictions gave mixed results. Thus, the SAR and the QSAR findings should be used only as a guide since exceptions are obviously present, as is often the case in bioactivity studies of natural products.

Stilbenol Activity Against Blue-Stain Fungi

The stilbenols **44** and **45**, as phytoalexins, are produced by pine trees in response to bark beetle attack (Hart 1981). Consequently, we measured the activity of selected stilbenols against two blue-stain fungi, *C. minor* and *C. clavigera*, associated with the southern pine bark beetle. The results, expressed as IC_{50} values, ppm, are given in table 2.

Table 2. *Fungal activity of selected stilbenols against blue-stain fungi*

Compound	IC_{50}, ppm	
	C. minor	*C. clavigera*
1 4'-H	>250	144
7 3'-Me	52	37
22 4'-H BB	57	32
35 3-OH	124	249
36 cis-3-OH	23	26
38 4-OH,3-OMe	>250	>250
43 cis-3-OH,4-OMe	85	56
44 3,5-diOH	293	>250
45 3-OH,5-OMe	385	>250
46 3,5-diOMe	>250	>250

The fungal activities of the three stilbenes produced by pine trees (**44-46**) were relatively low. This is in agreement with prior work on stilbenol activity against blue-stain fungi associated with the mountain pine beetle in lodgepole pine (Hart 1981). The production of stilbenols by a pine tree under bark beetle attack may be in response to the mechanical damage caused by the bark beetle, rather than a blue-stain fungi response.

The *cis* isomer **36** appeared to be more fungicidal than the *trans* isomer **35**. In addition, the bibenzyl derivative **22** was more active than the stilbenol analogue **1**. However, these results are both based on only one set of compounds, and thus are only tentative. Finally, it is interesting to note that one of the more active brown-rot stilbenols, the 3'-Me derivative **7**, was also fairly active against blue-stain fungi.

CONCLUSIONS: THE ROLE STILBENOLS PLAY IN NATURAL WOOD DURABILITY

We mentioned earlier that the role that stilbenols--and indeed extractives in general--play in the natural durability of wood is poorly understood for hardwoods. We proposed a hypothesis at the start of our work on stilbenols: When stilbenols are first formed by woody plants they have only moderate fungicidal activity but may be biomodified in vivo to give derivatives that have greater and/or broader fungal activities (Schultz and others 1990). Based on our work, it appears this hypothesis is correct in some cases. For example, partial biomethylation of stilbenols can result in compounds with increased hydrophobicity and thus greater brown-rot activity, as is the case with pterostilbene discussed earlier. In addition, the bioreduction of a stilbenol precursor to form a bibenzyl may give an extractive some white-rot activity. The C-isoprenoid substitution of a stilbenol can result in significantly greater fungicidal activity against some fungi (Kindl 1985, Schultz and others 1994).

When natural stilbenols are compared to a commercial biocide, however, it is readily apparent (table 1) that most stilbenols have low fungal activity as measured by the agar plate and also soil block tests (table 3). The same result has also been found for most wood extractives (Rudman 1963).

Table 3. *Representative soil block results*

	Compound	Retention kg/m^3	Relative % weight loss *G. trabeum*	Relative % weight loss *T. versicolor*
1	4'-H	7.2	38	-
7	3'-Me	3.9	3	-
22	4'-H BB	7.4	12	-
44	3,5-diOH	9.6	70	81
45	3-OH,5-OMe	8.3	53	75
48	Chlorophorin	6.9	-	91
48	Chlorophorin	9.6	103	-
47	IPBC (toxic threshold, kg/m^3)		(0.4)	(0.6)

North American hardwoods that have exceptionally high heartwood durability are black locust, red mulberry and osage-orange. When we studied osage-orange (Schultz and others 1994), we found that all extractives had relatively low fungicidal activity. However, high levels (about 40 kg/m^3) of two extractives were found. In a review of prior work, high levels (about 40 kg/m^3) of one or two compounds were also reported in red mulberry (Venkataraman 1972) and black locust heartwood (Hart 1989). These compounds all had low fungicidal activity, but were present at high levels. Thus, one possible explanation for the exceptionally high durability of some hardwoods may be the presence of high levels of one or two monomeric compounds (Schultz and others 1994). Other factors may also contribute to heartwood durability, such as synergism (Hart 1989).

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Identification of a Host Compound and Its Practical Applications: 4-allylanisole as a Bark Beetle Repellent[1]

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ABSTRACT

Gas chromatography/mass spectrometry analysis of resin collected before and after injection of loblolly pines (*Pinus taeda* L.) with a fungicide mixture known to make pines more "attractive" to southern pine beetle, *Dendroctonus frontalis* Zimm., resulted in the identification of 4-allylanisole as a likely candidate for repellent effects. The phenylpropanoid, 4-allylanisole (Chemical Abstract 140-67-0), is a compound produced by many conifers, including loblolly pine, an abundant species in southern pine forests and a preferred host of the southern pine beetle. The repellency of 4-allylanisole to southern pine beetle was demonstrated in laboratory behavioral assays and in natural populations by comparing its effects with those of the beetle-produced inhibitory pheromone, verbenone. Responses of other North American scolytids and associates were also determined. Additionally, responses of southern pine beetle to various chemical analogues of 4-allylanisole were tested. The response in the field of southern pine beetle to its attractant pheromone in funnel traps was significantly reduced by simultaneous release of either 4-allylanisole or verbenone, which did not differ from one another in repellency. Both compounds together did not significantly further reduce trap catch. The response of a major predator, *Thanasimus dubius* (F.), to the attractant pheromone of southern pine beetle did not differ with the simultaneous release of either compound. The results of preliminary field tests with 4-allylanisole, in which lightning-struck pines were protected from southern pine beetle attack, are presented and discussed in relation to implications for development of a practical tree protection tactic.

Keywords: Analogue, Coleoptera, *Dendroctonus frontalis*, 4-allylanisole, host compound, inhibitor, *Pinus*, repellent, Scolytidae, semiochemical, sodium-N-methyldithiocarbamate, verbenone.

INTRODUCTION

The southern pine beetle, *Dendroctonus frontalis* Zimmermann, is the most destructive insect to pine forests in the Southeastern United States. Since 1960, the southern pine beetle has been responsible for the loss of nearly a billion dollars of forest resources southwide (Price and others 1992). Management options for reducing southern pine beetle losses are limited, especially for individual tree protection and in areas with multiple management objectives.

Physical and chemical qualities of oleoresin are important in the ecological interactions between bark beetles and their coniferous hosts. It is well known that volatile components of the host resin can be important semiochemicals (i.e., message-bearing chemicals) for bark beetles (Raffa and others 1993, Wood 1982). In the southern pine beetle/southern yellow pine system, a-pinene is a predominant component of loblolly oleoresin and has been shown to act synergistically with the beetle's primary aggregation pheromone, frontalin, to enhance beetle aggregation. However, it has generally proven very difficult to quantitatively relate beetle behavior to host chemistry. For example, stressed trees, such as lightning-struck trees, are vulnerable to attack by bark beetles, but the reasons for preferential selection of these host trees are not well understood (Hodges and Pickard 1971). Previous work has also shown that trees treated with a formulation of the fungicide,

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sodium-N-methyldithiocarbamate (MS), and the carrier, dimethyl sulfoxide (DMSO), are very susceptible to attack by the southern pine beetle (Dalusky and others 1990, Miller and others 1994, Roton 1987).

In an effort to better understand the host selection process of the southern pine beetle, we utilized the apparent "attractiveness" of MS+DMSO-treated trees. Oleoresin, phloem, and wood were sampled before and after treatment, continuing until trees were successfully attacked by the southern pine beetle. Results were then related to the timing of southern pine beetle attack to determine whether there were quantitative or qualitative changes in chemical constituents that may be responsible for the observed change in host susceptibility.

IDENTIFICATION OF THE COMPOUND

In the spring of 1991, 30 loblolly pines (*Pinus taeda* L.) from a single site in Camp Beauregard, Rapides Parish, Louisiana were selected for study based on similar diameter and crown characteristics. Three treatments were randomly assigned to individual trees: (1) No treatment, (2) water treatment, and (3) MS+DMSO (4:1 v/v) treatment. Application of treatments was accomplished using a modified "hack and squirt" method (Dalusky and others 1990, Roton 1987). Hacks were made around the circumference of each tree, leaving 2 to 5 cm between them. Into each hack 8 to 10 ml of water or MS+DMSO were released and allowed to passively infuse. Thus, the number of hacks and total tree dosage were dependent on tree circumference.

Oleoresin was sampled prior to treatment and weekly thereafter following the methods of Lorio and others (1990). A 1.27-cm arch punch was used to remove the outer and inner bark and the injury was allowed to drain resin into a collection vial for 24 h. Phloem and wood were sampled in a like manner, with tissue being removed from the punch and placed immediately into vials. To minimize the potential influence of previous sampling, each new wound was offset horizontally and vertically by approximately 5 cm from the previous sample. Collected resin was stored in an ultracold freezer (-70 °C) until prepared for chemical analysis.

To determine whether MS-DMSO injection induced the formation of abnormal or unusual chemical compounds, sample extracts from the phloem and wood samples were analyzed with a Kratos MS80 gas chromatograph/mass spectrometer. Extraction was accomplished with a soxhlet-type apparatus using ethanol and benzene (1:1) as the solvent. A 0.5-ml aliquot was reacted with diazomethane/ether and the total volume adjusted to 1.0 ml. The gas chromatograph conditions were as follows: initial temperature 60 °C, hold time 2 min, program rate 6 °C per minute, final temperature 280 ° C, hold time 20 min. A representative total ion chromatogram of phloem tissue extract with a J & W DB-5 column is shown in figure 1.

For the quantitative determination of oleoresin components, 25 mg of oleoresin was diluted to 10 ml with 9 ml of benzene and 1 ml of 1.0 mg/ml diphenylmethane in benzene. A 0.5-ml aliquot of this solution was reacted with 0.5 ml of diazomethane/ether. Oleoresin samples were analyzed with a Hewlett-Packard HP5840A gas chromatograph equipped with a J & W DB-5 column and operated as described above. Average response factors were determined using five different standard levels in the concentration range of 10 to 250 µg/ml. The chemical identity of the oleoresin components was confirmed by gas chromatography/mass spectrometry using selected samples. Camphene, 4-allylanisole, and diphenylmethane were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI), and were utilized without further purification. Abietic acid was obtained from Aldrich and was recrystallized before use. Limonene, α-pinene, and myrcene were obtained from Sigma Chemical Company (St. Louis, MO), β-pinene was obtained form Pfaltz & Bauer, Inc. (Waterbury, CT). Pimaric acid, isopimaric acid, palustric acid, levopimaric, and neoabietic acid were obtained from Dwayne Zinkel, USDA Forest Service, Forest Products Laboratory, Madison, WI.

Results of the resin analysis showed that three volatile compounds--myrcene, β-pinene, and 4-allylanisole--had changed dramatically in MS-DMSO-treated trees by week 3 after treatment (table 1), a time coincident with southern pine beetle attack. Of these, the compound showing the highest degree of repellency to southern pine beetle in preliminary assays was 4-allylanisole. Further, the ratio of α-pinene (an important component in southern pine beetle aggregation) to 4-allylanisole rose dramatically (fig. 2), suggesting that 4-allylanisole may be involved as a **deterrent** in the beetle's host selection process. Therefore, laboratory and field assays were undertaken to determine the repellent properties of 4-allylanisole to southern pine beetle and related insects. Commonly known as methyl chavicol or estragole, 4-allylanisole (Chemical Abstract 140-67-0) (fig. 3), is known from numerous pine and other conifer species (Drew and Pylant 1966, Mirov 1961). Although there is considerable intra- and interspecific variation, 4-allylanisole is a consistent component in the oleoresin of southern yellow pines, usually making up 1 to 5 percent of the turpentine yields (Drew and Pylant 1966, Mirov 1961, Sutherland and Wells 1956).

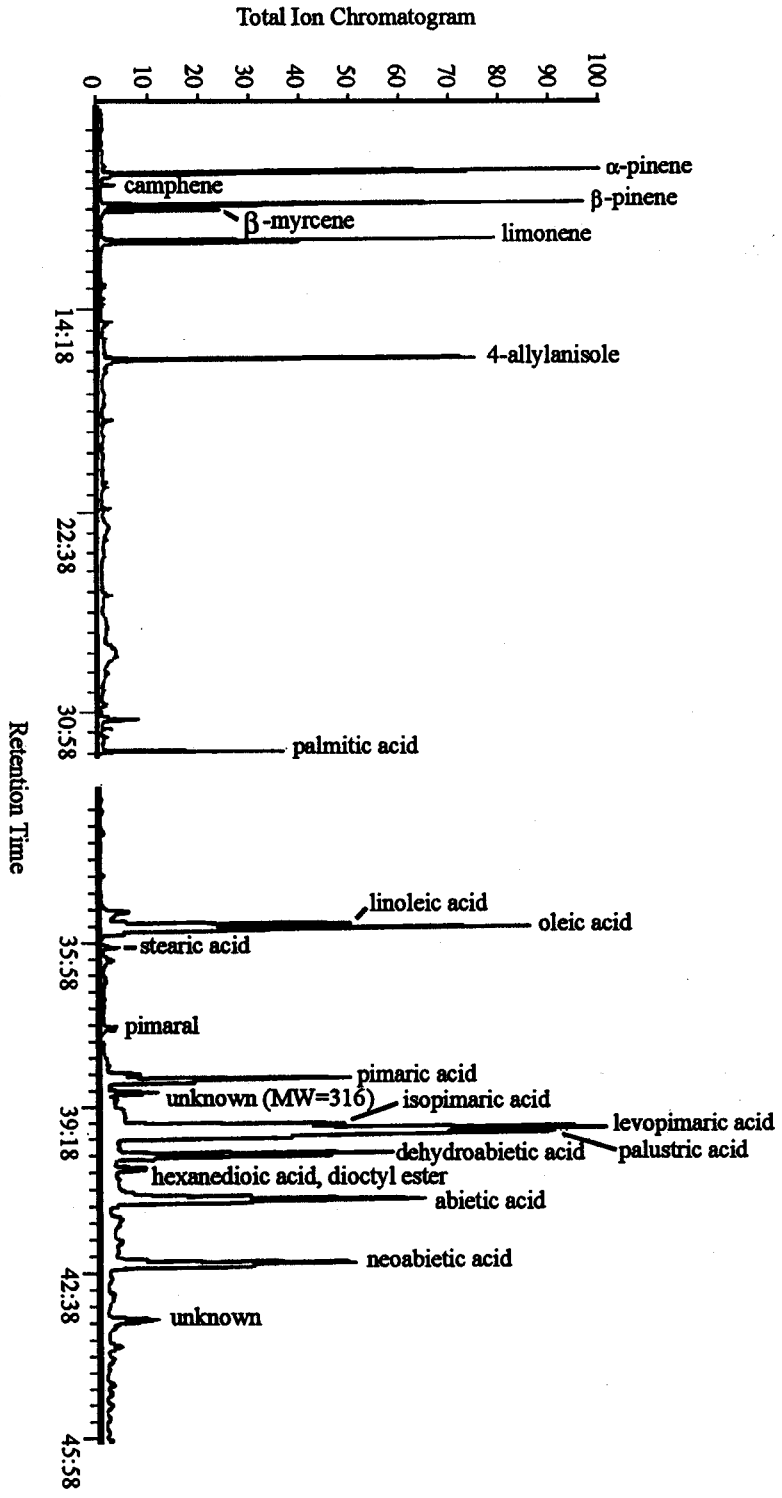

Fig. 1 A representative total ion chromatogram of phloem tissue from MS-DMSO injected trees.

Table 1. Concentration of chemical components of the oleoresin prior to treatment with MS-DMSO (week 0) and when trees were most likely to be attacked (3 weeks). Water-treated and untreated were combined for the control group. Percentage change is defined as: (week 3 - week 0)/week 0.

	Concentration					
	week 0		week 3		Percentage change	
Compound	Control	MS-DMSO	Control	MS-DMSO	Control	MS-DMSO
α-pinene	15.94	12.44	14.70	12.73	-7.8	2.3
Camphene	0.52	0.05	0.25	0.05	-51.9	0.0
β-pinene	7.74	6.54	7.38	4.67	-4.7	-28.6
Myrcene	0.63	1.03	0.77	0.34	22.2	-67.0
Limonene	1.64	0.81	1.74	0.90	6.1	11.1
4-allylanisole	**1.57**	**1.39**	**1.84**	**0.53**	**17.2**	**-61.9**
Pimaric acid	3.87	3.69	3.71	4.20	-4.1	13.8
Palustric & iso+levo Palmiric acids	34.39	35.43	33.96	36.06	-1.3	1.8
Dehydroabietic acid	6.85	6.48	6.83	10.15	-0.3	56.6
Abietic acid	14.10	15.41	14.26	17.54	1.1	13.8
Neoabietic acid	11.48	12.15	11.62	9.59	1.2	-21.1
Total	98.73	95.42	97.06	96.76		

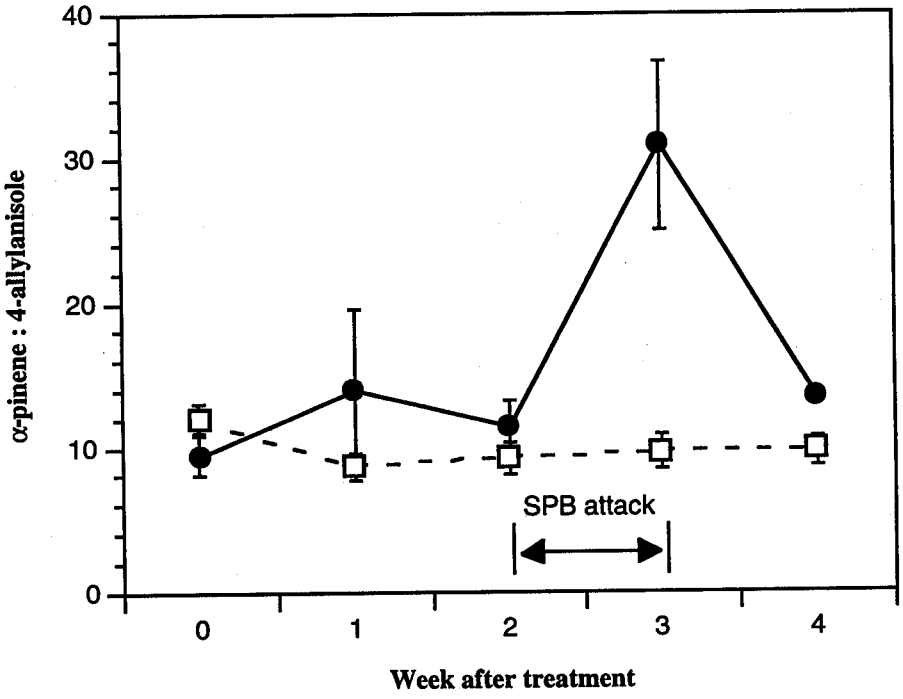

Figure 2. *The α-pinene: 4-allylanisole ratio changed significantly by week 3. Water-treated trees and untreated trees were not significantly different and were combined for the control treatment. Solid line is MS+DMSO; dashed line is control.*

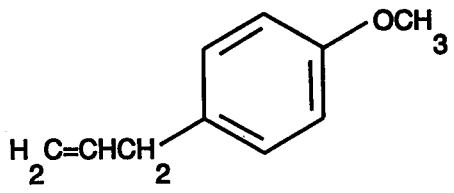

Figure 3. *Structure of 4-allylanisole.*

DETERMINATION OF REPELLENCY

With the exception of the analogue experiment, the experimental methods and results summarized below are described in greater detail elsewhere (Hayes and others 1994).

Lab Assay

A test of individual beetle response to 4-allylanisole vs. verbenone (a beetle-produced anti-aggregation pheromone) was conducted. A circle (17 cm diam by 5 mm wide) of 4-allylanisole or verbenone was "painted" with a camel' s-hair brush on a 28 by 21.5-cm piece of uncoated cardboard. After 3 min, beetles (2 to 5 individuals) were released in the center of the treated circle. Testing was conducted at room temperature with light supplied from an adjoining room. To prevent overwhelming photopositive responses, an object was used to cast a shadow over the test circle. Beetles were briefly refrigerated prior to testing to reduce their tendency to fly. Responses (<30 s exposure) were recorded as **not-repelled or repelled:** not-repelled beetles walked through the circle or stopped but proceeded across the circle within 30 s of exposure; and repelled beetles stopped abruptly, raised antennae (some "reared up" on hind legs), stood motionless and/or moved away from the circle (some moved abruptly in the opposite direction). Generally, beetles that were repelled by 4-allylanisole demonstrated a higher degree of alarm and more abrupt behavior than beetles repelled by verbenone.

Trials were conducted with newly emerged male and female southern pine beetles on three different dates from three different source populations; results of these trials (n = 300) were combined for presentation in figure 4. Trials were also conducted with a clerid beetle, *Thanasimus dubius* (F.), a common predator of southern pine beetle, and other scolytid species including: mountain pine beetle, *Dendroctonus ponderosae* Hopkins; western pine beetle, *Dendroctonus brevicomis* LeConte; spruce beetle, *Dendroctonus rufipennis* Kirby; pine engraver, *Ipspini Say*; the small southern pine engraver, *Ips avulsus* Eichhoff; and the six-spined ips, *Ips calligraphus* Germar, (fig. 4). In all trials, only apparently healthy beetles were used (n = 50).

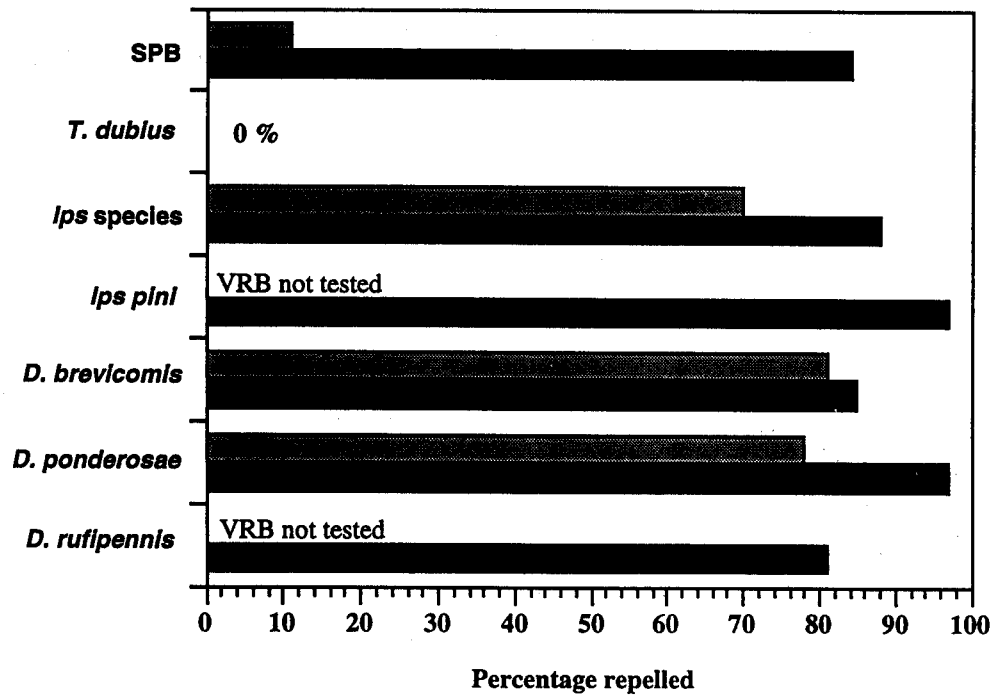

Figure 4. *The response of southern pine beetles, other scolytid species and the predatory clerid,* Thanasimus dubius *(F.), to 4-allylanisole (black bars) and verbenone (shaded bars) in laboratory assays. Response of southern pine beetles to frontalure was 0 percent.*

Behavioral assays with chemical analogues can provide information of ecological importance, as well as improve efficacy of control techniques. To determine the potential of analogues of 4-allylanisole for repelling the southern pine beetle, selected analogues were also tested using the assay described above (fig. 5).

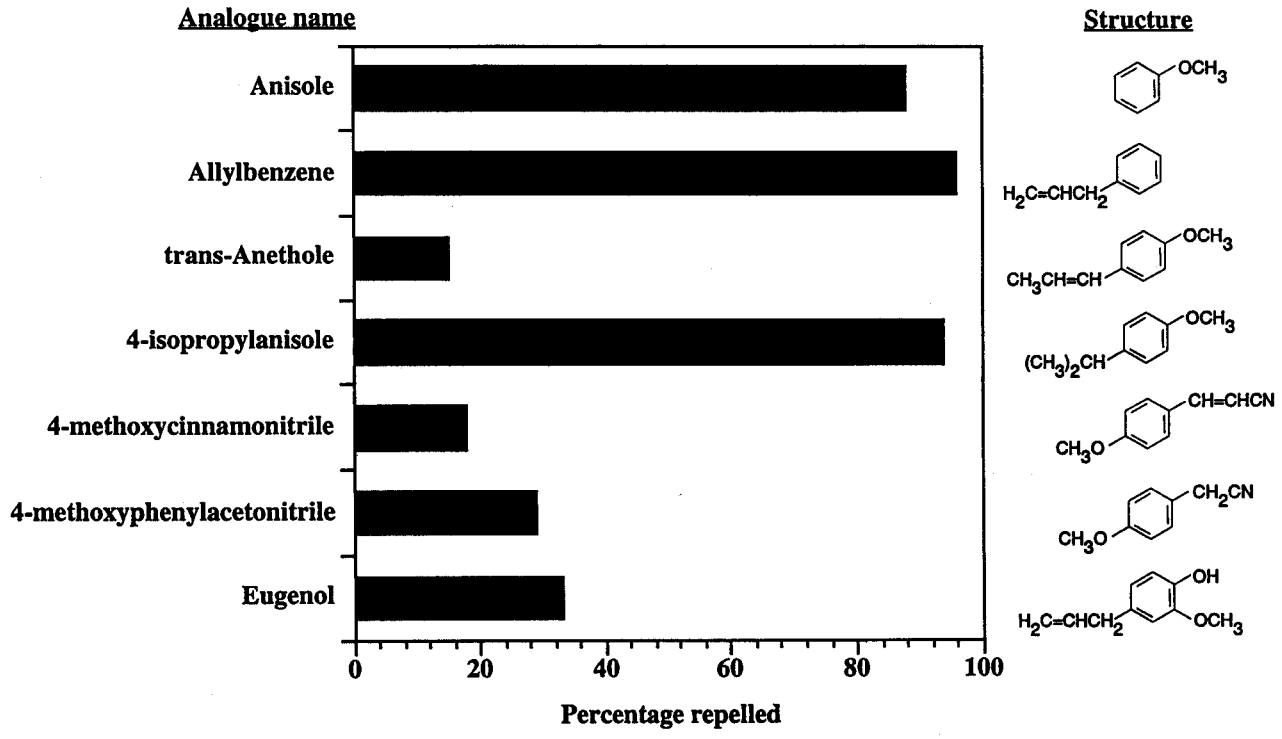

Figure 5. *Response of southern pine beetles to chemical analogues of 4-allylanisole. Analogue structures are also shown. Sample size for each analogue ranged from 45 to 53.*

Field Assay--A test of local southern pine beetle population's response to 4-allylanisole and to verbenone [vs. the attractancy of frontalure, the southern pine beetle aggregation pheromone frontalin + α-pinene (1:2)] was conducted using baited funnel traps (Lindgren 1983) placed in active southern pine beetle infestations in the spring (6 replications) and fall (7 replications) 1992. Traps were baited (2 traps/treatment) with frontalure, frontalure + verbenone, and frontalure + 4-allylanisole. Trap position was randomly assigned and changed daily in a sequential order for 6 days. The number of southern pine beetle and clerids were recorded daily (figs. 6, 7).

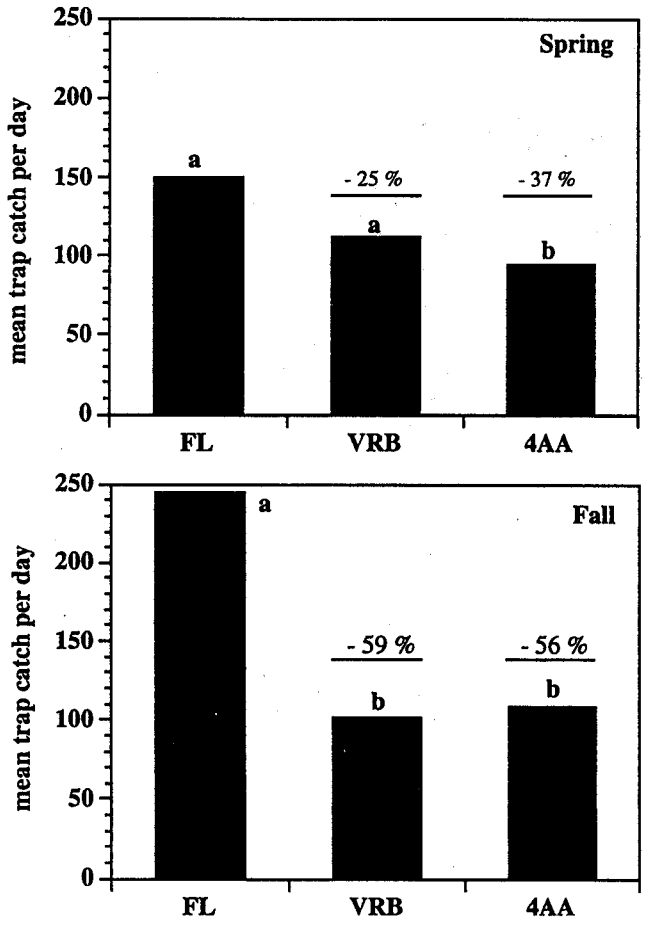

Figure 6. *Capture of southern pine beetles in the spring and fall using semiochemically baited* funneltraps. *Different letters near bar margins indicate significant difference in trap catch within a season (P<0.05 LSD of transformed data, SAS Institute, Inc. 1988). Percent change in catch relative to frontalure is shown above bars. FL = frontalure, VRB = verbenone, 4AA = 4-allylanisole.*

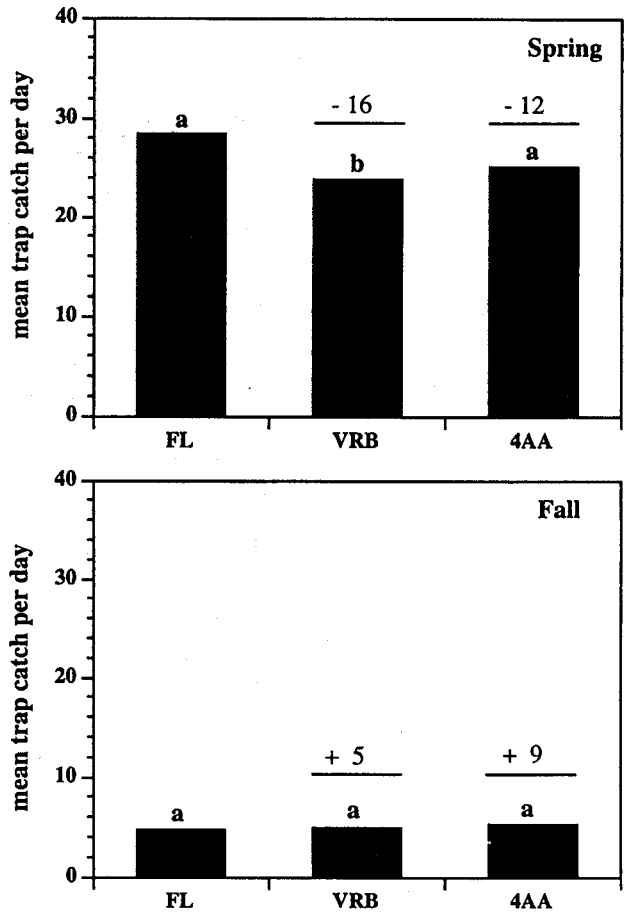

Figure 7. *Capture of the clerid,* Thanasimus dubius *(F.), in the spring and fall using semiochemically baited funnel traps. Different letters outside bar margins indicate significant difference in trap catch within a season (P<0.05 LSD of transformed data, SAS Institute, INC. 1988). Percent change in catch relative to frontalure is is shown above bars. FL = frontalure, VRB = verbenone, 4AA = a-allylanisole.*

Dose-response experiments (five replications) were conducted to test response to frontalure given increasing numbers of 4-allylanisole elution devices. Traps were baited (two traps per treatment) with frontalure alone or frontalure + one, two, or four 4-allylanisole elution devices. Trap position was randomly assigned and changed daily in a sequential order for 8 days. The numbers of southern pine beetles and clerids were recorded daily (fig. 8).

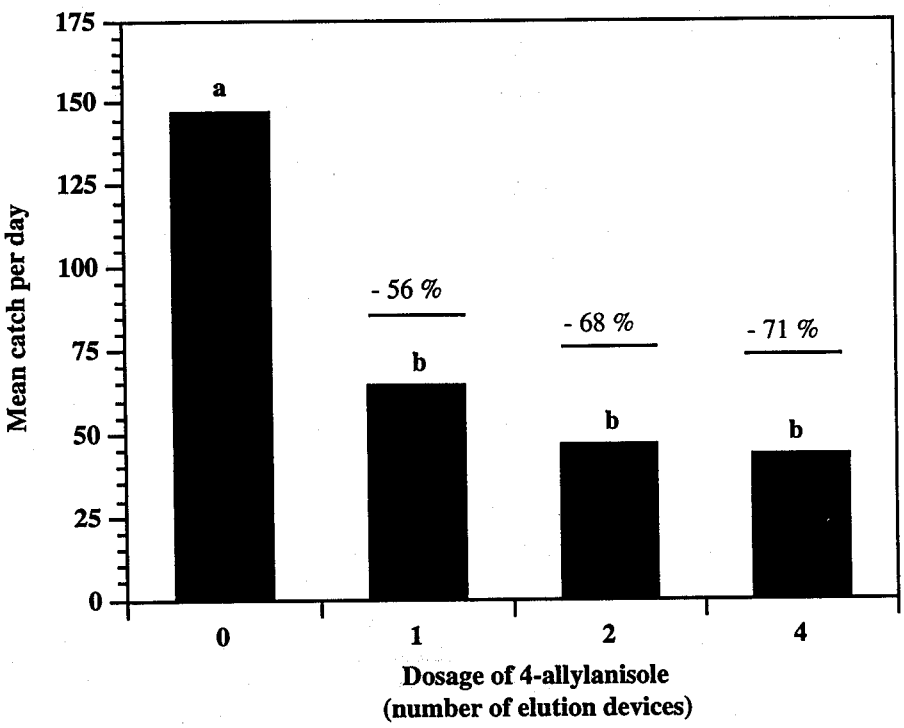

Figure 8. *Effect of 4-allylanisole dosage on the capture of southern pine beetles in funnel traps. Dosage is defined as the number of 4-allylanisole elution devices (wicked vials) in a trap. All traps also included the aggregation pheromone frontalure. Different letters near bar margins indicate significant difference in trap catch (P<0.05 LSD of transformed data, SAS Institute, Inc. 1988). Percentage change in catch relative to frontalure is shown above bars.*

RESULTS OF REPELLENCY TESTS

(1) Male and female southern pine beetles were repelled when exposed to 4-allylanisole in laboratory assays; higher percentages of all categories were repelled by 4-allylanisole than verbenone using the same assay method (fig. 4).

(2) Other scolytids, including local and nonresident species, were also repelled when exposed to 4-allylanisole in laboratory assays; for those species tested, equal or higher percentages were repelled by 4-allylanisole than verbenone (fig. 4).

(3) Significantly fewer southern pine beetles were captured in the spring and fall in traps baited with 4-allylanisole + frontalure than frontalure alone; trap captures did not differ between 4-allylanisole- vs verbenone-baited traps (fig. 6).

(4) Clerid beetles showed no repellent response when exposed to 4-allylanisole or verbenone in laboratory assays (fig.4) and were apparently unaffected by the addition of 4-allylanisole or verbenone to traps baited with frontalure (fig. 7).

(5) The repellent effect of 4-allylanisole on southern pine beetles was not significantly enhanced by the addition of more than one elution device (vial with 20 ml 4-allylanisole) (fig. 8); nor did one or more 4-allylanisole elution device impact clerid attraction to frontalure.

(6) Southern pine beetles were repelled by three of the chemical analogues of 4-allylanisole--allylbenzene, 4-isopropylanisole and anisole--to a degree at least equal to that of 4-allylanisole (fig. 5).

PROTECTION OF INDIVIDUAL TREES

For wildlife, cultural, and recreational resource management, as well as in suburban and urban settings, there is a need for the protection of individual trees threatened by bark beetles. Current control methods are based on stopping the spread of an infestation after it begins and usually requires the sacrifice of a large number of trees in the surrounding area (USDA 1987). Although two insecticides are registered as tree protectants for southern pine beetle, increasing environmental concerns may curtail their future use, indicating that additional tactics need to be developed. The results of our repellency assays suggest that 4-allylanisole is a candidate for a biologically-efficient tree protectant.

Preliminary Field Trials

Trees previously determined to be at high risk for southern pine beetle attack were selected to test the efficacy of 4-allylanisole for protecting individual trees.

Lightning Strikes

Loblolly and longleaf (*P. palustris* Mill.) pines struck by lightning were treated with 4-allylanisole within 48 h of being struck. The treatment consisted of placing nine 20-ml polyethylene vials with cotton wicks evenly spaced from the ground to 8 m up the tree bole on the damaged side. Trees of the same species and struck by lightning in the same storms were also located to serve as untreated controls. At day 30, numbers of southern pine beetle attacks were counted in a 15.2-cm- wide band and around the tree circumference at 2 and 4 m up the bole (table 2). In two other noteworthy instances, lightning-struck loblolly pines in residential settings were treated as described above. In both cases, the trees were protected from southern pine beetle attack for 30 days, until the 4-allylanisole was removed.

Table 2. ***Paired lightning-struck trees treated with 4-allylanisole (4AA) or untreated. Total number of southern pine beetle attacks was measured at 2 m and 4 m up the tree bole. Tree fate is the apparent condition 30 days after treatment began, at which time, treatments were removed***

Lightning strike date	Pine species	D.b.h. —cm—	Treatment	Number of attacks/m^2 —2m—	—4m—	Tree fate
6/1/92	Loblolly	49.3	4AA	0.0	0.0	Alive
6/1/92	Loblolly	40.9	Untreated	38.8	86.1	Dead
6/28/92	Loblolly	45.7	4AA	0.0	0.0	Alive
6/28/92	Loblolly	53.3	Untreated	86.1	150.7	Dead
7/1/92	Longleaf	51.3	4AA	16.1	6.9	Alive
7/1/92	Longleaf	43.2	Untreated	96.9	148.5	Dead

Red-Cockaded Woodpecker Trees

Currently 4-allylanisole is being tested for its operational efficacy as a bark beetle repellent on cavity trees of the endangered red-cockaded woodpecker (*Picoides borealis*). Mortality patterns of these trees suggest that they are susceptible to attack by southern pine beetles (Conner and others 1991, Mitchell and others 1991), and the importance of these trees for the management of this endangered species qualifies them for treatment with 4-allylanisole. A large study, funded by the National Center for Forest Health Management, involving over 30 red-cockaded woodpecker clans is underway on the Vernon Ranger District, Kisatchie National Forest, Louisiana.

CONCLUSIONS

Results presented in this paper indicate that 4-allylanisole may provide the basis of tactics for protection of high-value single trees and possibly stands from southern pine beetle attack. The results of the laboratory and field assays indicate the consistent repellent properties of 4-allylanisole to southern pine beetles (and other scolytid beetles) throughout the year. The fact that clerids are not repelled by 4-allylanisole provides further evidence for use of this semiochemical to protect trees in natural settings with minimal disturbance. Although additional studies are needed, preliminary natural field trials further support the prospect of using 4-allylanisole in single tree and possibly stand protection strategies. Based on the results obtained to date, a patent application entitled "Scolytid Repellent" has been submitted to the U.S. patent office (08/113,709).

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Identification of a Host Compound and Its Practical Applications: 4-allylanisole as a Bark Beetle Repellent[1]

J.L. Hayes; L.L. Ingram, Jr.; B.L. Strom; and L.M. Roton[2]

ABSTRACT

The phenylpropanoid, 4-allylanisole (CAS.# 140-67-0), is a compound produced by many conifers, including loblolly pine (*Pinus taeda* L.), an abundant species in southern pine forests and a preferred host of the southern pine beetle (SPB) *Dendroctonus frontalis* Zimm. Gas chromatography/mass spectrometry analysis of resin collected before and after injection of loblolly pines with a fungicide mixture known to make pines more "attractive" to SPB resulted in the identification of 4-allylanisole as a likely candidate for repellent effects. The repellency of 4-allylanisole to SPB was demonstrated in laboratory behavioral assays and in natural populations by comparing its effects with those of the beetle-produced inhibitory pheromone, verbenone. Responses of other North American scolytids and associates were also determined. The response in the field of *D. frontalis* to its attractant pheromone in funnel traps was significantly reduced by simultaneous release of either 4-allylanisole or verbenone, which did not differ from one another in repellency. Both compounds together did not significantly further reduce trap catch. The response of a major predator, *Thanasimus dubius* F., to the attractant pheromone of *D. frontalis* did not differ with the simultaneous release of either compound. The results of preliminary field tests with 4-allylanisole, in which lightning-struck pines were protected from SPB attack, are presented and discussed in relation to implications for development of a practical tree protection tactic.

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Characterization of Wood Fiber/Urethane Composites[1]

R.E. Ysbrandy, T.G. Rials, M.P. Wolcott, and D.J. Gardner[2]

ABSTRACT

The combination of wood fibers and synthetic polyols may provide an opportunity to develop novel polyurethane composite systems with widely different material properties from rigid to flexible. Little research has addressed the chemistry of this novel composite system. The introduction of wood fibers may dramatically influence the curing chemistry and subsequent formation of the polymeric network. The influence of formulation variables such as wood fiber:synthetic polyol ratio, polyol type, and isocyanate index on network formation and structure was studied by differential scanning calorimetry and solvent extraction. In general, the wood fiber actively contributed to the curing reaction as it affected both the temperature profile and the degree of crosslinking in the urethane composite. In addition, infrared spectroscopy of the extracted material showed substantial differences in the chemical composition of the extracts for composites prepared with different polyols and different isocyanate indexes. These results indicate that wood fiber can be considered a reactive filler for polyurethane materials and may provide a means to further engineer the material properties of this versatile polymeric system.

KEYWORDS: Curing reaction, differential scanning calorimetry, extractables, infrared spectroscopy, network structure, reactive filler.

INTRODUCTION

Wood fibers are composed of three polymers (cellulose, lignin, and hemicellulose) that are rich in hydroxy functionality. As such, the wood fiber can be characterized as a multifunctional polyol that can react to various degrees with isocyanate groups to form durable polyurethane bonds (Galbraith and others 1992, Weaver and others 1992). Not surprisingly, research has addressed the use of polymeric isocyanates as adhesive binders for different types of conventional wood composites (Hawke and others 1992, Milota and Wilson 1992, Steiner and others 1980). Introducing synthetic polyols of various chain lengths and hydroxy-functionality into a wood-isocyanate system may provide a mechanism to enhance some of the composite's properties, such as stiffness and toughness. Alternatively, the addition of wood fiber to synthetic polyol (i.e., the other formulation extreme) may provide opportunities for novel fiber-reinforced plastic composites. The potential versatility of this polyurethane composite system may offer inroads to traditionally nonwood-oriented markets and processes. In this regard, products manufactured by techniques such as reaction injection molding (RIM) that are widely used in urethane processing could become of special interest.

Very little information is available on the chemistry and behavior of blends of wood fiber and synthetic polyols for the formulation of polyurethanes. The addition of wood fiber introduces a wide range of reactive groups due to the presence of various hydroxy functional groups (phenolic, primary, and secondary hydroxyls). As such, the effect on network formation in such fiber/urethane composites remains a subject of interest. The results of preliminary investigations are presented on the effect of formulation variables, including fiber addition, on cure characteristics, network formation, as well as chemical structure of wood fiber/urethane composites.

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$$\text{Isocyanate for wood} = \frac{(50)*(164.7)*(1.25)}{331.95} = 31.0 \text{ g}$$

$$\text{Isocyanate for long chain triol} = \frac{(50)*(164.7)*(1.25)}{1{,}001.8} = 10.3 \text{ g}$$

Thus, the total requirement of MDI amounts to 41.3 parts for 50 parts wood and 50 parts polyol to form the wood fiber/urethane composite system.

Composites were prepared using an isocyanate index of 1.25 at polyol to wood blend ratios of 100:0, 85:15, 75:25, 60:40 using a high speed mixer. For purposes of homogeneity, milled wood fibers passing a 2 mm screen were used after being dried in a forced-air oven at 120 °C. The fiber/urethane composites were prepared by precuring at ambient conditions for several days, followed by a postcure of 6 hours at 150 °C in a forced-air oven.

Composite Characterization

Differential scanning calorimetry of the wood/urethane composites was performed on a Perkin Elmer DSC-7 interfaced to an IBM computer. All samples were analyzed at a scan rate of 10 °C/min over the temperature range from 40 °C to 210 °C. Sealed, large-volume, stainless steel capsules (sample size = 40 to 50 mg) were used to minimize the effects of volatile emissions.

Soxhlet extractions on the cured composites were carried out with acetone on 4 grams of milled wood-urethane samples. Amount of extractables served as an indicator for the influence of wood content on the degree of isocyanate consumption for the various wood-urethane systems and the extent of polymer network formation. In addition, infrared spectra were collected on the extracted material of selected systems using a Nicolet Model 20 DXB fourier transform infrared spectrophotometer. Samples were prepared by solvent deposition of the soluble component on a KBr plate and analyzed (an average of 20 scans were collected) from 4,000 cm^{-1} to 400 cm^{-1} at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry

Results of differential scanning calorimetry for the reaction of each of the synthetic polyols with polymeric MDI are shown in figure 1. Interestingly, although each of the polyols are based on polypropylene oxide and contain secondary hydroxy functionality, the curing reaction was significantly different for each system. The long chain diol (LCD), incapable

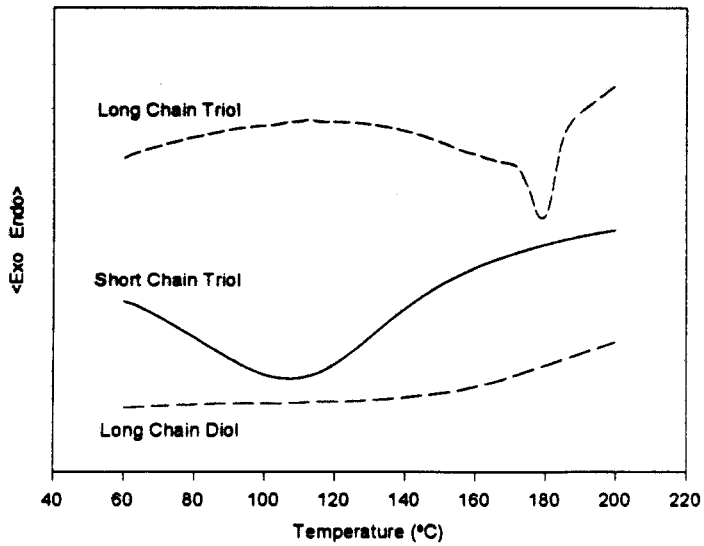

Figure 1. DSC thermograms of different synthetic polyols with MDI.

of crosslinking and thus remaining highly rubbery, showed the least exothermal behavior of the polyols with an estimated maximum for the curing exotherm at approximately 130 °C. In contrast, the short chain triol (SCT) cure was initiated at around 53 °C with a cure maximum for its broad exotherm at 108 °C and showed completion at 175 °C. The large heat of reaction (ca. 150 J/g) observed for this system reflected the high functionality of this polyol and its ability to form a highly branched crosslinked network on account of its low molecular mass. The long chain triol (LCT), being of moderate functionality and advanced molecular mass resulting in a less dense crosslinked network, had the highest temperature for a maximum of curing exotherm of 179 °C and a cure range from 110 °C to 190 °C. It is difficult to assign these differences in behavior to any one factor; however, it may be a consequence of differences in polyol hygroscopicity and contamination by atmospheric moisture. This behavior may also indicate differences in the compatibility and solubility of the polyol in the polymeric isocyanate. This is particularly true for the long chain triol system where the slow initial reaction may enhance the compatibility and result in a relatively sharp peak and rapid curing reaction near 180 °C. In effect, the wide temperature range of the curing reaction for these purely synthetic urethanes is consistent with condensation polymerization processes that typically yield broad molecular weight distributions.

The effect of wood on the curing reaction of the long chain triol system is shown in figure 2. Perhaps the most dramatic difference is a significant shift in both the onset of the curing reaction and the cure maximum temperature with the initial introduction of fiber. This is not altogether surprising given the range of hydroxy functionality available on the lignin-rich surface of the fiber, including more reactive phenolic and primary hydroxy groups. The reduced curing temperature, however, suggests that some reaction may have occured during the composite blending process, and prevents the quantitative analysis of the reaction. Also, at a ratio of 85:15 (P:W) a second peak is observed at 185 °C suggesting the cure of a pure synthetic urethane phase. This synthetic phase cure is still apparent at the 60:40 ratio, although at a much reduced intensity. The maximum of the curing exotherm decreased from 179 °C for the synthetic urethane system to 141 to 148 °C for the polyol-wood urethane blends. Interestingly, with the incorporation of wood into the system, the heat of reaction remained relatively unchanged. These results clearly demonstrate the dramatic effect that wood fiber has on the curing reaction, presumably as a result of increased heterogeneity of the chemical environment as well as the range of hydroxy functionality that is introduced into the system.

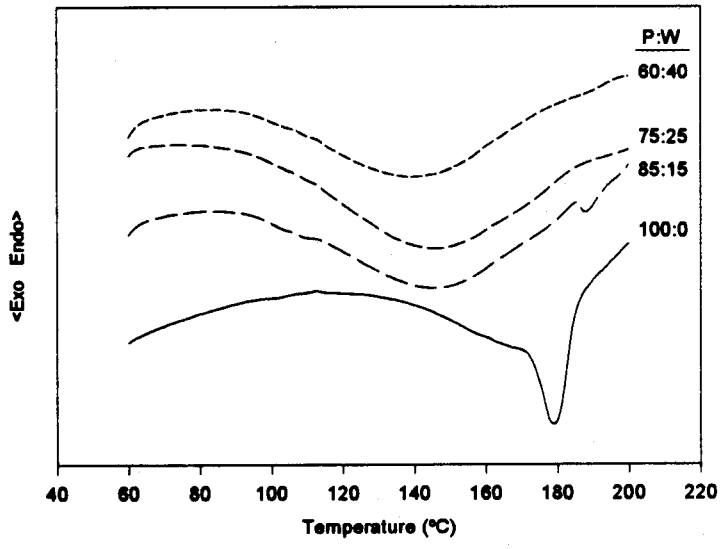

Figure 2. DSC thermograms of different long chain triol to wood ratios (P:W) with MDI.

Table 2 summarizes the DSC results for the wood fiber/urethane composites prepared with the different synthetic polyols. Introduction of wood at different ratios of polyol to wood into the long chain diol urethane system did not affect the cure range and the maximum of curing exotherm, but wood at least doubled the heat of reaction, which was an indication of an increased functionality of such a system leading to somewhat tighter crosslinking. In contrast, the wood/urethane system prepared with the short chain triol showed only slight reductions in the heat of reaction (ΔH_{rxn}) with the addition of wood fiber. This presumably reflected a slight reduction in the hydroxy functionality available for reaction and subsequent network formation. These observations suggest that the wood fiber can serve as a multifunctional polyol component that may dramatically impact the chemistry of the curing reaction.

METHODS

Raw Materials

The wood fiber used in this study was supplied by Temple-Inland Corporation, Diboll, TX. The woodpulp was a high-yield thermomechanical fiber generated by a Sprout-Waldron pressurized disc refiner. All of the synthetic materials were supplied by Miles, Inc., Pittsburgh, PA. The isocyanate crosslinking agent used was Blendur KU 3 5006, a diphenylmethane diisocyanate (MDI) prepolymer. Three synthetic polyols based on polypropylene oxide were selected that varied primarily in molecular weight and average functionality as summarized in table 1.

Table 1. Summary of characteristics for the polyols used in composite formulation.

Polyol ID	Code	Average functionality	Mol. Wt. (g/mol)	Hydroxyl number
Multranol-3600	LCD	2	2,000	53-59
Multranol-3400	LCT	3	3,000	54-58
Multranol-4011	SCT	3	300	530-570
TMP Fiber	TMP	***	***	169*

*Taken from Wang and others, in press.

Composite Formulation and Preparation

The stoichiometry of the urethane resin's reactive components is a particularly critical point of control of the final structure and properties of the composite. The ratio of isocyanate reactive groups (percent NCO-content) to polyol functionality (hydroxyl number) must thus be closely controlled in order to achieve the desired end properties. Isocyanates are typically defined by an isocyanate value, which is calculated here as:

$$\text{Percent NCO} = \frac{4{,}200}{\text{MDI equivalent weight}}$$

where

$$\text{MDI equivalent weight} = \frac{\text{MDI molecular weight}}{\text{functionality}}$$

Similarly, the hydroxyl number assigned to a certain polyol is defined as the amount of potassium hydroxide equivalent(mg) in 1 gram of polyol, where:

$$\text{Hydroxyl number} = \left\{ \frac{(\text{functionality})(\text{KOH molecular weight})}{\text{polyol molecular weight}} \right\} * (100)$$

With knowledge of the NCO-content of the isocyanate and the hydoxyl-number of the polyols, the polyurethane system can now be strictly formulated on an equivalent weight basis. For example:

Long chain triol equivalent weight=56100/56=1001.8
Wood equivalent weight=56100/169=331.9
Isocyanate equivalent weight=4200/25.5=164.7

Typically, an excess isocyanate (referred to as an isoindex) is used to ensure that all hydroxyl-groups react to form urethane linkages, since unreacted polyol tends to produce a sticky/tacky part. The isoindex used throughout this study was 1.25. Consequently, for a parts-by-mass ratio of wood to polyol of 50:50, the following amounts of isocyanate are required:

Table 2. Summary of DSC cure analysis of wood fiber-urethane systems formulated with different polyol:wood ratios and polyol types (isoindex = 1.25).

Polyol type	Polyol: wood	Wood content (Wt. percent)	Cure characteristics (°C)			Est. ΔH_{rxn} (J/g)
			T_{onset}	T_{final}	T_{max}	
LCD	100:0	0.0	70	170	130	13
	85:15	11.8	70	170	133	37
	75:25	19.1	70	170	132	35
	60:40	29.2	70	170	128	52
LCT	100:0	0.0	110	190	179	20
	85:15	11.8	85	195	148	23
	75:25	19.1	80	195	146	30
	60:40	29.2	85	195	141	19
SCT	100:0	0.0	53	175	108	150
	85:15	5.3	48	175	107	140
	75:25	9.4	53	180	107	129
	60:40	16.3	52	175	106	120
	40:60	27.5	45	180	102	123

Composites Extractives Content

Although the previous results indicated that the wood fiber actively participated in the curing reaction, additional information on network formation and structure was obtained by studying the acetone exctractables (sol fraction) of the cured composites. The influence of P:W ratios for the different urethane composites on extractives content is shown in figure 3. No marked change in the level of extractables, ranging between 1 percent and 2.8 percent, was observed for the urethane systems prepared with the short chain triol with increase in wood content. Because of its high functionality, the SCT forms a dense network with very little soluble material As such, the addition of wood did not contribute significantly, or interfere, with network development. This was also reflected by the somewhat reduced heats of reaction (table 2) as the wood content of the composite was increased. However, in the case of the long chain diol and triol, the lower amount of extractables with incorporation of wood reflected that wood could serve partly as a grafting site for the difunctional MDI, to which in all likelihood also synthetic polyol or even longer polyol-MDI chains were linked. Wood was invaluable in the crosslinking of the long chain synthetic polyol systems, which resulted in the formation of a more highly crosslinked network structure.

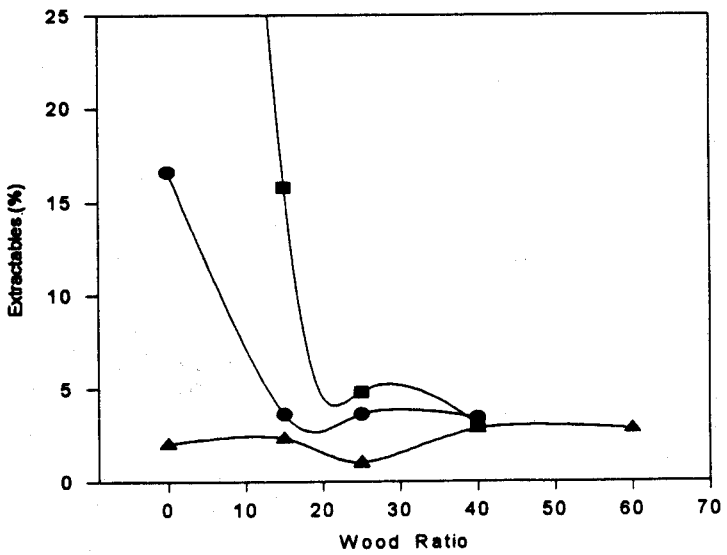

Figure 3. Variation of wood-urethane extractables (percent) for different polyol:wood ratios from materials prepared with the long chain diol (■), long chain triol (●), and short chain triol (◆).

In order to verify such speculation, two blends of synthetic urethanes, each with a ratio of 92:8 of long chain diol and triol with short chain triol, were prepared as to establish how extractable content would be affected. In this case, the amount of short chain triol represented the actual amount of wood present by mass for a polyol to wood ratio of 85:15 in a final wood-

urethane composite, which was 11.8 percent (table 2). Both systems showed a noticeable decrease in extractables with respective figures of 1.2 percent for the long chain triol and 18.3 percent for the long chain diol system, which was indicative of the formation of tighter crosslinked networks with introduction of short chain triol. These results confirmed that wood, already at low addition levels, could function effectively as a crosslink densifier in synthetic urethane systems similar to a short chain triol.

The effect of isocyanate index on extractables for composites prepared with polyol to wood ratios of 75:25 are shown in table 3. Clearly, an isoindex of 0.5 was insufficient for proper network development and resulted in high extractable content of polyol (note: the long chain diol system forms a linear polyurethane and, theoretically, is completely soluble in acetone; i.e. 100 percent extractables). At isoindexes of 1.0 and 1.25, there was a remarkable drop noticeable in the amount of extractables, which further decreased steadily with an increase in isoindex. This indicated that for all practical purposes an excess of 25 percent MDI would be sufficient for crosslinking the polymer network.

Table 3. Wood-urethane extractables (percent) from materials prepared at different isoindexes for the different polyols.

Polyol	Polyol:	Wood content	Extractives content (Wt. percent)				
type	wood	(Wt. percent)	I=0.5	I=1.0	I=1.25	I=1.5	I=2.0
LCD	75:25	19.1	***		4.8		3.4
LCT	75:25	19.1	39.0	4.8	3.6	0.97	0.2
SCT	60:40	16.3	44.8		2.8		3.0

Infrared Analysis

Additional information on network structure can be derived by characterizing the chemical structure of the soluble component that was not successfully incorporated into the network. Infrared spectra of acetone extractables from wood-urethane systems based on long chain triol to wood ratios of 75:25 for isoindexes of 0.5, 1.25, and 2.0 are shown in figure 4. There are three distinct regions in the spectra that are characteristic of this system. First, the absorption peaks found at 3,400 cm^{-1} and 3,300 cm^{-1} are due to free hydroxyl groups and the urethane's amide stretch, respectively. Second, the strong peaks found at about 1,800 cm^{-1} and 1,500 cm^{-1} are due to the carbonyl stretch of the urethane and urea groups (Steiner and others 1980).. Also, the strong absorption band centered at 1,150 cm^{-1} originates from the ether linkage of the polypropylene oxide polyol. A steady growth in intensity of the N-H stretch at 3,300 cm^{-1} with an increase in the isoindex from 0.5, 1.25, to 2.0 was noticeable and was indicative of an increase in urethane formation. This observation was also

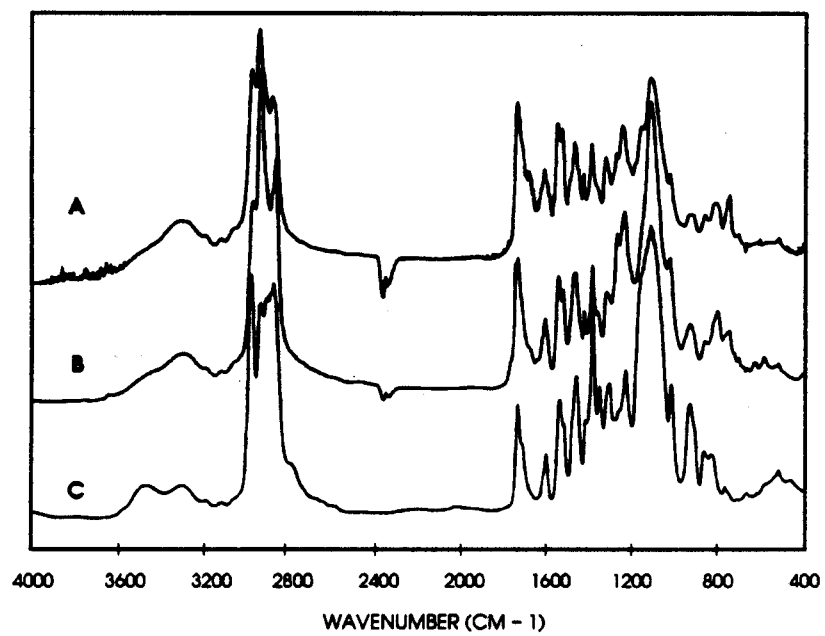

Figure 4. Infrared spectra of wood-urethanes of long chain triol to wood ratios of 75:25 prepared at different isoindexes (I): {A) I = 1.50; (B) I = 1.25; (C) I = 0.50.

supported by the spectral change of the intermolecular hydrogen stretch, related to the hydroxy groups present in the long chain triol, in that the individual peak was clearly visible for an isoindex of 0.5 at 3,600 cm-1, but disappeared at the higher indexes of 1.25 and 2.0. Further evidence of urethane formation was presented by a decline in peak intensity at around 1,150 cm^{-1}, arising from the ether linkage of the polypropylene oxide chain, with increasing isoindex. This chemical evidence, along with the results for extractable content, supported the observation that with an increase in amount of isocyanate more polyol was indeed incorporated into the network structure. Spectral changes in the 1,800 cm^{-1} to 1,500 cm^{-1} were related to amide and carbonyl stretches, which pointed toward different stages of urethane formation and the initial formation of some polyurea at 1,600-1,670 cm^{-1}. The latter was attributed to the presence of atmospheric moisture contamination during the mixing operation.

Figure 5 compares the infrared spectra over the region 2,000 cm^{-1} to 400 cm^{-1} for the extractables of the composites prepared with the three different synthetic polyols (isoindex = 1.25). Interestingly, the spectra are very similar for the LCD and LCT sol fraction. The carbonyl absorption found at 1,800 cm^{-1} and the strong ether band located at about 1,100 cm^{-1} indicate that the extractable material is primarily composed of an oligomeric urethane. In contrast, the SCT system's extractables show an intense peak at about 1,700 cm^{-1} and a dramatic reduction in the absorption of the ether peak. This led to the conclusion that the sol fraction of this composite system was dominated by a polyurea component resulting from the reaction of the isocyanate with moisture. These results highlighted the dramatic impact that moisture can have on the development of crosslinking in this filled polymeric network.

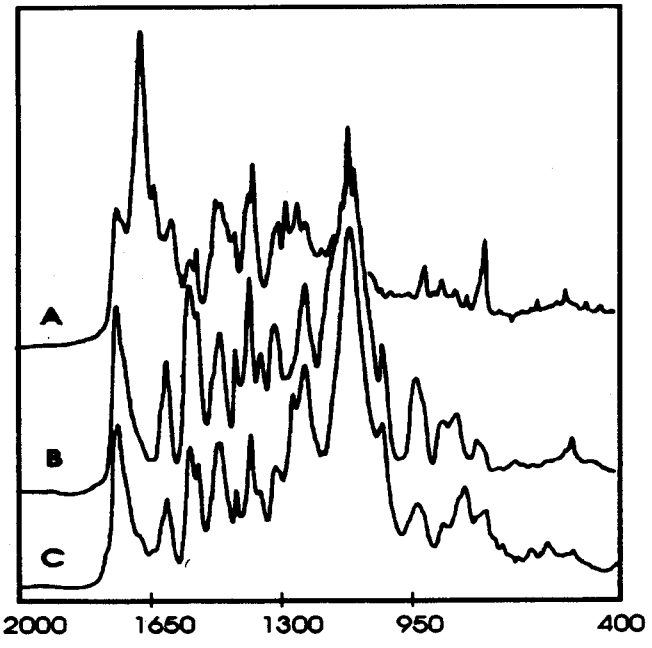

Figure 5. Infrared spectra of wood-urethanes of polyol to wood ratios of 75:25 prepared at isoindex 1.25, using different synthetic polyols: (A) short chain triol (SCT), (B) long chain diol (LCD), (C) long chain triol (LCT).

CONCLUSIONS

These experiments indicated that the wood fiber could actively contribute to network development in polyurethanes and effectively functioned as a reactive filler for these material systems. Differential scanning calorimetry showed that the addition of wood fiber significantly affected the temperature profile of the curing reaction and the heat of reaction, although some dependence on the type of synthetic polyol was found. Determination of the sol fractions of the urethane systems confirmed network formation and defined the degree of crosslinking relative to the type of polyol used. Generally, an increase in functionality of the synthetic polyol resulted in lower sol fractions and a higher degree of crosslinking, as would be expected. Analysis by infrared spectroscopy revealed that chemical composition of the extractives was influenced both by the isocyanate index and type of synthetic polyol. In conclusion, the combination of wood fiber and synthetic polyols

offers considerable potential for the development of novel composite materials that can be engineered to meet a wide range of properties for new products.

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Preparation of Lignin Models and Dehydropolymers by Transition Metal Oxidation of Coniferyl and Sinapyl Alcohols[1]

Lawrence L. Landucci[2]

Treatment of coniferyl and sinapyl alcohol (or their mixtures) with iron or manganese salts yielded low molecular weight products (dimers and trimers) and/or higher molecular weight products (oligimers and polymers). The dimers and trimers proved to be valuable lignin models for C9 units in lignin because they could be completely characterized by NMR spectroscopy. The polymers proved to be similar to conventionally prepared dehydropolymers (DHP's). Reaction conditions could be altered to favor low or high molecular weight products. Investigation of different oxidation conditions is expected to provide clues leading toward the preparation of DHP's that are much more representative of native lignin than DHP's prepared by conventional enzymic techniques. The relative contents of beta-0-4, beta-5 (coumaran), and beta-beta (resinol) units in the polymer could be altered by changes in experimental parameters such as the metal ion, the rate of addition, and pH.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Dendrochemistry of Loblolly Pine and Cypress Cores: Initial Results for Forest Health Monitoring[1]

C.E. Thomas, S.D. Latimer, and O.P. Mills[2]

ABSTRACT

Chemical analysis of tree cores provides the possibility of a retrospective view of environmental pollution or condition. Analysis of trends or changes in levels of inorganic chemicals in rings over time could yield information pertaining to both elemental levels in the environment and changes in tree growth rates. Costs of analyses for multiple elements in multiple years can quickly escalate. Development of methods that aggregate rings, preserve the sample for future analyses, and characterize both nutrient and possible pollutant elements will provide an important baseline for monitoring changes in the chemical environment of important tree species in the Southern United States. Achieving the current goals of dendrochemistry is not simple. Among problems the new field must address are the development of methods for recognizing and correcting for natural physiological translocation of chemicals across rings. Translocation may confound measurements of elements absorbed in the period of interest. Initial x-ray spectrochemical methods and analyses of core samples are described. Preliminary analyses of (1) aluminum-calcium ratios from 50 year periods are depicted for cypress cores, and (2) five-year increment means and sampling errors for concentrations of aluminum, potassium, and magnesium in loblolly pine cores are presented.

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Human Drugs from Trees: Effect of Isoprenoid Substitution on the Antimicrobial Activity of Aromatic Secondary Metabolites[1]

T.P. Schultz, W.B. Harms, T.H. Fisher[2]

ABSTRACT

The search for higher plant extractives (secondary metabolites) to treat human pathogens is expensive and time consuming. Only one out of a million compounds tested is used commercially. Determination of structure-activity relationships (SAR) to identify compounds that have pharmacological potential would prove beneficial. The objective of this study was to test the hypothesis that isoprenoid-substituted secondary metabolites (prenylated or gernaylated) may have greater bioactivity than similar nonisoprenoid-substituted aromatic extractives.

Eleven compounds were isolated from trees of the family Moraceae. Isoprenoid-substituted and similar but nonisoprenoid-substituted stilbenes, xanthones, and flavonoid were isolated. Structures were determined by 1- and 2-D nuclear magnetic resonance (NMR) techniques, mass spectrometry (MS), and literature comparison.

In the case of two flavonoid, designated F2I and F3I, complete structure elucidation could not be made. The hydrophobicity of F2I and F3I was similar to that of the other isoprenoid-substituted compounds, however. The extractives were screened for antimicrobial activity against a standard set of human pathogenic micro-organisms using the agar well diffusion assay. Compounds that showed good activity against a particular micro-organism were further studied by measuring the minimum inhibitory concentration (MIC).

The geranylated stilbene chlorophorin showed good fungal activity against an AIDS opportunistic yeast. The geranylated flavonoid Rubraflavone A and the hydrophobic flavonoid F2 I and F3 I were all active against four of the five bacteria examined. The nonisoprenoid-substituted flavonoids and stilbene showed minimal or no activity. All xanthones, both isoprenoid- and nonisoprenoid-substituted, showed minimal or no activity. The results suggest that isoprenoid-substituted stilbenes and flavonoids appear to have greater bioactivity against selected human pathogenic microorganisms than similar nonisoprenoid-substituted derivatives.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Research and the National Environmental Policy Act: What Every Chemical Scientist Should Know[1]

Tim Mersmann and Jerry Ingersoll[2]

ABSTRACT

The process for complying with the National Environmental Policy Act (NEPA) has grown in scope and application in the 24 years since the law's passage. Full integration of the NEPA process into planning of Federal research is required and inevitable. A brief introduction to NEPA, its goals and procedures, is provided. Examples are shared of NEPA's application to research from our experience with the Ecosystem Management Research Program on the Ouachita and Ozark National Forests. This experience has shown NEPA's application to research to be feasible and valuable, as well as legally required. Scientists are urged to take a proactive approach to NEPA compliance.

Keywords: Ecosystem management, environmental analysis, herbicides, NEPA, Ouachita National Forest, science.

INTRODUCTION

The National Environmental Policy Act, or NEPA, has become a way of life for those in Federal government charged with making decisions about the Nation's natural resources. From the perspective of National Forest managers, it is a first thought whenever a decision must be made, be it policy, program, or project. It is an administrative approach that affects almost everything we do. It is the way forest managers do business.

The comprehensive process that today represents NEPA compliance has grown and evolved in the 24 years since the legislation's passage (Culhane 1990).[3] This growth reflects societal trends. In the presence of abundant information, citizens are increasingly aware of environmental effects caused by increasing human populations and resource use. They desire and feel competent to be intimately involved, individually or through powerful interest groups, in much of their government's decisionmaking.

Researchers and research are not immune to these trends. Increased public scrutiny of research can be expected to continue as researchers work closer with policymakers and managers to develop solutions to environmental issues. Research methods, as well as results, will receive increased scrutiny, especially as experiments expand in scale to capture whole-system responses.

Chemical scientists should be particularly prepared for public scrutiny and challenge. Many chemicals being studied have potential for environmental effects. Many citizens are passionately concerned about toxic effects of synthetic chemicals in their environment and are willing to take legal action to reduce their use (O'Brien 1990).

As a legal requirement, NEPA provides the framework through which citizens will express their concerns and become more involved in research planning. National Environmental Policy Act is the forum for researchers and citizens to establish a necessary and continuing dialogue. All researchers who work for, or with, or receive funding from, the Federal government should be familiar, if not proficient, with the requirements of this Act.

Our objectives here are (1) to introduce NEPA to those not well acquainted with it, and (2) to stress the importance of meeting NEPA's requirements when planning Federal research. We will draw on our experience in applying NEPA to Ecosystem Management Research on the Ouachita and Ozark National Forests.

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[1] Paper presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

[2] Forester, USDA Forest Service, Ouachita National Forest, Hot Springs, AR 71902; Forest planner, USDA Forest Service, Ouachita National Forest, Hot Springs, AR 71902.

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AN INTRODUCTION TO NEPA

What is NEPA?

NEPA is the National Environmental Policy Act of 1969, signed on January 1, 1970. It arose from the same growing environmental consciousness that resulted in the first Earth Day 4 1/2 months later. The declared purpose of the Act is to "encourage productive and enjoyable harmony between man and his environment,...prevent or eliminate damage to the environment... [and] enrich the understanding of the ecological systems and natural resources important to the Nation" (Sec. 2 of the Act).

The National Environmental Policy Act's most important provision (Sec. 102(2)(C)) for accomplishing these goals is to direct "all agencies of the Federal Government" to include in every proposal "for legislation and other major Federal actions significantly affecting the quality of the human environment" a detailed statement that includes:

"(i) The environmental impact of the proposed action,
(ii) Any adverse environmental effects which cannot be avoided should the proposal be implemented, [and]
(iii) Alternatives to the proposed action... "

This "detailed statement" is the well-known Environmental Impact Statement. Notably, the Act also requires all agencies to "utilize a systematic, interdisciplinary approach" in planning and decisionmaking when impacts to the environment may occur (Sec 102.(2)(A)).

National Environmental Policy Act also created the Council on Environmental Quality (CEQ) to advise the President on environmental issues. Using results of several years of judicial review, CEQ wrote broad regulations (40 CFR 1500-1508) for implementing NEPA. Agencies were directed to develop more specific, supplemental policy to guide NEPA implementation within their jurisdictions. Therefore, in most cases, NEPA requirements are defined by the Act itself, CEQ regulations, and agency policy. In addition, evolution through judicial review continues.

Through regulation, policymaking, and case law, the basic mandate from the Act itself has become a comprehensive administrative scheme with a variety of objectives (Culhane 1990). Goals inherent in this scheme include improving rationality of agency decisionmaking, encouraging application of state-of-the-art scientific understanding in agency decisionmaking, checking agencies' administrative authority, improving citizen access to agency decisionmaking, and building public support for agency decisions. Whether any or all of these goals are met depends on the attitudes and knowledge of both agency personnel and involved citizens, as well as the nature of the proposed project (Funk 1990).

In a nutshell, the Supreme Court has ruled this legislation has two primary aims: that Federal decisionmakers consider the environmental consequences of their actions, and that they inform citizens of these consequences.[3]

Who is Subject to NEPA?

National Environmental Policy Act's coverage is broad. The environmental analysis requirements of NEPA are directed at "all agencies of the Federal government" (Sec. 102.(2)). The whole of regulations and case law make it clear that all Federal actions are to be examined within a NEPA context. Federal actions, as defined by regulation, are those "which are potentially subject to Federal control and responsibility," and include "new and continuing activities, including projects and programs entirely or partly financed, assisted, conducted, regulated, or approved by federal agencies (40 CFR 1508.18)."

Courts have defined actions subject to NEPA as those enabled by Federal funding or under Federal control (*Almond Hill School* vs. *United States Department of Agriculture*, 768 F.2d 1030; *Enos* vs. *Marsh*, 769 F.2d 1363), although they recognize that Federal participation in some projects is one of degree and clear standards are not defined (*Almond Hill School* vs. *United States Department of Agriculture*, 768 F.2d 1030). One such gray area occurs when Federal agencies cooperate with State or private organizations, a common arrangement for research projects. The concept of "connected actions" can be useful in assessing whether NEPA applies in these cases[4].

Connected actions are those that "(i) automatically trigger other actions..., (ii) cannot or will not proceed unless other actions are taken previously or simultaneously, [and] (iii) are interdependent parts of a larger action and depend on the larger action for their justification" (40 CFR 1508.25). In most cases, NEPA compliance would be necessary for actions connected to Federally funded or controlled actions.

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[4] Carbone, J. 1993. Personal communication with the author. On file with: USDA Forest Service, Ouachita National Forest, Hot Springs, AR 71902.

For example, if a scientist uses Federal funds to research the efficacy of herbicide, **applied as part of normal operations** by an industry cooperator on industry land, that herbicide application would not be subject to NEPA (the study itself would, but could probably be categorically excluded--see later discussion). On the other hand, industry application of herbicides on industry land **specifically for the Federally funded research** would be subject to NEPA, because the herbicide application would be connected to the Federal action; i.e., it would not have proceeded without the study, and is an interdependent part of the study.

How Much Public Involvement is Required?

The mandate for involving the public in the NEPA process was defined broadly in CEQ regulations (40 CFR 1506.6). Agencies are directed to:

"(a) Make diligent efforts to involve the public in preparing and implementing their NEPA procedures.
(b) Provide public notice of NEPA-related hearings, public meetings, and the availability of environmental documents so as to inform those persons and agencies who may be interested or affected....
(c) Hold or sponsor public hearings or public meetings whenever appropriate....
(d) Solicit appropriate information from the public.
(e) Explain in its procedures where interested persons can get information...on environmental impact statements and other elements of the NEPA process.
(f) Make environmental impact statement, the comments received, and any underlying documents available to the public ....

It is important to involve the public as early as possible when planning an action. Council on Environmental Quality directs that "[t]here shall be an early and open process for determining the scope of issues to be addressed and for identifying the significant issues related to the proposed action" (40 CFR 1501.7). This process, termed "scoping," is aimed at surfacing and hopefully resolving issues early. Early responsiveness to issues can greatly facilitate a project's implementation.

Because of these broad requirements for public involvement, truly successful use of the NEPA process requires a special form of social facilitation and awareness (Drtina and Lundstedt 1982). It usually requires doing more than the minimum required; it often can be approached productively as an "entrepreneurial" opportunity (Shannon 1991).

Of course, public involvement is not free; it can require a great deal of time, effort, and patience. Rewards, however, can be increased mutual understanding leading to improved relevance of research and broad support for research programs. Given the changing role of forest scientists, there may be no other rational choice than to invest in public involvement: short-cuts in public involvement often prove to be anything but short.

How Much Analysis and Documentation is Required?

Three classes of NEPA documentation are defined, each associated with a different level of analysis. They are: Environmental Impact Statements (EIS), Environmental Assessments (EA), and Categorical Exclusions (CE). One of the most fundamental decisions an agency must make in complying with NEPA is determining which class of documentation is appropriate for the action in question.[3] This decision is based on the potential of the action to affect the human environment--greater potential impacts generally require greater analysis and higher classes of documentation.

Environmental Impact Statement

An EIS is required for those Federal actions that may "significantly" affect the quality of the human environment. (Criteria for "significance" are given by CEQ at 40 CFR 1508.27.) It serves to document and disclose to the public those significant impacts that an agency readily admits may occur if an action is taken. Producing an EIS requires publishing a notice of intent in the Federal Register and release of a draft EIS for public review and comment. Although CEQ envisioned EIS's as usually being less than 150 pages (40 CFR 1502.7), in practice, many are much larger with extended appendice.[3]

Council on Environemntal Quality regulations provide a format for writing an EIS (40 CFR 1502.10). They suggest an EIS be composed of the following elements:

"(a) Cover sheet,
(b) Summary,
(c) Table of contents
(d) Purpose of and need for action,
(e) Alternatives including proposed action....
(f) Affected environment,
(g) Environmental consequences....
(h) List of preparers,
(i) List of Agencies, organizations, and persons to whom copies of the statement are sent,
(j) Index
(k) Appendices (if any)."

Some flexibility in format is provided however. The Council on Environmental Quality further prescribes the process for preparing EIS's: "Environmental impact statements shall be prepared using an inter-disciplinary approach which will insure the integrated use of the natural and social sciences and the environmental design arts..." (40 CFR 1502.6).

Most research programs and projects will not require an EIS, but some have. Forest Service examples include EIS's for a project on the Starkey Experimental Forest in the Pacific Northwest, which required 40 miles of fencing to exclude elk and deer, and one for a programmatic plan for the Luquillo Experimental Forest in Puerto Rico[5].

Environmental Assessment

Although not originally thus intended by CEQ, the EA has become the most commonly used NEPA document. Its role is to document that the agency has considered an action's potential for causing significant impacts. It is usually followed with a Finding of No Significant Impact, or FONSI, which, based on the EA, concludes significant impacts will not occur and, therefore, an EIS is not needed (if the EA cannot support a FONSI, then an EIS must be done).

Whereas an EIS admits significant impacts may occur, an EA and FONSI state that none will occur, shouldering a much greater burden of proof in the view of the courts. Meeting the court's expectations for rigorous disclosure of an agency's consideration of potential impacts has led to "legal armor plating" of EA's, ballooning them into documents far in excess of the 10 to 15 pages originally envisioned by CEQ.[3] Nevertheless, EA's still usually require less time and expense to produce than EIS's.

Environmental Assessments generally follow the format suggested by CEQ for EIS's. Environmental Assessments will be needed for many research projects involving treatments that modify the environment.

Categorical Exclusions

Agencies, on direction from CEQ (40 CFR 1500.5, 1508.4), have identified categories of actions exempt from requirements to prepare EIS's or EA's. These are categories of actions that the agency knows through environmental analysis and experience will not have a significant effect on the environment, absent "extraordinary circumstances. " To use a categorical exclusion (CE), one must be able to fit the action in question clearly into one of the categories defined by the agency with control of the action.

The Secretary of Agriculture has identified such categories (7 CFR 1b.3). They include: policy development related to routine activities such as personnel, organizational changes, and administration; activities dealing solely with program funding; education and information programs; law enforcement; consultation with other agencies and organizations; and trade representation and market development. Notably, the Secretary also includes "[i]nventories, **research activities, and studies, such as resource inventories and routine data collection when such activities are clearly limited in context and intensity...."**

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[5] Granskog, J. 1993. Personal communication with the author. On file with: USDA Forest Service, Ouachita National Forest, Hot Springs, AR 71902.

The Forest Service also has identified categories for exclusion (FSH 1909.15), dividing them into two groups, the first of which (31.1b) requires no documentation. Actions in the first group include: short-term road or area closures; administrative actions; repair and maintenance of buildings, roads, trails, and boundaries; land acquisition, sale, or exchange; and minor short-term special uses of National Forest lands. This group specifically includes use of registered herbicides and pesticides to control pests at administrative and recreation sites and facilities.

The second group of categories (31.2) requires a Decision Memo and a project file. A Decision Memo is a Forest Service document that briefly describes the action to be taken and the reasons for its categorical exclusion from NEPA analysis, including a determination that no extraordinary circumstances exist. A project file contains, at a minimum, the Decision Memo, a list of those contacted when preparing for the action, a list of those informed of the decision, and copies of any notice used to inform interested parties. Actions in this group include trail construction, minor special uses on National Forest lands requiring less than five contiguous acres, small timber harvests (less than 250,000 board feet green, or 1,000,000 board foot salvage), regeneration and site preparation that does not result in vegetation type conversion or involve herbicides, and timber stand or wildlife habitat improvement that does not involve herbicides, such as prescribed burning. Note that **herbicide use is explicitly not included in these categories for exemption.**

Although CE's are an attractively simple means of meeting NEPA requirements, they should not be used lightly or inappropriately. The Department of Agriculture recently lost a case involving categorical exclusion of a research project.[6] In *Fund for Animals* vs. *Espy*, the plaintiff challenged the Animal and Plant Health Inspection Service's (APHIS) funding of research by Texas A&M University on brucellosis in bison near Yellowstone National Park. The judge rejected USDA's contention that the activity was categorically excluded, labeling it a "post hoc rationalization. He noted that "the record reveals no contemporaneous consideration by the administrative decisionmaker of the applicability of a categorical exclusion." The judge further found it unlikely that the action could fit within the defined categories in any event. This ruling reinforces the need for virtually all Federal actions to be examined for NEPA compliance, and for that examination to be timely, rigorous, and well documented.

What Happens if NEPA's Requirements Aren't Met?

The National Environmental Policy Act carries no criminal penalties; no one will go to jail or pay a fine for not writing an EA. But the penalty can be severe if you are trying to implement a project: delay, expensive rework, and possible litigation, and sometimes, where a window of opportunity is critical, project cancellation.

The Council on Environmental Quality regulations state that "the President, the federal agencies, and the courts share responsibility for enforcing the Act. . . " (40 CFR 1500.1(a)). To meet their responsibilities, many agencies have set up systems of internal review to check for NEPA compliance. In addition to internal reviews, the Forest Service has an administrative appeal process, whereby interested citizens can seek review of decisions by a higher official. A large part of this review is a check on NEPA compliance. Failure to meet full compliance results in reworking of the analysis and project delay.

Courts, of course, can provide a final review where compliance is challenged through litigation. While the goals of NEPA are substantive (e.g., "... encourage productive and enjoyable harmony..."), National Environmental Policy Acts mandate is procedural. Courts generally have not examined the "correctness" of the decision reached, only whether the correct process was used in reaching the decision.[7] Not using the correct procedure can result in an injunction, rework, and delay.

NEPA AND RESEARCH

A Focus on Impacts

Since its passage, NEPA has been applied unevenly across the Federal government. This is due, at least in part, to its evolving interpretation and to each agency having its own implementing policies. It is also because some agencies have not

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[6] Gippert, M.J. 1993. Unpublished memorandum for Mark Reimers, Deputy Chief, Programs and Legislation, and Jerry Sesco, Deputy Chief, Research. APHIS loses Categorical Exclusion case: *Fund for Animals vs. Espy*, Civil No. 93-0360-LFO (D. Mont. February 24, 1993). Dated 4 March 1993. On file with: Office of the General Counsel, Suite 600, 1371 Peachtree St., N.E., Atlanta, GA 30367.

[7] Cohen, W.M. 1991. Practical considerations in litigating cases under the National Environmental Policy Act. Unpublished mimeo from a course on the National Environmental Policy Act, School of the Environment, Duke University, November 4-8, 1991. On file with: Office of the General Counsel, Suite 600, 1371 Peachtree St., N.E., Atlanta, GA 30367.

been sufficiently challenged legally to spur full compliance. Many government employees continue to be unaware that NEPA applies to them. Their awakening, like that of APHIS in *Fund for Animals* vs. *Espy*, could occur in court.

Federal researchers have often been among those unaware of NEPA's relevance. Some researchers have thought that research, as a broad class of actions, was exempt or categorically excluded from NEPA compliance, possibly believing such exemption is justified by the higher purpose of research--that of generating knowledge. Or they have assumed that NEPA is not necessary because research projects are usually small in scope, and have usually not been controversial or challenged by public. Statute, regulation, and case law do not support these notions.

Research is not specifically mentioned in the Act or CEQ regulations, indicating no special-case intent for research by the law's framers. The focus is on environmental impacts controlled by the Federal government that might result from **any kind of action.**

Research is mentioned at the level of agency policy. As indicated earlier, research is mentioned in one of the Secretary of Agriculture's categories for exclusion (7 CFR 1b.3). When viewed in context with the other categories listed by the Secretary, **this exclusion for research appears to cover only those research projects involving primarily measurement, with little or no treatments that alter the environment.** This exclusion, as CEQ intended (40 CFR 1508.4), is based on the minimal impacts expected, not on the purpose of the action.

Final Forest Service categories for exclusion do not specifically mention research, but an early draft of this policy did. Originally, "research-related activities were placed in a category for exclusion based on their research purpose, rather than based upon their possible effect on the environment" (USDA FS 1992). After review of these draft guidelines, it was decided that "[c]ategories of actions should address the potential physical and biological effects of proposed actions rather than their intended purpose" (USDA FS 1992). Therefore, this category was eliminated. Again, it is the level of impact that keys NEPA compliance.

NEPA and Research in Practice

A few examples from the Ecosystem Management Research Program on the Ouachita and Ozark National Forests illustrate NEPA's application to research.

The Ecosystem Management Research Program is a cooperative venture among the Ouachita and Ozark National Forests, the USDA Forest Service, Southern Forest Experiment Station, and area universities. The program's original charge was to examine ecosystem response, at the stand scale, to reproduction cutting alternatives to clearcutting and planting. Over 60 scientists are involved in research on topics including vegetation diversity, tree regeneration, tree growth and yield, soil nutrients, erosion and compaction, visual quality, insect diversity, bird and small mammal populations, herbicide movement, and logging economics.

In the first phase of this project, examples of alternative silviculture were implemented as quickly as possible to serve as demonstrations and to allow researchers to pilot test research methods. To avoid delay, timber sales for these demonstration stands were chosen (and slightly modified in some cases) from sales already through the NEPA process as part of ongoing National Forest management. Little additional NEPA work was needed.

To meet researchers' needs for a scientifically rigorous study, the next phase of the program had to start from scratch. The research team proposed an operational-scale, replicated study design, including random stand selection and assignment of treatments. Fifty-two stands, representing 4 replicates of 13 treatments, were selected and proposed for treatment. Timber sales for this study covered over 2,000 acres on two National Forests. Sales resulted in the harvest of 17.2 million board feet of timber, sold for over $2.2 million.

This project obviously required more than a Categorical Exclusion. We wrote an Environmental Assessment. We were able to arrive at a Finding of No Significant Impact (and thereby did not have to do an EIS) because, despite the project's large size, the activities were dispersed and represented only a small portion of the overall management program for the Forests. Impacts from the overall Forest management program had been previously disclosed in EIS's associated with Forest Plans. By "tiering," or incorporating by reference, these broader analyses we were able to save effort on the project analysis. Tiering is encouraged by CEQ (40 CFR 1508.28). The EA was approximately 100 pages plus appendices and included information gathered from all the districts involved. Public involvement was extensive, with special effort directed at involving those who frequently appeal Forest Service decisions.

All participants learned a great deal through the NEPA process (Mersmann and others 1994). Researchers generally found interested citizens to be reasonable and intelligent, and research plans have benefitted as a result of their suggestions. We found that it is possible to find environmentally preferable alternatives that still meet research needs. In fact, the alternative

chosen for implementation was not the original proposed action, but was one modified in response to citizen concern about some environmental impacts. Through this process, by listening and responding, we have created a better project and achieved widespread public support. The process was not quick or easy, however; it required approximately 6 months of very intensive work.

Two side studies have required additional NEPA compliance and are of particular interest to chemical scientists. In one case, a cooperating scientist proposed to sample canopy arthropods on National Forest land using a pyrethrum insecticide fog. A maximum of 24 trees was to be sampled.

Although the proposal was small in scope, we did not feel comfortable claiming an exclusion. The project involved a pesticide, automatically making it an environmental concern for many citizens, and this was a pesticide with which we had little experience. As part of our research program, this project would likely receive a lot of attention. Again, we chose to write an EA.

Whereas herbicide applications on the Forest have been analyzed programmatically, allowing tiering to an EIS (USDA FS 1990), we had no such analysis for this pesticide. Therefore, to adequately assess impacts from this proposal, a review of literature on animal and human toxicity was required and conducted in this case by the cooperating scientist. Most research involving application of uncommon synthetic chemicals to the environment will require this type of indepth literature review because of the lack of programmatic analyses. Due to the low toxicity and persistence of this pesticide and the small scope of the project, we were able to arrive at a FONSI. We worked intensively with several key citizens and were able to meet NEPA compliance and gain citizen consent within a few months. This EA was 18 pages long.

Actions involving synthetic chemicals often require a more careful application of the NEPA process due to public concern about toxic effects on humans and wildlife. Impacts to health are typically analyzed using risk assessments, as was crudely done in this case. Chemical scientists conducting these analyses should be aware, however, that many in the environmental community are critical of the quantitative risk assessment approach (O'Brien 1990). Other recurring public concerns center on the unknown composition and effect of inerts in formulated pesticide products, and the potential for synergism of synthetic chemicals in the environment. Because of their nature, these concerns can be difficult to satisfy through NEPA analysis.

A third example illustrates the difficulty sometimes present in deciding how much analysis is needed. Researchers proposed to apply common forestry herbicides, mimicking a site preparation treatment, on two 0.25-acre plots specially instrumented to measure subsurface herbicide movement. The project was to occur on an experimental forest within the National Forest.

Can this project be categorically excluded? Some argued that it could, under the Secretary's category mentioned earlier, because it was a "research activity...clearly limited in context and intensity...." Others argued that it couldn't, because Forest Service categories explicitly stated that site preparation involving herbicide could not be excluded, and the Secretary's category implied, through context, categorical exclusion of research that involved measurement only.

As leaders of the process, we chose the second position and wrote a brief EA (11 pages) including limited public involvement. If an error were to be made, we chose to err on the side of overcompliance. However, we do recognize the apparent inconsistency that allows categorical exclusion of herbicide use in recreation areas, but not small-scale use within the forest as was proposed here. Time and costs for this EA were minimal (3 weeks); however, taking this route did open the project to delay from a citizen appeal. Fortunately, none were filed.

CONCLUSIONS

NEPA's full integration into the planning of Federal research is legally required and inevitable. The same social forces that have expanded NEPA's role across the Federal government will bring it into the research arena. Our experience with the Ecosystem Management Research Program has shown NEPA's application to research to be feasible, profitable, and sometimes even pleasant.

Many view and fear the NEPA process as a frustrating political battlefield, armed by entrenched obstructionists. It can be that. But researchers have a great opportunity to nurture a different kind of NEPA experience. In general, the public views researchers positively, as objective experts searching for the truth. This stance gives researchers an opportunity to use NEPA proactively as a forum for initiating positive and educational dialog with interested citizens. This kind of dialog can only help increase the social relevance of research. It also can create a network of relationships that may provide support for research programs.

To capitalize on this opportunity, researchers must become students of NEPA. National Environmental Policy Act training opportunities designed specifically for Forest Service scientists are currently being planned[8]. However, full success with the NEPA process will require forest scientists to go beyond becoming proficient NEPA technicians; they must be willing to become active participants in the social dialog that is stimulated through the NEPA process. Society and the forestry profession need to hear the voices of scientists now more than ever.

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[8] Granskog, J. 1993. Personal communication with the author. On file with: U.S. Department of Agriculture, Forest Service, Ouachita National Forest, Hot Springs, AR 71902.

Fumigant Application to Reduce Inoculum of Laminated Root Rot: from Stumps to Living Trees[1]

Walter G. Thies[2]

ABSTRACT

Laminated root rot caused by *Phellinus weirii* (Murr.) Gilb. is the most damaging root disease of conifer forests in the northwestern United States and Canada. The pathogen survives in dead trees and stumps and later spreads through root contacts to and among trees in the succeeding stand. This paper reviews four field studies used to develop application techniques and establish dosages for the use of chloropicrin, methylisothiocyanate (MITC), and Vorlex in reducing inoculum of *P. weirii* in stumps or in living Douglas-fir without killing the trees.

Keywords: Chloropicrin, methylisothiocyanate, *Phellinus weirii*, Vorlex.

INTRODUCTION

Phellinus weirii (Murr.)Gilb., which causes laminated root rot, is widespread throughout the range of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco. Douglas-fir is the most economically important host, but nearly all conifers appear to be susceptible to some degree. The disease reduces forest productivity annually by about 4.4 million m^3 in western North America (Childs and Shea 1967, Nelson and others 1981). It has been estimated that laminated root rot occurs on 10 percent of the commercial forest land in Washington and Oregon and causes a 50- to 70-percent reduction in wood volume on the areas affected.[3]

When infected trees die, the pathogen continues to live saprophytically in infested butts and large roots for as long as 50 years (Childs 1963; Hansen 1976, 1979) and in relatively small diameter roots for at least 8 years (Thies and Hansen 1985, Wallis and Reynolds 1965). Infection in a young stand begins when roots of young trees contact residual infested stumps and roots from the preceding stand. The infection spreads between living trees through root contacts. As the fungus advances along a tree's roots, the roots distal to the fungus are killed, denying the tree water and nutrients necessary for growth. Biology, distribution, and impact of the disease, relative susceptibility of host species, and options for management have been reviewed (Hadfield 1985, Nelson and Sturrock 1993, Thies 1984, Wallis 1976).

Immediate succession by Douglas-fir or other highly susceptible species on a site infested with *P. weirii* often results in more disease and heavier losses in the new stand (Wallis and Reynolds 1965). Strategies to manage the disease have focused on changing species composition or reducing inoculum before a susceptible stand is regenerated. Physical removal of inoculum from the soil may effectively reduce or prevent future infections on the site (Thies and others 1994); however, stump removal may not be feasible or may be edaphically and visually objectionable on many sites.

During examination of excavated *P. weirii*-infested stumps, extensive stain at the stump top indicated either advanced decay or hollowing at the root collar contiguous with advanced decay and stained wood near the center of major roots. The presence of decay columns forming "ducts" to infected portions of the root system suggested stump fumigation as a means of eradicating *P. weirii* from infested stumps. Fumigant application to soil as well as directly to wood to destroy particular fungi has been reviewed previously (Filip 1976, Thies and Nelson 1982).

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[1] Paper presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

[2] USDA Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, Corvallis, OR 97331.

[3] Goheen, Don. 1993. Personal communication with the author. On file with: U.S. Department of Agriculture, Forest Service, Region 6, 333 SW 1st Avenue, Portland, OR 97208.

FUMIGATION STUDIES

The remainder of this paper deals with four studies (three concluded and one ongoing) involving fumigants and attempts to reduce *P. weirii* inoculum in Douglas-fir trees or stumps. All studies were conducted in the coast range of Oregon or Washington, in naturally regenerated, naturally infested mature stands of predominantly Douglas-fir.

Study 1--(Thies and Nelson 1982)

Forty infected stumps were selected and stratified into eight groups of five stumps each, based on similarities in stump top diameter and amount and stage of decay. The five treatments (chloropicrin, allyl alcohol, Vapam, Vorlex, and an untreated control) were assigned at random to the five stumps in each stratum.

All fumigants were applied at the rate of 1,000 mL/stump. A minimum of eight holes was drilled into each stump top with a 2.5-cm diameter drill. After fumigant application, each hole was plugged with a hemlock dowel dipped in asphalt roofing compound. Each stump top was then coated with roofing compound.

Treatments were applied in October 1978 and stumps sampled in October 1979. Stumps were bulldozed from the soil so as to retain the maximum number of major roots. Roots were cut at 30-cm intervals from the root collar, perpendicular to the root axis. When *P. weirii* stain or advanced decay was found, a sample disk was removed. Root disks were evaluated for viable *P. weirii* by attempted isolation of the fungus from stained or decayed wood on freshly split faces of each disk. Additionally, sample disks were incubated at ambient temperatures (5 to 20 °C) for 4 to 8 weeks to allow the fungus to express itself by growing on the disk surface. A disk was recorded as having viable *P. weirii* if the fungus was found either in the associated culture tubes or on the incubated disk. If *P. weirii* was not recovered from a symptomatic disk, the treatment was assumed to have killed the fungus. Treatment success was judged by the percentage reduction of the stained and decayed portion of the root system with living *P. weirii*.

The first study determined that all of the fumigants tested could greatly reduce or eradicate *P. weirii* from infected root systems and that the effective dose was related to stump size. Eradication appeared nearly complete for stumps with a diameter of 48 cm or less.

Study 2--(Thies and Nelson 1987a)

Building on study 1, this study attempted to refine the dosage and application techniques needed to eradicate *P. weirii* from infected stumps, using chloropicrin, Vorlex, and methylisothiocyanate (MITC). Chloropicrin and Vorlex were selected because they were effective against *P. weirii* in wood, they were widely used in agriculture, and they were easy to detect at low concentrations. MITC is the stated active ingredient in Vorlex and, because it is a solid, offers some advantages in handling and weight.

Sixty stumps from recently harvested live trees were stratified into 5 groups of 12 stumps each based on stump diameter and stage of *P. weirii* decay observed on the stump top. Thus, there were 11 treatments and an untreated check for the live tree stumps with five replications. The dose for each stump was based on the estimated treated biomass, belowground root and stump biomass plus aboveground stump, estimated from the stump diameter. Local data were used to establish a relationship between basal area at the stump and at breast height and then reported relationships between diameter at breast height (d.b.h.) and belowground biomass (Gholz and others 1979) were used to make estimates of treated biomass. The minimum effective dosage (standard dosage) was taken to be 1,000 mL/stump for a stump diameter of 48 cm. A standard dosage (D) was 6.7 ml of chloropicrin, 6.7 ml of Vorlex, or 1.5 g of MITC per kilogram of treated biomass. The MITC in a 1.0 D dose was equivalent to the MITC in a 1.0 D dose of Vorlex.

Stumps were created in the fall of 1981, treated in March 1982, and excavated and evaluated in October and November 1983. Treatment application was much the same as in Study 1. Treatment holes, 3.2 cm in diameter, were drilled vertically into each stump top at stained areas with a minimum of one in each quadrant. A dose of fumigant was distributed equally to all holes in the stump, and each hole was plugged tightly with a dowel 3.3 cm in diameter. One end of each plug was beveled to facilitate driving the plugs into the holes. The beveled end was dipped into resorcinol glue (to resist passage of the fumigant), and the glue was allowed to harden before the plug was used. Stumps were removed with an excavator, and roots were sectioned, sampled, and evaluated for viable *P. weirii* as in Study 1.

After two seasons, all fumigants had reduced the amount of inoculum, but MITC was the least effective, the 0.5-D dosage was not detectably different from the other two, and sealing the stump top did not improve fumigant effectiveness. After 20 months, chloropicrin and Vorlex had eliminated the fungus from the stumps and reduced the volume of roots supporting vigorous *P. weirii* to about 7 percent of the prefumigation volume.

Labeling was approved by the Environmental Protection Agency in January 1989, for the use of chloropicrin to reduce inoculum of laminated root rot in Douglas-fir stumps. The label dosage is approximately 3.3 ml of chloropicrin per kilogram of treated biomass (0.5 D dosage).

Study 3--(Thies and others 1991, study in progress)

During the first two studies, individual stumps were treated with fumigants, and success was based on reducing pathogen viability. Had the stumps remained in the soil, the fumigants and the various microbial antagonists recolonizing the roots might have eliminated the pathogen. In this study, treatments are on an area basis with all stumps within a plot being treated. Treated stumps will remain in the soil, and success will be based on disease development in the replacement stand.

This study was established to determine the cost and degree of reduction in the reappearance of laminated root rot in a replacement stand using chloropicrin as a stump treatment.

The stand was cut in July 1988, stumps were fumigated in October 1988, and the area was planted with Douglas-fir in March 1989. Evaluations are ongoing.

The study area was subdivided, systematically searched, and the location of each *P. weirii*-infested stump mapped. Using a map depicting the locations of infested stumps, circular, 0.04-ha, nonoverlapping treatment plots were established in the study area in locations to include concentrations of infested stumps. An inoculum index (INOC) was calculated for each infested stump, based on stump diameter and stump condition, and summed to get a total INOC for each plot. Based on total INOC, plots were stratified into eight blocks of four plots each. Treatments involved application of chloropicrin at either 100 percent of the labeled dosage (about 3.3 ml/kg of stump and root biomass), and either all stumps were treated or only those with stain (or advanced decay) typical of *P. weirii*. Three chloropicrin treatments and an untreated check were randomly assigned within each group of four plots in a block: (1) Check (nothing done to the stumps), (2) 100 percent, all stumps, (3) 20 percent, all stumps, (4) 100 percent, stain-only stumps. Treated stumps were drilled, treated, and plugged as in study 2 above; however, no effort was made to seal the stump tops. An apparatus used to measure and dispense chloropicrin, using compressed nitrogen to move the fumigant, has been described (Thies 1990).

There is an ongoing effort to evaluate the bioresponses of nontarget organisms to the chloropicrin treatment. Samples have been collected 1, 2, 3, and 5 seasons after treatment both around selected stumps and on an area basis and evaluated; that effort has been previously described (Ingham and others 1991).

Study 4--(Thies and Nelson 1987b; Thies and Nelson 1994, in press)

This study was begun in 1981 to test the hypothesis that a soil fumigant (chloropicrin, MITC, or Vorlex) injected into live Douglas-fir could reduce the volume of root wood occupied by *P. weirii* without killing the treated tree. There is now supporting evidence that live Douglas-fir trees tolerate injection of fumigants into the bole (Goodell and others 1984, Morrell and Newbill 1990).

Dominant and codominant Douglas-fir were classified by probability of *P. weirii* infection: I, infected; II, probably infected, and III, probably noninfected. Each infection class was separated (blocked) into five groups of nine trees each based on similarities of size and tree location. Study trees ranged in d.b.h. from 27.4 to 62.2 cm. Treatments were randomly assigned to trees within each group. It was assumed that the dose tolerated by a tree is proportional to the estimated biomass (based on d.b.h.) of its major roots and first 2.4 m of bole. Dosage rates were the same as those used in study 2.

In March 1982, seven treatments were applied: Check; chloropicrin 1D, 0.5D, 0.25D; MITC 1D, 0.5D, 0.25D. Two additional treatments were applied in April 1983: chloropicrin 0.125D and Vorlex 0.5D. Fumigant was applied to holes drilled down (45° below horizontal) toward the center of the tree at 15-cm intervals around the tree. Each hole was tightly plugged with a dowel as before. Check trees were not altered. Trees dying over the course of the study and all trees remaining after 10 growing seasons were felled and their stumps and roots removed from the soil and cleaned. Roots were evaluated as in Study 1.

This study demonstrates that fumigation can effectively reduce *P. weirii* in living Douglas-fir without killing the tree. Most (73 percent) of the treated trees survived 10 growing seasons. Of the 32 treated trees that died, 25 died within 2 years of treatment. All 45 methylisothiocyanate-treated trees survived to harvest. In contrast, 16 of 45 chloropicrin-treated trees and 12 of 15 Vorlex-treated trees survived at similar dosages. Fumigant killed *P. weirii* in much of the colonized root wood thus reducing inoculum. The percentage reductions of inoculum volume (combining dosages D, 0.5D, and 0.25D) follow: chloropicrin--73; MITC--83; Vorlex--81; and Check--9. The amount of inoculum reduction for all fumigant treatments was significantly different from the check treatment. Each fumigant totally eliminated the pathogen from some infected root systems: chloropicrin--16 of 31 trees; MITC--10 of 21 trees; Vorlex--4 of 6 trees.

CONCLUSIONS

In summary, the four studies described in this paper demonstrate that soil fumigants could be used to reduce inoculum from laminated root rot in both stumps and living trees. While silvicultural manipulations will remain the most widely used tool to manage laminated root rot, fumigation will be an additional tool available to the land manager. Initially, fumigation will likely find its greatest use in areas where site disruption associated with stump removal is unacceptable such as steep terrain, in areas of fragile soils, or in the treatment of relatively small areas or areas of high value.

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Invasive Treatments to Control Laminated Root Rot in Douglas-fir: Effects on Tree Growth and Survival[1]

C. Harrington and W.G. Thies[2]

An experimental approach is presented on the control of laminated root rot (*Phellinus weirii* (Murr.) Gilb.)--injection of biocides or chemical fumigants into living trees--and the effects of these biocides on subsequent growth of Douglas-fir trees. Fifteen trees were randomly assigned to each of nine treatments. Two fumigants chosen for treatment--chloropicrin and methylisothiocyanate (MITC)--were each applied at several dosages, whereas Vorlex (active ingredient MITC) was applied at one dosage.

During the 9-year study period, the control treatment had 80 percent survival. The higher dosage chloropicrin treatments caused substantial mortality (<30 percent survival). Some treatments increased survival; the lowest dosage chloropicrin treatment and all MITC treatments had 100 percent survival. All of the injection treatments killed some tissues around the injection sites. Death of the vascular cambium was more common than death of the cork cambium resulting in fluted lower boles. The lowest dosage chloropicrin and the three MITC treatments resulted in the least damage.

Height growth was greatest for untreated trees; however, growth in the lowest dosage chloropicrin and MITC treatments was not significantly less. Upper stem growth was greatest for the untreated trees and least for the high chloropicrin and Vorlex treatments. At the same dosage, chloropicrin and Vorlex had significantly greater negative effects on tree height and diameter growth than MITC.

MITC increased survival of infected trees and at low dosages was effective in reducing infection levels without causing significant growth reductions. Future trials with MITC are clearly warranted.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Therapeutic Treatment of Douglas-fir with Chloropicrin, Methylisothiocyanate, or Vorlex to Reduce Root Colonization by *Phellinus weirii*[1]

W.G. Thies and E.E. Nelson[2]

In 1982, 135 Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, trees were segregated into three infection classes based on signs and symptoms of infection by the pathogen *Phellinus weirii* (Murr.) Gilb. Eight fumigation treatments and one control treatment were applied to five replicate trees within each established class. Fumigant was applied to 3.3-cm-diameter holes drilled down (45° below horizontal) to the pith in a helical pattern around the base of the tree. Measured volumes of fumigant were placed in the holes, which were then sealed with resin-coated wood dowels. Untreated trees were not drilled. The dose of fumigant for each tree was based on formulas relating the biomass of roots and lower bole to tree diameter. Stumps of trees killed before 1991 and stumps of all remaining live trees harvested in 1991 were excavated and their roots dissected and sampled for viable *P. weirii*. Twenty-four of the 30 trees treated with the two highest doses of chloropicrin were killed, presumably by the fumigant. None of the 45 trees treated with methylisothiocyanate (MITC) and only 3 of the 15 trees treated with Vorlex died (as did 3 of the 15 untreated controls). The volume of stained and decayed roots occupied by viable *P. weirii* was reduced 80 to 90 percent by MITC or Vorlex. This compares with reductions of 52 to 66 percent by chloropicrin at the two lower, less phytotoxic doses and 9 percent for untreated controls. This study demonstrates that fumigation can effectively reduce *P. weirii* infection in living Douglas-fir trees without adversely affecting the host tree.

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[1] Poster presented at Research and Applications of Chemical Sciences in Forestry; 1994 February 1-2; Starkville, MS.

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Do You Dig Roots?[1]

Walter G. Thies[2]

ABSTRACT

A technique is described for extracting stumps and roots of second-growth Douglas-fir for research purposes. The technique, using an excavator commonly used in industrial forestry operations, can be divided into three steps: (1) undermine the stump and root system with an excavator, (2) split the stump into sectors separating major roots, (3) extract the sectors from the soil and shake.

Keywords: Biomass.

INTRODUCTION

Excavation of stumps and roots is inevitable for those who study root diseases of forest trees. Usually, stump removal for management purposes is done with the goal of getting the wood out of the ground or breaking it up into pieces small enough to prevent problems. Researchers may wish to examine the roots or study a belowground process and to do so may need to recover all roots from a particular stump relatively undamaged. In addition to manual excavation or bulldozing, other techniques have been used in the Pacific Northwest to expose root systems for study: hydraulic excavation (McMinn 1963, Reynolds and Bloomberg 1982), bulldozing in combination with hydraulic cleaning (Thies 1980), compressed air (Weir 1964), log forks (Thies 1984), a vibrating stump puller (Arnold 1981), and explosives (Bertagnole and Partridge 1988, Hertert and others 1975).

The extraction technique must be tailored to the needs of the study being conducted. Some studies may require the capability to collect undamaged roots of 100 or more second-growth Douglas-fir stumps in a few weeks. Many of the previously described techniques are best used for special objectives or for a few stumps, and some have significant drawbacks: manual systems have a high labor cost; heavy equipment (bulldozers and log forks) often removes bark and smashes roots; a vibrating stump puller works best on healthy roots and is in short supply in the Pacific Northwest; hydraulic excavation works best on a slope, requires a good source of water, and must be located so that soil-laden runoff does not get into a stream. In this paper is described a technique using an excavator, commonly used in industrial forestry operations, to quickly recover undamaged roots of mature Douglas-fir for examination.

TECHNIQUE

The excavation technique can be divided into three steps: (1) undermine the stump and root system by using an excavator, (2) split the stump into sectors separating major roots, (3) extract the sectors from the soil and shake.

Undermining Stump and Roots

For easiest extraction, select a stump separated from other stumps by at least 2 m. Position the excavator so that the target stump is between the tracks with at least 8 m of open ground beyond the stump. Extend the excavator arm across the stump to place the bucket as far beyond the stump as possible. The dig starts by removing shallow (30 to 45 cm) scoops of soil successively in a line toward the stump. The scoops are taken by putting the teeth of the bucket perpendicular to the ground, pushing the teeth and bucket into the soil, and rotating the bucket toward the stump to scoop soil. This will allow small roots to pull out of the soil mass and not be crushed or broken off as would happen if the bucket were dragged toward the stump. When small roots from the target stump are exposed, begin to dig the hole deeper as far from the stump as necessary to avoid the exposed roots. Deposit the soil in one or two piles. As each scoop of soil is deposited, it spills down the pile making possible recovery of inadvertently severed roots. Expand the hole right and left by repeating the above procedure. Two observers should work with the excavator to assure that roots pulled from the root system are noted as missing and located.

Depth of the hole depends on the rooting depth of the subject tree, but in most cases, the hole will be 3 to 4 m deep.

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With the elbow of the excavator arm beyond the edge of the hole, remove soil from under the root system. As the root system is undermined, soil falls away from the roots and can be removed. If exposed roots are at risk of being crushed by the excavator arm, remove the roots and label them. Continue this process until the cavity under the roots extends nearly to the stump. Soil may be further encouraged to fall away from the root system by either pounding on the top of the stump with the excavator bucket or by collecting a heaping scoop of soil in the bucket and gently lifting up under the root system. The intent is to loosen soil packed around the roots and to cause the soil to fall away from the root system so that major roots can be easily detected.

Splitting the Stump

Based on a "best guess" of major root locations, mark the stump top along three to five radii each dissecting two major roots. With a chainsaw, cut a groove along each marked radius to provide a starting place for a wedge and to better assure that the stump will split along that line. Place the edge of the wedge into the groove and tap the wedge with the bottom of the bucket. After driving the wedge into the stump, remove the wedge and repeat at the other grooves. The wedge can be removed by hooking a tooth of the bucket on a bar welded on the top of the wedge. Splitting will be easier if all grooves are split before the stump is undermined. If the stump is large or appears difficult to split, cut the grooves deeper. If a groove is nearly perpendicular to the axis of the excavator arm, it may be possible to split the stump by using the teeth on the bucket instead of the wedge.

Extracting the Stump Sectors

Extract first the sector that has been undermined. After the stump is opened by the wedge, insert the teeth of the bucket into the split. Pry the stump apart through a combination of pressure and rocking of the bucket. The split must be opened wide enough to both assure separation and allow that sector of the stump to be gripped between the hydraulically operated thumb and the teeth of the bucket. It may be necessary to pry on the second split associated with the first sector of the stump to be removed.

Grip the first sector of the stump between the thumb and bucket and lift the stump sector and associated roots free. Suspend the roots over the pile of soil, and shake them until all loose soil has fallen away from the roots. Any roots that break off will be easily found on the pile of fresh loose soil. The sector can then be labeled and placed in a convenient location for further examination.

Each remaining sector of the stump can be removed in turn. Each sector can be split from the remainder of the stump by prying with the teeth of the bucket. Before removing the second and successive sectors, additional undermining may be necessary. If the roots of a sector are still partially buried, the sector must be pulled so that the roots move toward the stump with minimal lifting until the roots are free of the soil. While each sector is suspended, it can be examined for evidence of broken roots. Lost root pieces are easily found by excavation and examination of additional scoops of soil.

One variation on the system involves using a choker to grip a sector when it is difficult to use the thumb and bucket. Use a choker if the sector slips and falls into the hole or if the bucket does not have a thumb. It is best to notch the stump portion of the sector to receive the choker cable. The cable can be put around one or more main roots; however, it may cut into the root or knock off bark, which may be undesirable depending on the needs of the study. A sector held by a choker can be violently shaken by quickly tipping the bucket in small increments.

Equipment Specifications

It is important to have a large enough excavator to easily undermine the root system and to be able to pull and lift the stump sectors. Following are the specifications for the smallest excavator we have used to successfully extract second-growth Douglas-fir root systems: weight 20,000 kg; 118 hp; hydraulic activated thumb; bucket capacity 1 m^3; bucket width 1 m with a reach of 10.6 m; tracks 80 cm wide and 3.2 m between the outside edge of the tracks. The bucket has a hook welded to the bottom to attach a choker. A larger excavator would be acceptable, but a bucket exceeding 1.75 m^3 may get in the way.

The wedge is not commercially available and must be fabricated. The wedge used is made of 32-mm thick T-1 steel with a piece of 6-cm-square carbon-steel bar welded to the top edge. The bar extends 6 cm beyond the width of the wedge on both sides. The wedge is 37 cm wide (leading edge), 46 cm long, and weighs about 30 kg. The lower 40 mm is tapered to form a keen edge. The wedge needs to be wide enough and thick enough to cause a split but light enough to be easily handled in the field.

The choker cable is 16 mm in diameter and 5 m long.

Time

Time needed to excavate the roots of 140 stumps ranging in diameter from 30 to 70 cm averaged about 45 min per stump, including time to move the excavator from stump to stump.

Safety Considerations

There is significant risk in working beside or behind an excavator. Modern excavators turn very rapidly, are noisy, and limit the view of the operator. An individual working around an excavator must be alert and guard their own well-being. Of particular concern is the rear-mounted counterweight. The back of the machine swings faster than anticipated and can unexpectedly hit an individual walking around the machine. The operator has a good field of vision to the front but is blind to the back and has a restricted view to the sides. Hence, it is important that anyone working around an excavator be aware of its movement at all times and that individuals not required to work with the machine maintain a distance of at least 30 m from it.

Safety equipment in the form of hardhats, steel-toed boots, safety glasses, and hearing protection should be worn at all times when working near an excavator. When soil is being shaken from the roots, chunks of soil, rocks, or pieces of root can dislodge and fly with enough force to cause injury. Also, rocks excavated with the soil may roll down the soil pile and injure a foot.

SUMMARY

Excavators can be successfully used for the removal of stumps and root systems in both a research and an operational setting. In many instances excavators are the only practical and cost effective way to recover complete root systems with a minimum of damage to the recovered roots.

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This technique has evolved during work on several studies over the past 12 years with much trial and error and many contributed ideas from folks with significant experience to offer--the machine operators. It is with appreciation and respect that I extend thanks to the following operators: K.C. VanNatta of VanNatta Brothers Tree Farm, Ranier, OR; Larry Bair of Bair Logging Company, Vernonia, OR; Don Bird and Bruce Graves of Miami Corporation, McMinnville, OR; and John Richards of Laughlin Logging Company, Yamhill, OR.

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Environmental Stresses and Reproductive Biology of Loblolly Pine (*Pinus taeda* L.) and Flowering Dogwood (*Cornus florida* L.)[1]

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The U.S. Department of Agriculture, Forest Service and Mississippi State University have begun a cooperative study to determine the effects of temperature and CO_2 on the reproductive cycle of loblolly pine (*Pinus taeda* L.) and flowering dogwood (*Cornus florida* L.). Test branches on study trees were enclosed in unheated chambers (the reference chambers), heated chambers (+2 °C above reference), and there were chamberless ambient controls. In the first year of the study, the effects of increased temperature on flowering and fruiting in loblolly pine were measured. The second year of the study will examine the effects of both temperature and CO_2 on loblolly pine and temperature alone on flowering dogwood. In the third year, CO_2 treatments will be added to the dogwood study. Experimental measurements include vegetative/reproductive and female/male bud ratios, phenological patterns of flowering and pollination, pollen quality/viability studies, and seed quality/viability tests. Data from the loblolly pine study indicate that pollen from branches in heated chambers sheds up to 3 weeks in advance of that from outside branches and that female flowers in heated chambers become visible 3 weeks before those on outside branches. Pollen from ambient branches and from branches in heated chambers has viability over 75 percent, while that from branches in unheated chambers drops to 55 percent.

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Chemical Characterization of Smoke from Wildland Fires[1]

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ABSTRACT

Fires in the open environment produce a diversity of combustion products. Special techniques are needed to characterize the emissions ranging from microcombustion-evolved gas analysis to airborne monitoring of the full-scale phenomenon. This paper discusses the advantages and disadvantages of each technique and provides a discussion of the measurement needs for more fully understanding the release of smoke emissions from wildland fires.

Keywords: Aerosols, combustion, emission factors, methane emissions.

INTRODUCTION

Fires in biomass fuels emit a complex mixture of particulate matter and gases into the atmosphere. Globally, the diversity of combustion products results from wide ranges in fuel types and fire behavior induced by the large variations in ecosystems and weather conditions. Fires in tropical ecosystems consume as much as 80 percent of the total biomass burned (approximately 6×10^{15} g yr^{-1}) on a global basis (Crutzen and Andreae 1990, Hao and others 1990). In contrast, fires in the United States produce only a small percentage of the total emissions (less than 2 to 3 percent) (Ward and Hao 1991). Most estimates of global emissions have been based on a few observations from laboratory-scale fires and ground and airborne measurements from fires of different fuel types in tropical countries and North America. The purpose of this paper is to review the methodology used to characterize emissions from fires over a broad range of ecosystems and to discuss results.

COMBUSTION PROCESSES OF FIRES IN BIOMASS FUELS

The complex nature of free-burning fires has made both fire behavior and smoke emissions difficult to model. Because of the complex chemical reactions occurring during the fires, it is important to have a clear understanding of the combustion process before attempting to model the fire/emissions phenomena. Fires in biomass fuels typically progress through three stages. First, the fire advances through a process of fuel preheating--the volatilization of free water and low boiling-point hydrocarbons, which is the beginning of pyrolysis. Second, a flaming combustion zone is formed in which the pyrolyzed products of hemicellulose, cellulose, lignin, and volatile hydrocarbons are rapidly oxidized. Third, a postflaming combustion mode (undoubtedly in a low-oxygen-content environment), called smoldering combustion, continues to produce aerosols and gases that do not enter the flaming combustion zone.

The dynamics of the flame structure and the extent of smoldering combustion are influenced by the distribution of different size classes of biomass and the packing density of the fuel complex. In addition, the moisture content gradients within strata of biomass affect the reaction rate and ultimate temperature within the oxidation zone. Technically, fires in biomass fuels range from laminar diffusion flames (candles) to highly turbulent flame structures (fast-moving, wind-driven fires). Compounding this is the wind's influence on the transport of oxygen to the volatilized fuel gases.

An advancing flame front interacts with the fuel and the atmosphere to produce smoke. The major processes affecting smoke production and character of emissions are outlined:

- fuel preheating zone
- fuel vaporization zone
- variable fuel-to-oxygen ratio inside and outside the flame zone
- air entrainment or quenching zone
- combustion product equilibration zone and photochemical reactions in the free atmosphere
- prompt processing of the emissions occurring within zones of water condensation (cloud processing).

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These zones often are not clearly delineated. Some of the research discussed was designed to answer questions concerning processes of emissions formation. Generally, sampling methods are devised to examine concentrations of emissions and ratios of emissions occurring outside the quenching zone or in areas of temperatures <100 °C. Airborne systems have been used to measure the emissions preceding and following cloud processing. Specialized experiments are needed to study the interactions between these six zones and the effect each has on the release of smoke emissions or a specific emission and the ultimate fate of the emissions.

FUNDAMENTAL PARAMETERS AND DEFINITIONS

Rothermel (1972) and Albini (1976) describe a series of models useful for predicting characteristics of the flame front: flame length, flame depth, residence time, rate of spread, and rate of heat release. The source strength for a fire is closely coupled to the rate of spread and the rate of consumption of biomass by the fire. Generally, the emissions released through the flaming consumption of biomass are more fully oxidized than emissions from smoldering combustion. The models of Rothermel (1972) and Albini (1976) do not describe the characteristics of the smoldering combustion of biomass fuels. Methods for estimating source strength of emissions are usually based on separate analyses of the flaming and smoldering combustion processes.[4] Our work involves the development of models for estimating emission factors and for evaluating ratios of emissions useful for estimating the total production of emissions. Here, we will use a model to evaluate important parameters for assessing source strength as follows:

$$q_{yn}=(EF_{yn})w_y r \quad (1)$$

where:

q_{yn} = source strength of emission n, g m^{-1} s^{-1};
n = specific trace gas or particulate matter emission; e.g., CO, CH_4, CH_3Cl, etc.;
EF_{yn} = ratio of the mass of emission n released to the mass of biomass consumed, g kg^{-1};
w_y = mass of available fuel per unit area, kg m^{-2};
r = rate of spread of the fire, m s^{-1}; and
y = if present, equals the phase of combustion, either flaming (f) or smoldering (s), if absent, the variable is assumed to apply for the fire.

Each of these variables should be evaluated to estimate the smoke emissions released from the burning of biomass. Often, the resolution needed only requires a measurement of the total area burned by the fire; it is not necessary to quantify the rate of spread of the fire. In this case, when a general estimate of emissions released on a regional scale is adequate, EF_n, w, and the area burned by the fires are the only parameters evaluated. This technique is used in making estimates of the release of emissions to the atmosphere on a global basis. For purposes where a specific q_{yn} rate is needed, r must be quantified to describe the rate at which the fire is moving into unburned fuel. Fire spread models of Rothermel (1972) and Albini (1976) can be used for this purpose, or estimates of rate of ignition can be made from empirical observations.

The mass of available fuel per unit area, w, is equal to the total mass of biomass above ground times a ratio, termed the **combustion factor**. The combustion factor is usually determined empirically or estimated from empirical data. Others have developed systems for evaluating the consumption of woody fuel, litter fuel, and duff (Brown and others 1991).

The adoption of **carbon mass balance** (CMB) methods of ratioing the emissions released to the biomass consumed (Radke and others 1990a, Ward and others 1979) has expedited techniques for empirically evaluating EF_{yn}. Generally, laboratory experiments (Nelson 1982) and some field measurements (Ward and Hardy 1991) use the method for quantifying EF_{yn} ratios for the flaming and smoldering combustion phases and the carbon released during each combustion phase. The application of airborne systems for measuring average EF_n ratios has been greatly improved through the application of CMB methods (Radke and others 1990a). There are limited cases where EF_n measurements have been made only for the flaming phase using airborne systems (Einfeld and others 1991, Radke and others 1991). Usually, after the first few minutes of a fire, the emissions from the flaming and smoldering combustion processes become intermixed, and it becomes difficult to assess which process (flaming or smoldering) is dominating the emissions being measured. An average EF_n ratio can be computed for the fire based on the carbon released during each phase of combustion:

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[4] Ward, D.E.; Susott, R.A.; Doughty, C.B. [and others]. [In preparation]. Combustion efficiency and smoke emissions from fires in selected savanna ecosystems of South Africa and Zambia. Journal of Geophysical Research.

$$EF_n = \frac{(EF_{fw})w_f + (EF_{sm})w_s}{w_f + w_s}. \quad (2)$$

Progress in modeling the release of emissions has come through the adoption of a measurement of **combustion efficiency** (η) defined as the percent of carbon released in the form of CO_2 (Ward and Hardy 1991). In the combustion literature, η is based on the ratio of the actual heat released in a combustion process to the heat of combustion. The measurements of η based on either CO_2 or on heat released by the oxidation process are closely correlated. The η can be computed by:

$$\eta = \frac{[CO_2a]}{[CO_2t]} = \frac{EF_{CO_2a}}{EF_{CO_2t}} = \frac{CO_2\text{-}c}{CO_2\text{-}c + CO\text{-}c + HC\text{-}c + PM\text{-}c} \quad (3)$$

where:

CO_2a	=	actual concentration or mass of CO_2 released by the fire,
CO_2t	=	theoretical limit of concentration or mass of CO_2 if all carbon is converted to CO_2,
CO_2-c	=	mass of carbon or molar concentration of carbon where "-c" denotes carbon content of the molecular species for CO_2, CO, hydrocarbons (HC), and particulate matter (PM).

An approach often used is to correlate dependent variables with the independent variable CO/CO_2. This ratio is correlated with η ($r = 0.99$) over a narrow range of η from 0.8 to 1.0. The CO/CO_2 ratio can be normalized by dividing by the sum of carbon contained with the CO and CO_2 and becomes linearly correlated with other products of incomplete combustion as suggested by Ward and Hao (1991). They proposed that this parameter be defined as the modified combustion efficiency ($\hat{\eta}$) as follows,

$$\hat{\eta} = \frac{CO_2\text{-}c}{CO_2\text{-}c - CO\text{-}c}. \quad (4)$$

Figure 1 shows the CO/CO_2 ratio, η, and $\hat{\eta}$, correlated with CO concentration. Hence, the more fundamental variable is recommended for more general use in correlating carbon emissions to a property of the combustion system. The $\hat{\eta}$ has been used as an estimator of η from the data of Ward and Hao (1991) where:

$$\eta = \frac{(\hat{\eta} - 0.15)}{0.85}. \quad (5)$$

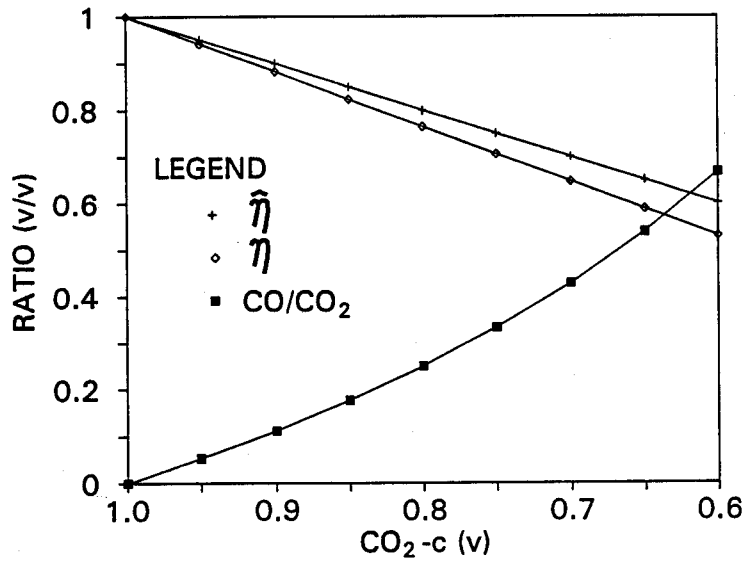

Figure 1. *A comparison of the linearity of the ratio of CO/CO_2 to CO_2 concentration, combustion efficiency (η), and modified combustion efficiency ($\hat{\eta} = CO_2\text{-}c/(CO\text{-}c+CO_2\text{-}c)$).*

METHODS USED TO MEASURE EMISSIONS RELEASED FROM BIOMASS FIRES

Extremes in measurement scale are often used to circumvent the hostile environment of the open burning fire and to reduce the variance encountered in the range of natural conditions. Laboratory and microcombustion techniques have been used on one end of the scale and on the other end, airborne and satellite technology. The discussion in the following sections progresses from the smallest scale to the largest scale methods of making measurement. Table 1 summarizes examples of research performed in the laboratory using milligram- to kilogram-sized samples of biomass fuel and makes comparisons to systems used in the field to characterize the full-scale to near full-scale phenomena.

Table 1. *Scale of fire studies in developing and characterizing emissions from fires in biomass fuels*

	ADVANTAGES	DISADVANTAGES
1. MICROCOMBUSTION SYSTEMS (mg quantities of fuel)	Pyrolysis products Mechanisms Replication of experiments Environment Temperature of fuel	Scale Fuel arrangement Reaction pathways Quenching of reactions Vessel walls
2. FLOW-REACTOR SYSTEMS (pyrolysis products and fuel vapors)	Scale Closed system Controlled environment Quenching Diffusion and/or premixed flames	Reaction pathway Flame scale all losses Fuel arrangement
3. COMBUSTION LABORATORY (kg quantities of fuel)	Scaling similarity for low intensity Modeled fuel beds Lifted fuel beds Lighting similarity Replicated studies Environment control Quenching control Complex train of analyzers and Close proximity to gc/ms	Wall losses Reaction pathway Sample zone temperature Costly to maintain Fuel moisture gradients Representativeness
4. FIELD (SURFACE BASED) (Small area and piled material studies)	Near full-scale experiment Replication of tests Less costly than aircraft Fuels, fire behavior, CMB	Temperature limitation Height limitation Reaction pathway
5. FIELD (AIRBORNE) (full-scale fires and large areas)	Full-scale Plume dynamics (trajectory analysis) Multiple sensors in same sample space CMB Cloud/emissions interaction studies	Weather (clouds) Lower limit of plume Mountainous terrain Large test areas Costly Replication is difficult Fuel and fire behavior

Microcombustion

In a microcombustion experiment using thermogravimetric-evolved gas analysis, Clements and McMahon (1984) burned 10-mg samples of ground-up pine needles to evaluate emission factors for total hydrocarbons (THC), CO, CO_2, volatile organic compounds, and thermoparticulates. The matrix of tests included oxygen and nonoxygen (nitrogen) environments and simulated pyrolytic processes associated with smoldering combustion. This study found that EF_{PM} values averaged 85 g kg^{-1} in an oxygen environment ($\eta = 0.67$). In another experiment using the same apparatus and techniques, Clements and McMahon (1980) found the production of oxides of nitrogen (NO_x) to be linearly proportional to the level of fuel nitrogen content and to agree well with values (NO_x emission factors range 1 to 10 g kg^{-1}) found from larger scale fires. They concluded that most of the NO is produced from the prompt release of bound nitrogen from the fuel substrate.

Kuhlbusch and others (1991) studied the nitrogen balance and found that only a small percentage of total fuel nitrogen could be accounted for by measuring NO_y. By using an argon or helium atmosphere, they deduced that an average of 36 percent of the fuel nitrogen is released in the form of molecular nitrogen, and most of this is emitted during the flaming phase.

Benner and others (1977) used a closed combustion vessel for testing the photochemical potential of smoke from burning of biomass fuels. They found that nonozone oxidant formation always preceded ozone formation when smoke from small samples of pine needles was irradiated with ultraviolet light.

Edye and Richards (1991) designed a bench-scale combustion experiment, using an inverted funnel and radiant heated petri dish to study the smoldering combustion of ponderosa pine and cottonwood. The compounds investigated include greenhouse gases and air toxics; i.e., CO_2, CO, CH_4, C_2 alkanes and alkenes, low molecular weight organic acids, and the oxidized compounds from cellulose and lignin in the biomass (Edye and Richards 1991; McKenzie and others, in press). The correlation among emitted volatile and condensable compounds was studied. This research was extended to study the emissions from the smoldering combustion of other classes of forest fuel including bark, humus, and litter. Using CMB methods, the results of this research will be compared with those derived from larger scale combustion systems research.

Ward (1979) proposed that volatile materials contained in the forest fuel complex contribute significantly to the emissions produced and that oxygen content of the fuel is inversely correlated with particulate matter emissions. A flow reactor-combustion chamber system was developed for burning single and multiple component fuels introduced into the combustion chamber in a gaseous phase. Particulate matter emission factors for fuels of methane, propane, heptane, benzene, ethanol, ethylene glycol, and α-pinene were evaluated. A model was advanced suggesting that particulate matter emissions for laminar-diffusion flames are a function of the flame envelope volume. Wildland fires are a turbulent diffusion flame process. A system has been developed to pyrolyze pulverized solid fuels in a controlled environment combustion chamber so that emissions tests can be performed for fuels of different chemical composition under controlled conditions (Corlett 1993). At this time, no emissions experiments have been performed with the apparatus.

Highly controlled combustion experiments can be very effective in testing hypotheses concerning processes affecting the release of emissions. In addition, these systems may be useful in establishing response functions for the release of various emissions as a function of combustion efficiency or other variables that then can be adjusted to the full-scale phenomena through a limited field research project.

Controlled Environment Combustion Laboratory

Feldstein and others (1963) and Darley and others (1966) pioneered research for evaluating emissions from fuel arrays arranged in different combinations using funnel devices for channeling and concentrating emissions. Fritschen and others (1970) and Vines and others (1971) used microcombustion hoods for burning small samples of forest fuels and deduced the approximate mixtures of carbon-containing gases and particulate matter. These data were used to optimize airborne sampling systems. Lobert and others (1991) and Jenkins and others (1991) used similar open-hood systems for studying emissions from fires in different types of fuels. Large-scale, controlled environment, combustion laboratory experiments have been extensively used whereby kilogram-sized samples of forest fuels are arranged (includes different levels of loading, packing ratios, moisture content, etc.) in fuel baskets on weighing platforms and burned; the emissions are exhausted through a hood-stack arrangement. McMahon and Tsoukalas (1978) studied the emissions of particulate matter and polynuclear aromatic hydrocarbons from the combustion of pine needle fuels burned using simulated heading fires (where the fire moved upslope) and backing fires (where the fire moved downslope). They found very high benzo[a]pyrene to PM ratios for backing fires 98 to 274 μg g-1 PM) with much lower values for heading fires (2 to 3 μg g^{-1} PM). These results were not substantiated under field research conditions, and no explanation for the discrepancy was reported (Ward 1989, White 1987).

Ward and others (1980) concluded that similarity of scaling of the fuel bed and flame structure of the combustion laboratory fires to that of the field was necessary for achieving agreement among different results. More recent results of

Hao and Ward (1993), Ward and Hao (1991), and Ward and others (1993) comparing controlled environment combustion laboratory, combustion hood experiments, and field measurements suggests that η is the principal controlling variable and ratios of products of incomplete combustion remain constant over a wide range of η conditions (Ward and others 1992, Weise and others 1991).

Griffith and others (1991) used a Fourier transform infrared (FTIR) system in a combustion laboratory and in field studies to compare emissions of compounds that are difficult to sample and quantify by GC; e.g., NH_3, N_2O, and CH_2O. The FTIR results for the field and for the laboratory were consistent for low-intensity smoldering combustion fires. The FTIR results obtained for NH_3 were higher than the NH_3 results obtained by GC. This is not surprising given the above-mentioned difficulty of sampling for NH_3. More FTIR-based studies are needed to better quantify the emissions of NH_3 and other reactive compounds that pose sampling problems.

Sandberg and others (1975) developed an inverse relationship between PM emissions and the rate of heat release by fires in modeled biomass fuel beds. They studied both the emissions of PM and HC as a function of treatment with a flame-retarding chemical, diammonium phosphate (DAP). This study showed no significant reduction in PM emissions as a result of treating with the chemical. On the other hand, Philpot and others (1972) found a significant increase in PM emissions using DAP, but a decrease in the total PM emissions resulting from the treatment of biomass with ammonium sulfate flame-retarding chemical.

The effect of wind on the combustion of forest fuels and resulting smoke emissions has been difficult to evaluate under field conditions. Jenkins and others (1993) constructed a chamber with a belt apparatus to position a fuel bed so that the flame structure is maintained under a combustion hood. They found that emission factors for particulate matter varied depending on fuel type (e.g., agricultural residues such as rice straw, wheat straw, and almond and walnut tree prunings) and for different wind conditions. The flame structure was also examined through measurements of local temperatures, gas concentrations, and soot volume.

Opportunities exist for designing experiments that can be used for establishing response functions for emissions difficult to characterize under field conditions. Combustion laboratory methods were used to study the effect of chloride salts on the release of CH_3Cl. Reinhardt and Ward[5] studied emissions of CH_3Cl using a combustion hood device and concluded that most CH_3Cl is released during the smoldering combustion of biomass fuels and is correlated to the content of the Cl ion in or on the biomass fuel particles.

The advantages of using combustion laboratory facilities include the opportunity to study emissions released as a function of flaming and smoldering combustion using sophisticated instrumentation. The components of a fuel complex (fine fuels, coarse fuels, leaves, twigs, etc.) can be stratified and mixed to model natural fuel complexes. In addition, the mass balance between the fuel and the emissions can be studied in detail. A disadvantage is the inability to duplicate the full-scale phenomena except for the lowest intensity fires.

Ground-Based Sampling

Sampling near the fire at downwind locations (Bonsang and others 1991, Fritschen and others 1970) or holding a sampling probe over a fire (Bonsang and others 1991, Crutzen and others 1985, White 1987) will not always provide representative samples of the flaming and smoldering phases. Sampling must account for the rapid rate of fuel consumption during the flaming phase (Ward and others 1992) and weight the emissions produced by phase of combustion by the carbon released during each phase (equation 2). Ideally, to evaluate an emission factor or ratio that is representative of both the flaming and smoldering combustion phases, the emissions must be sampled at a rate proportional to the rate of carbon release over the duration of the fire. In practice, the emissions are usually sampled by phase of combustion.

Ward and Hardy (1991), using towers and cable systems to support sampling apparatus over fires, sampled the rate of release of carbon and emissions for a wide range of fuel and weather conditions. These data illustrate the dependency of carbon-based combustion products on η (fig. 2). Ward and others (1992) used portable, computer controlled, in situ, Fire Atmosphere Sampling System (FASS) packages for field measurements in North America and in tropical ecosystems. The FASS packages contain real time analyzers for measuring each second CO_2, CO, and NO concentrations and three-dimensional winds and temperatures near the ground and near the sample inlets. Variables are measured at an adjustable rate of sampling from once per second to once per minute. The FASS packages are installed in the fire with the primary sampling ports positioned 3 to 10 m above the flames. The FASS packages are triggered automatically by an increase in temperature or increase in CO above a threshold value. The infire location avoids the potential for edge effects. Figure 3

[5] Reinhardt, T.E.; Ward, D.E. [In preparation]. Factors affecting methyl chloride emissions from burning forest biomass. Environmental Science and Technology.

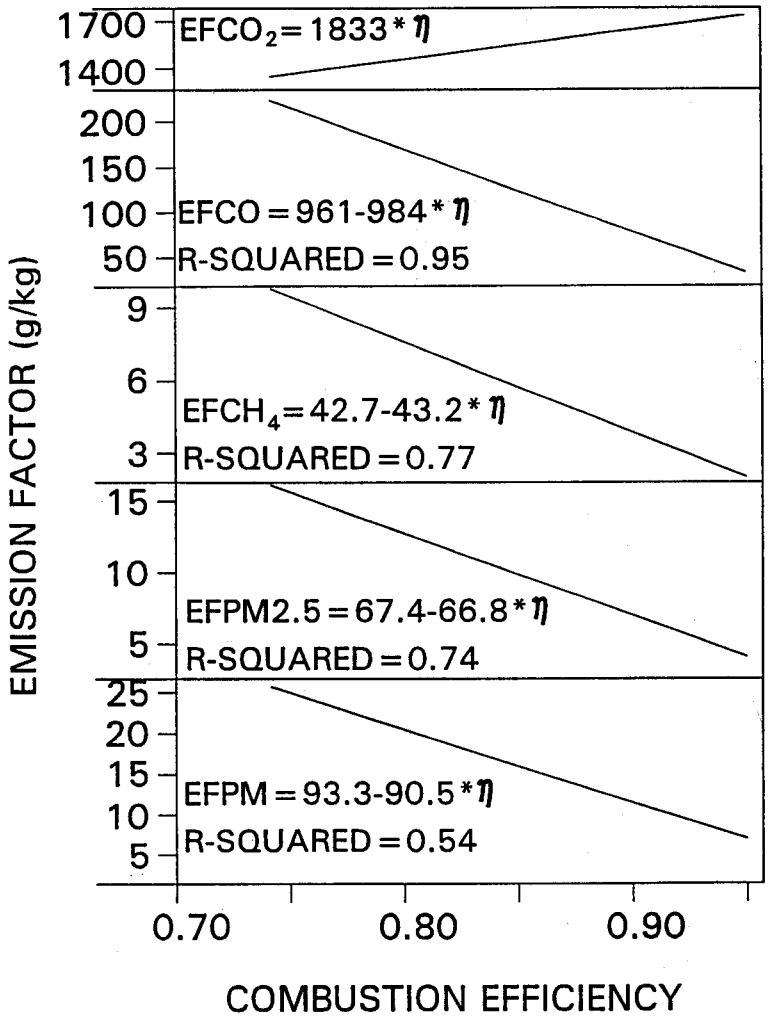

Figure 2. *Relation of emission factors for CO_2, CO, CH_4, particles less than 2.5 μm diameter, and total particulate matter without regard to size with η (Ward and Hardy 1991).*

shows models for emissions of particles less than 2.5 μm diameter (PM2.5), CO, CH_4, and H_2 for fires in tropical ecosystems. The models compare favorably with those of Ward and Hardy (1991) for logging slash fires of the Pacific Northwest (fig. 2). These models have been extended using data from Bonsang and others (1991), Crutzen and others (1985), and Greenberg and others (1984) to demonstrate the dependency of emission factors for different ecosystems on combustion efficiency. The pronounced difference due to considering combustion efficiency is illustrated by the results of Ward and others (1992) for CH_4.

White (1987) studied emissions of CO, PM, and B[a]P from the combustion of biomass fuels in the Southeastern United States. The handheld apparatus was used near the flamefronts for sampling emissions of particles onto filters and gas into bags for spreading fires--both heading and backing fires. The measurements of B[a]P emissions (ranging from 7 to 58 μg g^{-1} of PM) were considered more accurate than the laboratory experimental fires of McMahon and Tsoukalas (1978). Ward (1989) corroborated this, reporting emission ratios for prescribed fires in logging slash of 13±7 μg B[a]P per g of PM.

Temperatures in excess of 1,200 to 1,400 °C are required to dissociate molecular nitrogen and form NO. The potential for NO generation from dry, upland savanna types, burning under high ambient temperature, low relative humidity, and brisk wind conditions need to be investigated. We have measured temperatures of >1,200 °C for fires in the miombo-wooded savanna ecosystem of Zambia. Since fires in savanna ecosystems account for ≈50 percent of the biomass consumed globally, even the dissociation of a small fraction of the molecular nitrogen entering the combustion zone would contribute a large

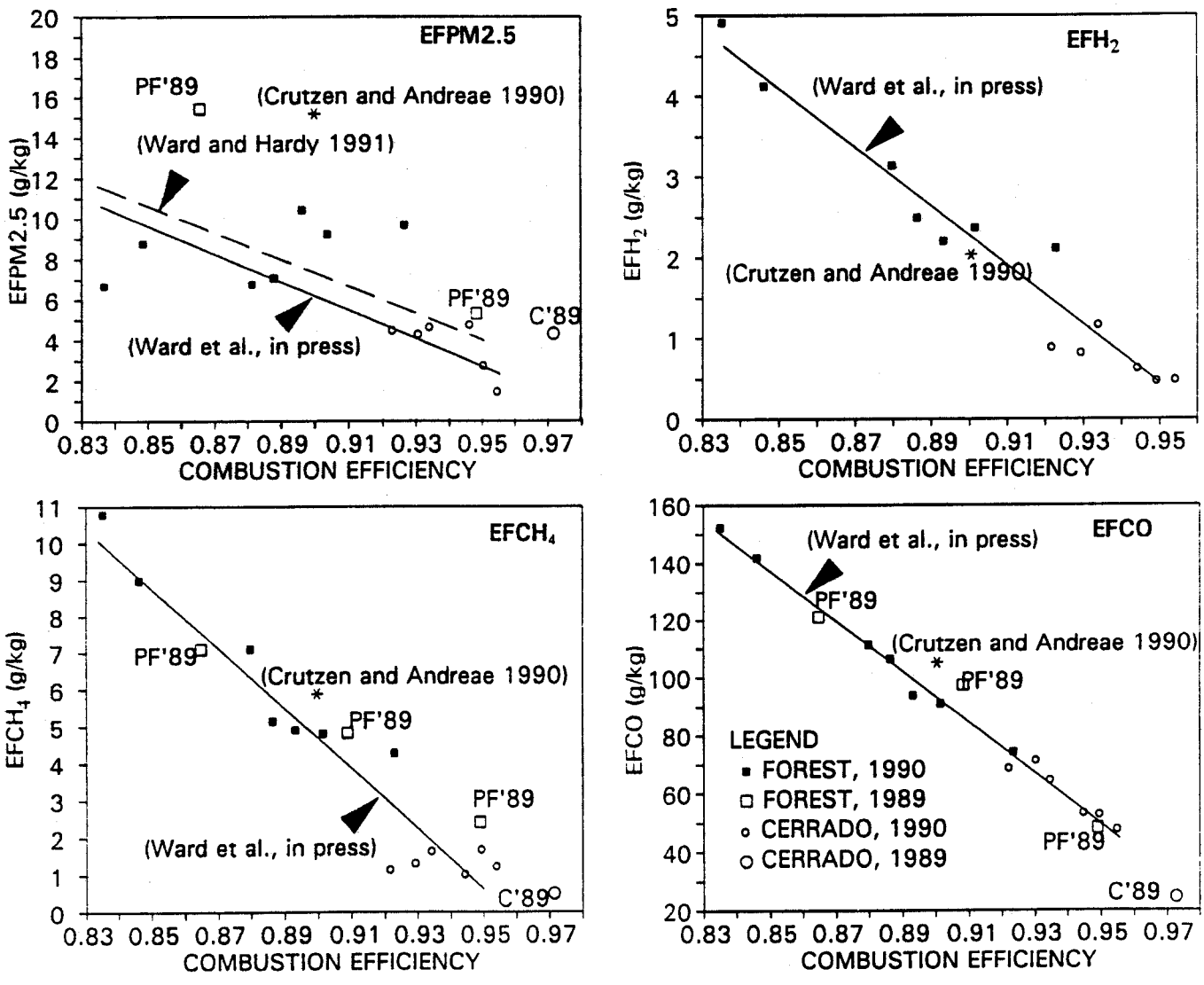

Figure 3. *Models developed for combined forest and grassland fuels of Brazil (Ward and others, in press). A comparison is made with average emission factors computed from Crutzen and Andreae (1990) and models for fine particles proposed by Ward and Hardy (1991).*

amount of NO to the atmosphere. Other high-intensity wildland fires may result in higher than normal combustion temperatures and the dissociation of molecular N_2 in larger quantities than would be predicted for lower intensity fires. A scaled combustion experiment would be valuable to demonstrate the temperature equilibrium for NO production from the dissociation of N_2 for fires burned in the open environment.

Several experiments have been performed where surface sampling from towers has been coordinated with airborne sampling techniques (Radke and others 1990a, Ward and Hardy 1991, Ward and others 1979). The results from both techniques agree reasonably well. Both tower and airborne sampling systems have benefitted from the use of CMB methods for characterizing the fuel consumed producing the measured emissions (Radke and others 1990a; Ward and others 1979, 1992).

Field sampling has many advantages including the sampling of emissions from natural fuel complexes burned in a full-

scale to near full-scale fire and the feasibility of characterizing the fuels and the fire behavior associated with the measured emissions. The experiments can be difficult to set up, relatively expensive, and for very high-intensity fires, the samples may be affected by the high temperatures.

Airborne Sampling

Airborne sampling of emissions from biomass fires is very useful, primarily because of the ability to transport many instruments to fires and because of the large scale of fires that can be examined. Sampling of a full-scale fire is necessary for verification and interpretation of findings from research using smaller scale fires. Airborne and/or remote sensing sampling techniques are the only methods that allow for measurements of smoke emissions from high-intensity, fast-moving wildfires. Measurements of emissions from large fires can be taken with minimal intrusiveness (Andreae and others 1988; Cofer and others 1988; Crutzen and others 1979, 1985; Fritschen and others 1970; Radke and others 1988, 1991; Stith and others 1981; Vines and others 1971; Ward and others 1979; Westberg and others 1981).

Vines and others (1971) computed emission ratios of PM to CO_2 and performed flux measurements from several fires of known rates of fuel consumption to estimate EF_{PM} ratios. The CMB method for field emission factor measurements was tested using ground-based and airborne methods (Ward and others 1979). The use of CMB methods for airborne sampling was perfected by Radke and others (1990a) in the early 1980's. Emission factors and ratios of emissions from biomass fires are important in studying atmospheric chemistry. A disadvantage in using airborne sampling methods is the considerable effort required to characterize fuels and fire behavior affecting the emissions (Ward and others 1992).

Airborne measurements of hydrocarbons were made by Westberg and others (1981) to demonstrate the change in the composition of reactive hydrocarbons with an increase in the concentration of O_3. They were able to show the rapid change of NO to NO_2 and the preferential loss of the more photochemically reactive hydrocarbons. Others have measured the increase in O_3 in plumes from forest fires (Evans and others 1977, Kaufman and others 1992, Radke and others 1991, Stith and others 1981).

The physical properties of aerosols have been studied by Radke and others (1991) under a wide range of fire conditions. Figure 4 illustrates the number of particles by size class over an extended range of diameter from 0.02 to 48.0 μm. The Myrtle Fire was a very active wildfire with a strong convection column during the time of sample collection; whereas, the Silver Fire was a very large fire but relatively quiescent during the periods of sample collection. For the Silver Fire of southern Oregon, Radke and others (1991) performed a Lagrangian study to examine the downwind change in the particle size distribution over a 2-day period. The results showed an increase in particle size and a decrease in the number of particles as the plume moved downwind. The rate of change of the particles was somewhat faster than simple coagulation modeling would suggest. Perhaps the strong, positively charged cloud observed over biomass fires may be an important factor in determining the rate of coagulation of particles (Latham 1991).

Improved methods are available for measuring the flux of emissions and for validating methods for predicting the flux of emissions from biomass fires. Equation 1 is a technique for estimating the flux of an emission (source strength, q). The q for particulate matter and other emissions can be measured directly if airborne systems can measure the concentration within a cross-section and the wind speed profile can be measured. Such direct in situ measurements have been made using multiple passes of an aircraft through the plume from top to bottom. An example is shown in figure 5 and Stith and others (1981) provide a detailed example.

An approach that greatly increases the efficiency of measurement utilizes airborne lidar. Here the entire plume cross-section (particulate matter) can be measured with a single pass of the aircraft above the plume or on smaller scales, a single scan from a ground based lidar (Banta and others, in press; Brock and others 1990; Radke and others, in press; Radke and others 1990b; Uthe and others 1982). Figure 6 shows a cross-sectional concentration drawing of an elevated biomass smoke plume (Radke and others 1990b). Work now completed relates the intensity of backscatter measured with an airborne lidar system to the mass concentration of the aerosols (Waggoner and others 1992). With available lidars and dropwindsonde equipment, a long-range aircraft should be capable of measuring the net particulate matter flux on continental scales (ignoring losses due to cloud processing, etc.) (Radke and Ward 1991). Such a capability is a vital part of verification of other predictive methods of estimating the flux of emissions from very large, distributed sets of biomass fires on continental scales.

Radke and others (1991) have conducted the unique study of examining the effect of water cloud processing and scavenging on the physical and chemical characteristics of smoke particles in plumes from fires in biomass fuels. These processes are particularly important in assessing the atmospheric impact of large fires since a significant fraction of large fires have plumes capped by cumulus clouds which, not infrequently, precipitate. Thus, a major fraction of the biomass smoke is exposed to cloud processing (Ward and Radke 1993). To evaluate processes occurring within the condensing cloud, several techniques are used:

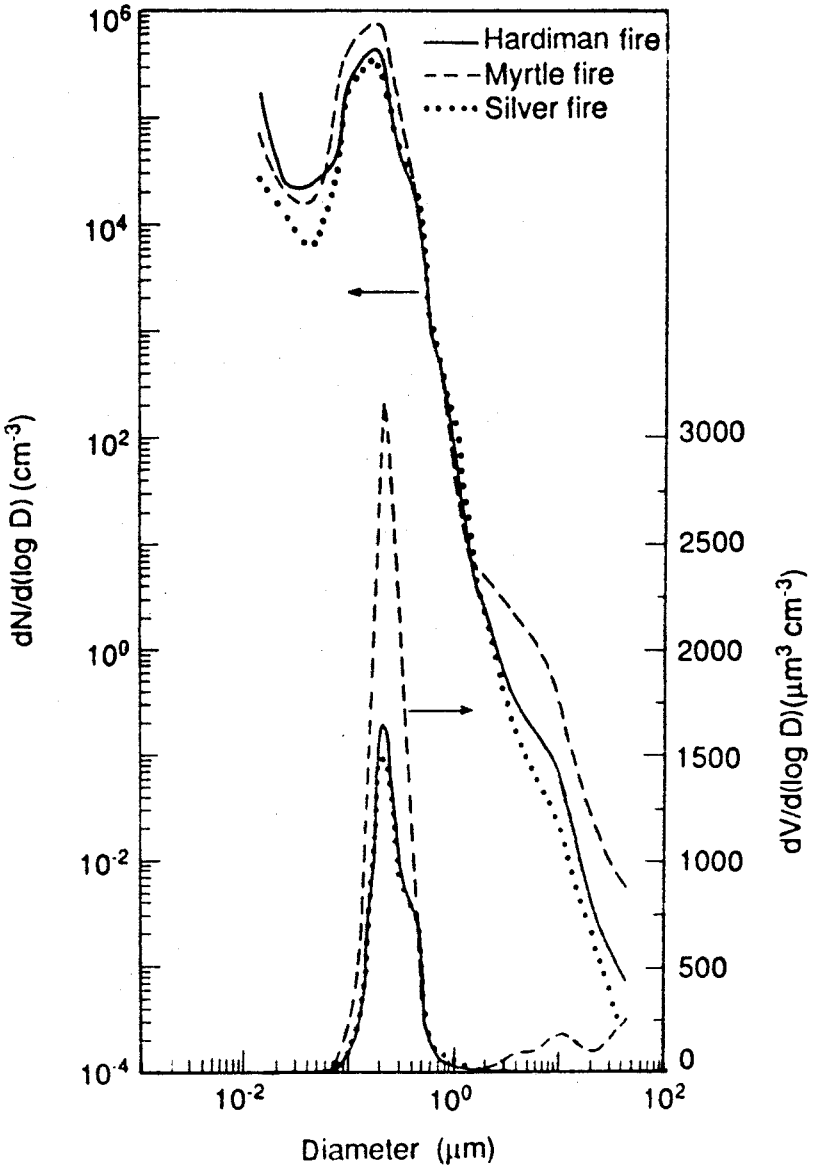

Figure 4. *Average number and volume distributions of particles by size class for smoke plumes of a prescribed fire in Ontario, Canada (Hardiman Fire) and two wildfires in Oregon (Radke and others 1991).*

(1) measuring the characteristics of the plume going into the cloud, then measuring the same features as the emissions exit the cloud,

(2) measurements of the effect of the cloud processing on emission factors measured below the cloud and on exit from the cloud,

(3) measurement of the ratio of smoke or a surrogate for smoke interstitial to the cloud droplets, and

(4) measuring in close time proximity the smoke concentration going into the cloud and the amount of smoke in the cloud water.

Most work performed by Radke and others (1991) has used method 1 above. Radke and Ward (1991) discuss the implications of emissions modification from cumulus cloud processing and how prescribed fire may be modified to enhance the cloud processing benefits from an air quality standpoint. Their work shows precipitating clouds remove nearly 100 percent of the supermicron smoke particles in all but the smallest capping cumulus clouds. In addition, the accumulation mode smoke particles (which represent the bulk of the smoke particles with potentially long atmospheric residence times) can enter cloudwater with significant efficiency (40 to 80 percent) and are removed from the cloud with equal efficiency (30 to 90 percent) by precipitating cumulus with depths greater than 2 km. No other research that we are aware of has been

performed that examines the fate of smoke emissions in the atmosphere (Ward and Radke 1993).

Airborne sampling techniques are expensive, but can be used to study the effect of atmospheric processing on the released emissions. Measurements from airborne sampling platforms should be directly correlated with spaceborne measurements. The capability of integrating samples across large areas makes airborne sampling especially attractive.

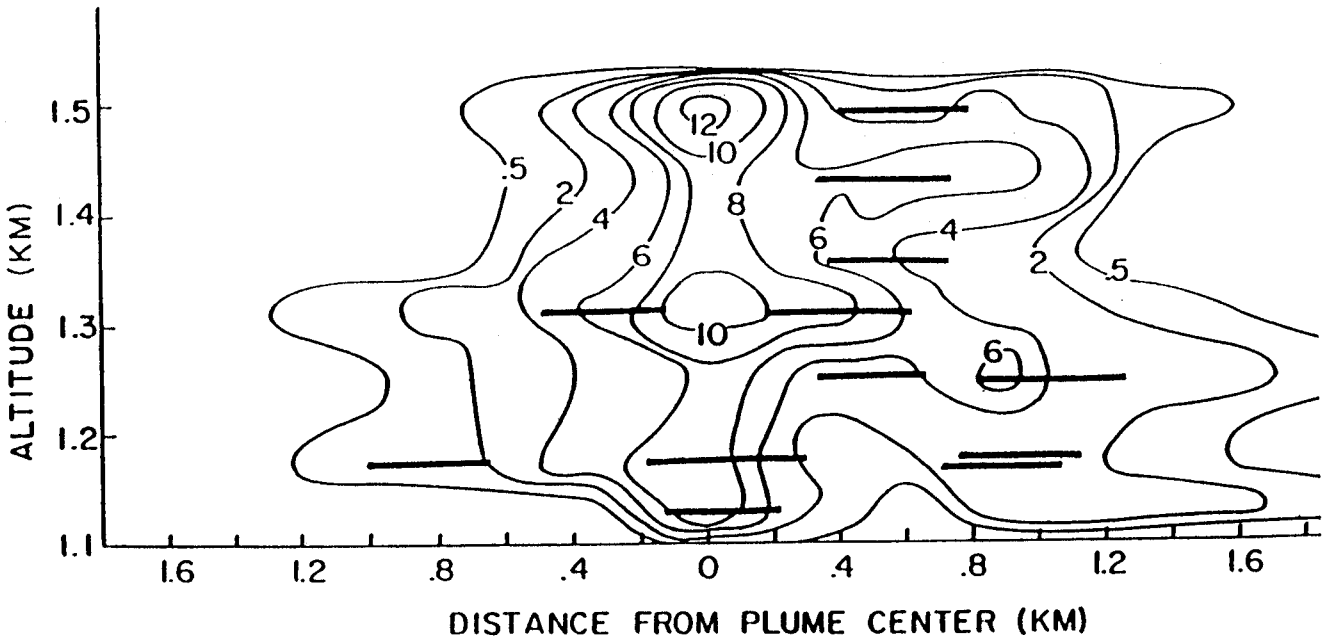

Figure 5. *Smoke plume cross-section of the light scattering coefficient (x 10^{-3} m^{-1} at 550 nm) measured perpendicular to the long axis of the plume using an integrating nephelometer. Airborne sampling was performed at multiple altitudes, and regions of equal values of light-scattering coefficients were drawn from the measurements.*

Figure 6. *Example of LIDAR sounding of the backscatter from smoke particles in a plume from a prescribed fire in southern British Columbia, Canada. New relations provide for the drawing of isopleth lines of equal particulate matter concentration from data collected during a single pass either above or below plumes from biomass fires.*

Satellite Techniques

It is important to develop better methods of measuring the spatial and temporal scale of biomass burning. Several parameters need to be quantified to make satellite techniques a viable method for estimating emissions production. For example, given that total biomass per unit of area data is available continentally or regionally, then measurements or algorithms for estimating combustion factors can be used for estimating the amount of biomass consumed. In North America, a few techniques for estimating combustion factors have been developed dependent on the amount and duration of precipitation and drying conditions (Brown and others 1991). As with other techniques for estimating emission factors, knowledge is needed regarding η for the conditions for the ecosystem and the meteorological conditions affecting the fire. A variety of remote sensing techniques is being explored.

Kaufman and others (1992) discuss the use of satellite data for assessing fires and the smoke from fires. Fires can be detected using the Advanced Very High Resolution Radiometer (AVHRR). This technique was developed at the Brazilian Institute for Space Research (INPE). The 1-km resolution imagery for the 3.75-μm and 11-μm channels can sense fires as small as 10 m by 10 m (although it provides no measure of fire size at scales below 1 km^2). The AVHRR imagery is being used to monitor the frequency of burning in Brazil. For example, in 1990 in Brazil there were 283,800 fires detected using this system from June 1 to October 1. In 1991, during the same period, there were 440,080 fires detected, or 55 percent more fires in 1991 than in 1990.[6] A correlation has been developed between the number of fires and the amount of deforestation as well as the fire count and the mass of emitted smoke particles (Kaufman and others 1992). A combination of the Landsat Multi Spectral Spectrometer (MSS) satellite data has been used through a double sampling procedure to account for the area of deforestation through burn scar analysis and fire counts.

Kaufman and others (1992) developed a technique for approximating the particle mass loading of the atmosphere from optical thickness measurements from AVHRR-visible and near-IR images taken over vegetated terrain. The correlation of smoke particles with trace gases then makes it possible to estimate the release of trace gases to the atmosphere from biomass fires. In the future, correlations may be possible between estimates of O_3 concentrations and the quantity of emissions produced from biomass burning (Fishman 1991) and through the use of higher resolution multispectral imaging that may significantly improve estimates of both optical depth and particulate matter mass loadings.

Spaceborne measurements are likely the only viable alternative for tracking the temporal distribution of the frequency of fires in many areas of the world. Coupling spaceborne, airborne, towers, laboratory, and benchtop studies provides a wide matrix of tools for examining fires from the process level to the global level. The results from each scale of measurement will provide the fundamental knowledge necessary for evaluating the impact of smoke emissions on a global-to-continental and subcontinental scale.

NEED FOR NEW TECHNIQUES IN ANALYZING SMOKE EMISSIONS

The very high exposures to smoke received by wildland firefighters from wildfires occurring in the Western United States during 1987 and 1988 underscored the need for more knowledge concerning the toxicological properties of smoke from fires in biomass fuels. This section summarizes two very promising instrumental approaches for the study of smoke emissions from biomass fires. Both techniques have application across the broad range of scales of research discussed in the previous sections. The feasibility of using Fourier Transform Infrared (FTIR) and Chemical Ionization Mass Spectrometry (CIMS) has been demonstrated in both the field and laboratory. Quality measurements of irritating and/or carcinogenic compounds have resulted that would be difficult to achieve by other techniques.

The vast majority of molecules absorb infrared (IR) radiation in the "fingerprint region" (400 to 4,000 cm^{-1}). For ambient measurements, H_2O and CO_2 block the transmission of IR across large portions of the electromagnetic spectrum. However, hundreds of important trace gases absorb in the "atmospheric windows" (where H_2O and CO_2 transmit well). These are the molecules that can be detected in smoke and air by FTIR, generally at ppb levels of concentration. The feasibility of using FTIR for smoke measurements in the field and laboratory environments was demonstrated by Griffith and others (1991). An FTIR system can best be used in a combustion laboratory setting in tandem with a number of real-time analytical instruments that can also measure the concentration of gases from experimental fires. The goals for such a study would include:

- Complementary path integrated measurements of gases now only measured at a specific point
- Simultaneous in situ measurements of reactive gases

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[6] Luduvice, Magna; Mello, Claudia Maria. 1993. Personal communication with D.E. Ward. On file with: U.S. Department of Agriculture, Forest Service, Intermountain Research Station, P.O. Box 8089, Missoula, MT 59807.

•Quantification of nitrogen compounds; e.g., NH_3 and HNO_3
•A technique to use in searching for chlorinated compounds.

The second technique discussed--now feasible, but very expensive (>$500,000)--is chemical ionization mass spectrometry (CIMS). Chemical ionization mass spectrometry can directly sample air with ppt sensitivity for a multitude of compounds. Chemical ionization mass spectrometry can measure some compounds that FTIR and GC techniques are not well suited for. Chemical ionization mass spectrometry is sensitive to ketone compounds that are poorly resolved using IR techniques because of blockage due to water lines, and also sensitive to compounds with molecular weights up to 200 amu whose IR spectra in air are intractable. In addition, CIMS is the most sensitive in situ technique in existence. High sensitivity is important for a number of reasons that should be of value in guiding the use of the method for characterizing smoke:

(1) Compounds with a long tropospheric lifetime, that can participate in catalytic O_3 destruction in the stratosphere, are at very low concentrations in the atmosphere and are expected to be produced from biomass burning in very low quantities (e.g., CH_3Cl and CH_3Br).
(2) Extremely toxic compounds (e.g., dioxin) require ultrasensitive sampling techniques.
(3) Chemical ionization mass spectrometry (CIMS) can measure OH radicals: the most important tropospheric catalyst and likely an important air toxic substance very near fires. In the troposphere, OH radicals are present in ppq concentrations.
(4) An aircraft-based deployment of CIMS offers the most powerful way to track chemical changes in the plume as it is dispersed.

Because of the very high cost of the two techniques listed above and the large amount of training required to produce useful results, groups need to work in collaboration.

CONCLUSIONS AND FUTURE RESEARCH NEEDS

- Whereas CO and CH_4, for example, are linearly correlated, the ratio of CO/CO_2 is nonlinearly correlated with both CO and CH_4 and many of the other hydrocarbons. In general, combustion efficiency (η) and modified combustion efficiency ($\tilde{\eta} = CO_2/(CO_2+CO)$) should be used as an independent variable in preference to CO/CO_2.
- CO_2 has been used to expand emission factor data for global estimates. However, combustion conditions that decrease the production of products of incomplete combustion tend to increase the production of CO_2. Serious errors may result from using a constant ratio.
- Although the main source of NO is from the oxidation of fuel-N, a second source of NO may result from the dissociation of molecular nitrogen under ideal burning conditions for very high-intensity fires.
- Progress has been made in the area of understanding the effects of cloud processing on smoke emissions. More work is needed, especially on the effects of water condensation processes on particles. The selective removal of both particles and gases needs further study.
- The electrical properties of aerosols may be of critical importance in understanding the life cycle of carbon particles in the atmosphere.
- The chemical composition of biomass is known to affect the release of trace emissions of nitrogen; however, little is known regarding other trace emissions either as the trace material may serve to catalyze the formation of products or lead to the formation of trace gases and particles.
- Although η and $\tilde{\eta}$ work well as integrators of fuel morphology and weather influences on fire behavior and resulting emissions, variables more closely coupled to parameters used for characterizing ecosystems are needed for evaluating emissions and combustion factors.
- In an effort to develop protocols for evaluating emissions based on a few measurements of stable products of combustion, more emphasis needs to be placed on cross-correlating stable compounds with those that are reactive or are difficult to sample and characterize under field conditions. Field experiments need to be coupled with closely controlled and designed laboratory-scale experiments to gain an understanding of the mechanisms affecting the release of trace emissions.

- A new generation of techniques is needed for gas measurements similar to the Fourier Transform Infrared (FTIR) system for quantifying emissions *in situ* rather than sampling, derivatization, or other difficult protocols. CIMS technology may appropriately be used to supplement the measurements possible using FTIR instrumentation. For particulate matter, lidar or other nonintrusive remotely operated systems are needed for measuring the vertical and/or horizontal profiles.

- New methods of characterizing plume particle size distribution, shape of particles in smoke plumes, and the light extinction, scattering and absorption characteristics of the particles are needed. Consideration needs to be given to the effect of wet atmospheres (where the relative humidity exceeds 60-95 percent) on properties of particles affecting the absorption and scattering of light.

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Cold Acclimation and Deacclimation of Pecan Trees[1]

Frank B. Matta and Amin F. Kawatin[2]

ABSTRACT

Differential thermal analysis, 3, 3, 5-tripheyletetrazoliam chloride stain, browning, and electrolyte conductance tests were performed on four pecan cultivars to monitor acclimation and deacclimation phenomenon. The highest critical temperature of -8.7 °C of 'Hughes' buds occurred in September. Lowest critical temperature of -17 °C occurred with 'Desirable' buds in December. Viability tests indicated that death of various tissue was related to a given critical temperature.

Keywords: Cold hardiness, viability test.

INTRODUCTION

Pecans, *Carya illinoensis* (Wangenh.) c. Koch, grow naturally from the mountains of central Mexico to northern Iowa and are considered a cold-hardy species. However, rootstocks and scions have been moved from their native habitat into other regions with new environmental conditions, and as a result, are less adapted to cold temperature.

Currently, Mississippi has 16,000 to 18,000 acres of pecan trees with an estimated annual production of 8 to 10 million pounds, but pecan production is marginally profitable in Mississippi. A survey conducted in 1990 showed that the wet spring of 1988 and late freezing temperatures in 1989, with drought in-between, was the major concern of every pecan grower in Mississippi (Rasberry 1990) .

Generally, cold injury occurs in freezing weather when the temperature of the tissue drops below the point of acclimation. As a result of these freezing temperatures, tissue cells either dehydrate or rupture after ice has formed inside them.

Cold damage is not limited to the spring; it can also occur in winter when trees face severe cold or in fall before trees enter dormancy and are acclimated to low temperature (Spark and Payne 1978). One way to reduce cold damage in Mississippi is by planting less cold-susceptible cultivars.

Freeze damage causes dramatic losses to fruit growers, exceeding the combined losses from diseases, insects, rodents, and weeds. The inherent variability of pecan trees offers hope in extending the range of adaptability and improving the yield of current cultivars (Spark 1992). Pecan trees can be improved significantly by choosing cultivars that are less susceptible to cold injury.

Using differential thermal analysis (DTA) as a direct and simple way of determining cold hardiness of pecan cultivars grown under Mississippi conditions, as well as conducting viability tests, will help to provide valuable information to growers and plant breeders. Therefore, the objectives of this study were to: (1) determine cold hardiness of buds and stems of four pecan cultivars grown under Mississippi conditions, (2) determine viability of tissues by visual browning, electrolyte leakage and triphenyle tetrazolium chloride tests, and (3) determine acclimation and deacclimation patterns of pecan buds and stems.

MATERIALS AND METHODS

Pecan bud and stem sections were collected from David Young's orchard, Starkville, MS. The four cultivars included in this study were Desirable, Owens, Jackson, and Hughes.

Samples were randomly selected in the morning from trees of each cultivar, wrapped in plastic bags, stored on ice, and moved to the cold-hardiness laboratory in the Horticulture Department at Mississippi State University. Cold-hardiness experiments were executed immediately.

Differential thermal analysis was performed at 7-day intervals from September 1 and continued until the end of March. In addition to the detection of supercooling points of different structures of the bud and stem, three viability tests (visual browning, electrolyte leakage, and tetrazolium tests) were performed on the 15th of each month.

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Differential thermal analysis was performed as follows: the thermocouple is composed of two wires; one wire was attached to the stem (1 cm long), or primary bud, using aluminum foil; the other wire of the thermocouple was used as the reference temperature.

The thermocouple, with samples attached to it, was placed in an insulated box, which was placed in a programmable freezer (Scien Temp Corp., Adrian, MI). The other end of the thermocouple was connected to a CR-7 Measurement and Control System, which dumps the data to a cassette recorder connected to it. By unloading the data from the recorder into a computer and using a special software program, a graph was generated showing the supercooling points and the exotherms of the frozen sample.

Viability Tests

Electrolyte leakage, tetrazolium, and Browning tests were conducted simultaneously. Each cultivar was assigned a number and each treatment a letter. Buds and stems of the four cultivars were frozen to (-5, -10, -15, -20, -25, -30, -35, -40 °C), starting in September and continuing through March. Three replications (single bud or stem) on each evaluation date (weekly) were executed.

Tetrazolium Stain Test

Samples were set in petri plates and placed in the freezer. At each temperature indicated above, samples were removed from the freezer and then placed in an ice bath for slow thawing. After thawing, a solution of 1 percent TTC (2, 3, 5-triphenyle-tetrazolium chloride) was added. Samples were incubated for 24 h in the dark. Visual determination for dead tissue (tissue not developing any color) was recorded.

Electrolyte Leakage Test

Samples were placed in test tubes and subjected to various low temperature treatments. After thawing, 20 ml distilled water was placed in each test tube. The tubes were incubated for 24 h, then Rl (conductivity of the solution) was measured using a YSI Scientific Model 35 Conductance Meter (YSI Scientific, Yellow Spring, OH). Samples were killed by placing them in an oven (70 to 80 °C for 30 min), then R2 (total leakage) was measured after 24 h of incubation. Leakage were determined by the following formula:

$$\text{Percent electrolyte leakage} = \text{Rl/R2} * 100.$$

Visual Browning Test

Samples were removed from the freezer at each test temperature and placed in an ice bath for slow thawing. Thawed samples were incubated at room temperature for 24 h. Buds and stems were then cut longitudinally to observe tissue browning.

Experimental Design

Differential thermal analysis was conducted using a completely randomized design (CRD) with three replications. Means were separate by the least significant difference (LSD) at 0.05.

Electrolyte leakage, visual browning, and tetrazolium stain tests were conducted and analyzed by using a repeated measure on a split plot in a CRD design with three replications. Means were separated by an LSD test at 0.05 or 0.01 level of significance.

RESULTS

All pecan cultivars studied showed supercooling. Supercooling varied depending on cultivar and was influenced by date. Hughes buds had the highest critical temperature of -8.7 °C in September, while Desirable buds had the lowest -17.0 °C in December (table 1).

Table 1. *Critical temperature of pecan buds measured by differential thermal analysis (DTA)*

Cultivar	September	October	November	December
	Critical temperature (°C)			
Hughes	-8.7*	-8.5	-10.8	-15.8
Desirable	-9.4	-11.0	-16.6	-17.0
Owens	-8.9	-11.3	-13.4	-16.3
Jackson	-9.6	- 9.3	-10.5	-16.9

* Average of four measurements (1 per week). Each measurement was replicated three times (single bud replicate).

Stems of Hughes showed the highest critical temperature at -12.8 °C in September. The lowest at -38.8 °C in December was exhibited by Owens (table 2). George and Burke (1977) showed that seasonal changes in supercooling corresponded to cold hardiness changes of wood of shagbark hickory. The same observations were also made in apple twigs (Quamme and others 1972).

Table 2. *Critical temperature of pecan stems measured by differential thermal analysis (DTA)*

Cultivar	September	October	November	December
	Critical temperature (°C)			
Hughes	-12.8*	-16.7	-22.1	-33.4
Desirable	-13.7	-17.1	-21.8	-34.3
Owens	-13.5	-17.1	-22.1	-38.3
Jackson	-14.0	-16.7	-20.5	-33.4

* Average of four measurements (1 per week). Each measurement was replicated three times (single bud replicate).

Low temperature exotherm (LTE) values for pecan buds ranged from -27.8 to -35.4 for Hughes in October and Owens in December, respectively (table 3). In 1973, Quamme and his associates showed that LTE's could also be detected in nonliving tissues.

Table 3. *Low temperature exotherm of pecan buds measured by differential thermal analysis (DTA)*

Cultivar	October	November	December
	Critical temperature (°C)		
Hughes	-27.8*	-30.7	-35.2
Desirable	-29.5	-33.1	-33.8
Owens	-29.0	-31.7	-35.4
Jackson	-28.8	-30.7	-35.2

* Average of four measurements (1 per week). Each measurement was replicated three times (single bud replicate).

None of the cultivars showed an LTE in September. In the third week of October, buds started to show an LTE. No relationship was found between the temperatures at which injury began and those at which LTE's occurred during the experiment.

Instem LTE's were related to xylem injury. This result was similar to that found in peaches (Quamme 1974).

The various structures of buds and stems varied in hardiness. The bud scales were the first to be frozen, followed by catkins and apical meristem. In stems, the pith was frozen first, followed by the bark, then the xylem. These results indicate that injury to the meristem concludes in the death of buds and injury to xylem tissue results in death of stems.

Viability tests indicated that the temperature at which injury or death occurred agreed with DTA results. Injury of the various tissue was associated with a given exotherm.

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Wintertime Anomalies in Ozone Deposition Above a Subalpine Spruce-Fir Forest[1]

K. Zeller and T. Hehn[2]

ABSTRACT

Ozone deposition wisdom tells us that ozone deposits on surface canopies. High rural concentrations of ozone are thought to be either stratospheric in origin or advected from some upwind urban source. In both cases, in addition to ambient production and destruction due to photochemical reactions, ozone is also expected to transport downward from within the atmosphere's surface layer to meet a chemical demise either within stomata or on leaf and mineral surfaces. Recent eddy correlation measurements of vertical ozone flux above a rural canopy *Picea engelmannii* (48 percent), *Abies lasiocarpa* (48 percent), *andPinus contorta* (4 percent) at 3,186 meters elevation in the Snowy Range Mountains of Wyoming demonstrate an upward flux during winter months. Upward ozone fluxes reaching 0.5 micrograms (μg) m^{-2} s^{-1} were measured during the 1991-92 winter season. These values, which convert to 10.8 kilograms (kg) Km^{-2} day^{-1}, were of the same order as the downward fluxes measured during the peak summer 1992 growing season. 'Counter-gradient' ozone profiles have been reported in Europe based on the assumption that ozone flux is downward; however, these are the first data to suggest that the associated ozone fluxes may not be counter-gradient. (Note: gradients or profiles are changes in concentration over distance, and fluxes are directional movements of mass.)

The explanation for these upward ozone fluxes is unknown; however, they suggest surface or canopy emissions of ozone. Forests are sources of natural nonmethane hydrocarbons, potential precursors to elevated ambient ozone concentrations, hence, potential ozone producers. The anomalous Snowy Range ozone flux observations occur in winter, and ozone production requires nitric oxide (NO) in addition to solar UV and nonmethane hydrocarbons--an unlikely winter scenario.

Keywords: Counter gradient, deposition velocity, eddy correlation, greenhouse gas, ozone flux, trace gas flux.

INTRODUCTION

Recent measurements of vertical ozone (O_3) profiles above forested canopies reported in the literature have claimed counter gradients (opposite to expected), where ozone concentrations are greater closer to the surface and decrease upward (Enders 1992, Fontan and others 1992). The counter gradient claim for ozone and other atmospheric constituents (Denmead and Bradley 1985) comes from the universally accepted idea that ozone 'deposits' toward the Earth's surface because the surface (plants, soil) breaks down ozone, and ozone is not generated at the surface. Hence, measured vertical gradients of ozone that decrease with height above ground would generate an upward movement of ozone mass or 'upward ozone flux' (Fick's Law), which is opposite the assumed downward ozone flux. Fontan and others (1992) have measured increases in ozone under forest canopies in isolated circumstances; however, they have not fully explained their measurements. Enders and others (1989) have also reported negative ozone deposition velocities based on occasional upward ozone fluxes. Gruzdev and others (1993) have measured higher surface ozone concentrations (single height measurements only) during winter months (relative to summer months) in the Antarctic above snowcover. They attribute these relatively higher ozone concentrations to stronger wintertime cyclonic activity aggressively transporting upper air ozone to the surface.

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[1] Paper presented at Research and Applications in Chemical Sciences in Forestry; 1994 January 31-February 2; Starkville, MS.

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High rural concentrations of ozone have been attributed to either advection from upwind urban source regions or the stratosphere. Local ambient production and destruction of ozone due to photochemical reactions is another reason for elevated ozone concentrations. Ozone is typically transported downward from within or through the atmosphere's surface layer and is depleted within plant stomata or on leaf and mineral surfaces.

The objective of this paper is to report measured O_3 fluxes and concentrations during different seasons in 1992 directly above a subalpine forest canopy to generate further discussion of these unusual measurements.

METHODS

Site

The U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station (RMFRES), conducts air-related ecosystem research at its Glacier Lakes Ecosystem Experiments Site (GLEES) in the Snowy Range of the Medicine Bow National Forest, Laramie District, Wyoming. The data presented in this paper were collected at the Brooklyn Lake tower site located within the GLEES area. A complete description of the GLEES complex can be found in Musselman (in press). The Brooklyn tower site at 3,186 meters in elevation is located approximately 3 kilometers southeast of the Snowy Range ridge (3,460 m average elevation). The tower is 29 m in height and is situated in a spruce-fir forest opening approximately 30 m in diameter. The major tree species in the forest are *Picea engelmannii* (48 percent), *Abies lasiocarpa* (48 percent), and *Pinus contorta* (4 percent). The average forest stand height surrounding the tower is 17 m. The terrain within 1 km of the tower slopes +2.5 percent from west to east and -9.7 percent from north to south. The terrain to the west-northwest, the predominant wind direction, is relatively flat. Three-meter ozone concentrations measured at the Brooklyn tower during 1990 ranged from 10 ppb to 80 ppb and peaked in May. These values are consistent with rural ozone concentrations measured at similar altitudes in Europe (Musselman and others 1992).

Instrumentation

The Brooklyn tower is instrumented with standard meteorological sensors at 29 and 10 m. Total solar radiation, precipitation, and soil temperature are also collected at the site. Complete details of the standard long-term meteorological measurements are given in table 7.2 of Fox and others (in press). The eddy correlation system used to measure ozone fluxes, sensible heat fluxes and momentum fluxes, as well as ozone concentrations, temperature, and wind speeds was located at 23 m on the Brooklyn tower (6 m above tree height). This system was developed and fully tested previously by the RMFRES at a prairie site (Zeller and others 1989). Table 1 lists the sensors that were used for the flux-related measurements. A three-axis Gill propeller anemometer was used because of its durability in extreme weather conditions.

Table 1.--*23-m instrumentation at Brooklyn Tower Site*

Instrument or model	Type	Parameter
TECO 49 O_3 Analyzer	UV adsorption	c: O_3 concentration
CAAM1 O_3 Analyzer	Chemiluminescence (fast response)	c': O_3 fluctuation
R.M. Young uvw	Gill propeller anemometer	w,w': vertical wind u,u': horizontal wind v,v': horizontal wind
AIR, Inc. FT-1A-T	Platinum resistance	T,T': temperature
Thermocouple	Copper constantan	Temperature lapse rate

Flux Measurements

Vertical flux measurement by eddy correlation involves interfacing sensitive micrometeorological and chemical sensors with real-time data processing. The two essential sensors used for the ozone measurements presented here were the Gill uvw anemometer for determining vertical wind fluctuations (turbulence) and a chemiluminesence ambient air monitor (CAAM1) for the O_3 fluctuation measurements (Ray and others 1986, Zeller and others 1989). These fluctuations or deviations are termed "eddies". The eddy deviations of ozone concentration, c', and three-dimensional vector wind component deviations (u', v', and w') are multiplied or correlated, then averaged over half-hour sampling periods to obtain the vertical flux, F_c (equation 1). This method is described by McMillen (1986):

$$F_c = \overline{w'c'} \qquad (1)$$

where:

F_c = The vertical flux of c in units of either parts per billion * m per second (ppb m s^{-1}) or micrograms per square meter per second ($\mu g\ m^{-2}\ s^{-1}$);

c' = $c - \overline{c}$, Ozone concentration, which is the instantaneous deviation between the instantaneous concentration, c (ppb), and the average concentration, $\overline{c}$ (ppb); and

w' = $w - \overline{w}$, the vertical wind component in m s^{-1} analogous to c'.

Here, positive F_c would indicate upward flux movement and negative F_C would indicate deposition or downward flux movement. (Note that a minus is often applied to the right hand side of equation 1 so that F_c, defined as deposition, can be reported as a positive value.)

Equation 1 is the essence of the eddy correlation measurement. Heat flux is also obtained using equation 1 replacing ozone with ambient temperature. Likewise, momentum flux is obtained by replacing ozone with the horizontal wind component. Certain meteorological and terrain conditions must be satisfied to obtain valid flux measurements: the terrain should be relatively flat and the variations in the meteorological and chemical parameters measured should be statistically stationary. When the site is not flat, a coordinate rotation is applied to the data, and fluxes are calculated perpendicular to the mean wind streamline (McMillen 1986) to account for irregular terrain . If the half-hour average vertical angle of the mean wind is reasonable (e.g., the mean wind streamline is within 20° of horizontal), measured fluxes are considered valid for this paper. Complete details and verification of the overall approach used are described by Massman and others (1990), Zeller (1993), and Zeller and others (1989 and 1990).

Fluxes were measured and calculated in real time utilizing equation 1. The Gill uvw anemometer data were corrected in real time for the inherent cosine response problem (Massman and Zeller 1988). Except for a few days, all periods presented here experienced west-northwest horizontal wind directions. The ozone flux data have not been corrected for vapor effects, instrument response, and instrument separation as described by Zeller and others (1989). Due to extreme weather conditions, only sporadic simultaneous measurements of sensible and latent heat fluxes are available to complete the flux corrections. However, because these corrections are positive in value (Zeller and others 1989), the combined effect of the corrections would typically increase the magnitude of the upward-moving ozone flux values slightly more than the downward-moving flux values. The overall results in terms of order of magnitude and flux direction are not affected by the lack of these corrections, and the upward flux values are conservative.

Trace gas deposition results are often reported as deposition velocity, V_d. Employing equation 2, by applying a deposition velocity to any concentration, site specific trace gas deposition can be estimated. By combining equations 1 and 2 using measured or modeled ozone flux and measured ozone concentration, V_d can be found:

$$F_c = -\overline{c}\, V_d \qquad (2)$$

Note that the minus sign in equation 2 provides for positive V_d in the downward direction, hence, a negative deposition velocity indicates upward movement. Enders (1989 and 1992) reported negative V_d values that indicate upward ozone fluxes similar to the results presented in this paper.

RESULTS AND DISCUSSION

The vertical ozone flux measurements ($\mu g\ m^{-2}\ s^{-1}$) demonstrate a consistent daytime upward flux during snow-covered winter months and downward ozone flux during the growing season with absence of snowcover. Upward ozone fluxes

reaching 0.5 μg m^{-2} s^{-1} were measured during the 1991-92 winter season. These values, which convert to 10.8 kg Km^{-2} day^{-1}, were the same magnitude as the downward fluxes measured during the peak summer 1992 growing season at the same location. Dramatic shifts in these diurnal ozone flux movement patterns occurred during the week snowmelt was completed: May 14, 1992 (Julian Day 135), and during the week snow again blanketed the forest floor: October 4, 1992 (Julian Day 278). Figures 1 through 5 give the ozone concentrations and ozone flux for five multiday periods from the end of 1991 through fall 1992.

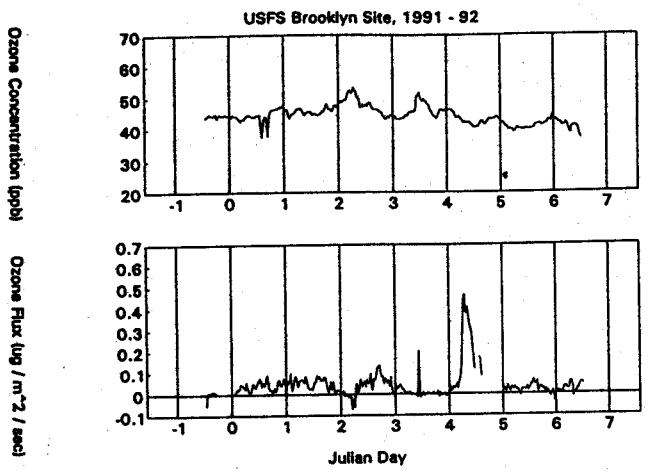

Figure 1. *Half-hour concentration (a) in parts ber billion (ppb) and ozone flux (b) in micrograms per square meter per second (μg m^{-2} s^{-1}) for the period December 31, 1991, to January 6, 1992.*

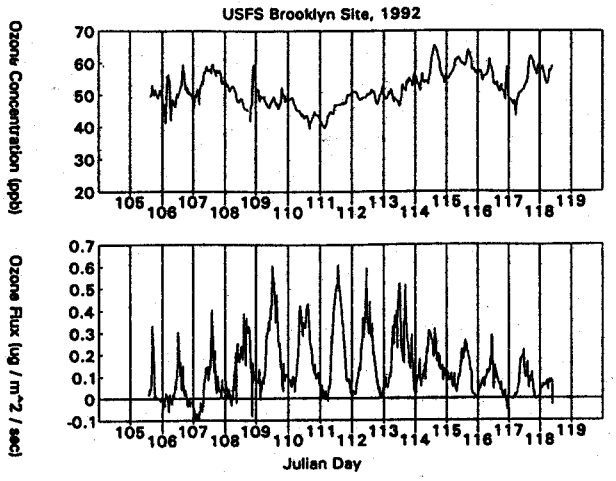

Figure 2. *Half-hour ozone concentration (a) in parts per billion (ppb) and ozone flux (b) in micrograms per square meter per second (μg m^{-2} s^{-1}) for the period April 14, 1992, to April 27, 1992.*

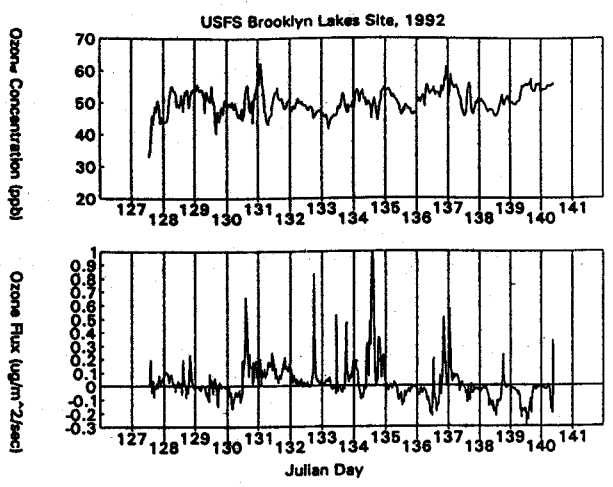

Figure 3. *Half-hour ozone concentration (a) in parts per billion (ppb) and ozone flux (b) in micrograms per square meter per second (μg m^{-2} s^{-1}) for the period* May 6, 1992, to May 19, 1992.

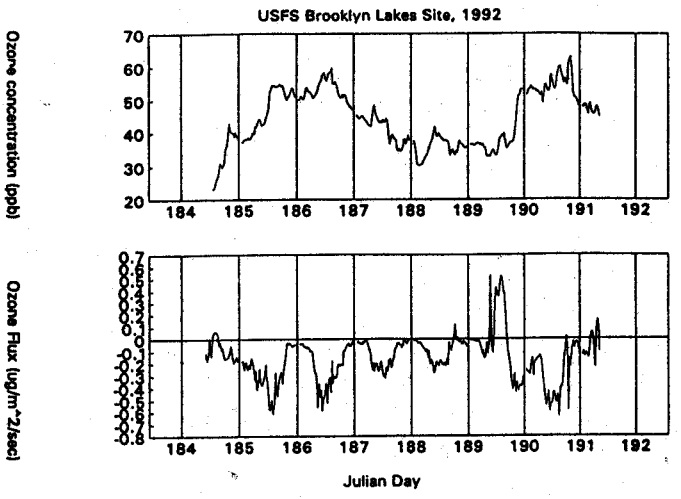

Figure 5. *Half-hour ozone concentration (a) in parts per billion (ppb) and ozone flux (b) in micrograms per square meter per second (μg m^{-2} s^{-1}) for the period* September 29, 1992, to October 9, 1992.

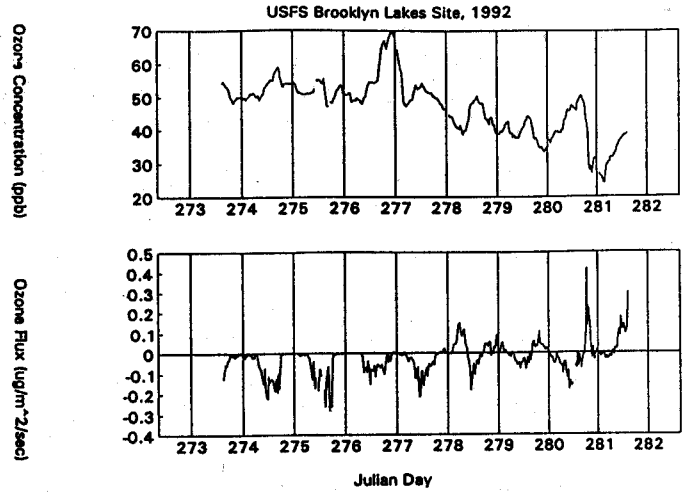

Figure 5. *Half-hour ozone concentration (a) in parts per billion (ppb) and ozone flux (b) in micrograms per square meter per second ($\mu g\ m^{-2}\ s^{-1}$) for the period September 29, 1992, to October 9, 1992.*

Figure 1, December 30, 1991, (Julian Day 364) through January 6, 1992, (Julian Day 6), was the first indication that ozone fluxes were positive at the Brooklyn tower. Although maximum downward ozone fluxes are associated with sunny days, the upward flux peak on January 4 occurred on a cloudy day. Temperatures were below freezing the entire period. Deposition velocities for the period ranged from -0.7 (upward) to 0.1 cm s^{-1}. Figure 2, April 14 to 27, 1992, demonstrates the day-to-day consistency of the upward ozone fluxes over several days. Temperatures ranged from 5 °C above zero to -10 °C during this period. Deposition velocities ranged from -0.8 to 0.2 cm s^{-1}. Figure 3, May 6 to 19, 1992, covers the period spring snowmelt ended and the diurnal ozone flux wave changed from upward to downward. Ozone fluxes ranged from positive to negative on any day during this period but remain predominately negative after May 17 (Julian Day 138). The temperature ranged from 3 to 10 °C above zero except for a brief nighttime excursion below freezing on May 10. Deposition velocities peaked at -1.5 cm s^{-1} on May 13 but generally ranged from -0.4 to 0.3 cm s^{-1}. Figure 4, July 2 through 9, 1992, shows the typical negative ozone fluxes that occur during the growing season. This diurnal pattern is briefly interrupted on July 7 (Julian day 189) when there was 0.3 mm of rainfall. The upward flux event is unusual for the summer period; however, similar events have been reported by Enders (Enders 1992, Enders and others 1989). Enders (1992) indicated that these upward fluxes were measured occasionally; however, consistent upward fluxes day after day as demonstrated in figure 3 were not reported. Temperatures ranged from 5 to 18 °C but did not go above 7 °C on July 7. Deposition velocities during this period ranged from -0.37 on July 7 when it rained to 0.4 cm s^{-1}. Figure 5, September 29 to October 9, 1992, shows the diurnal wave transition from negative ozone fluxes to positive fluxes. This transition is apparently seasonal. During this period, snow started accumulating on the ground. Temperatures ranged from 7 to 15 °C until October 4 when the range dropped to 0 to 5 °C then dipped below freezing on October 6. Deposition velocities ranged from -0.5 to 0.3 cm s^{-1} during this period.

The diurnal course of ozone concentration (ppb) at 23 m above the canopy at the Brooklyn tower does not show the daily maximum/minimum sine wave pattern typical of urban, photochemically dominated air masses at any time during the year. There are some days in all seasons that show a small 1- to 5- ppb diurnal swing with midday maximums. Data taken at 3 m height at the same Brooklyn tower site (Musselman and others 1992) does show the diurnal sine wave pattern: maximum ozone values are no higher than those shown in figures 1 through 5; and minimum ozone values fall as low as 10 ppb at night. This difference can be explained if near the ground ozone is chemically destroyed at night and not replenished from above due to stable air and lack of daytime turbulent wind energy.

Table 2 gives a summary of recent forest ozone deposition experiments to provide a comparison to results given in this paper. Flux measurement techniques represented in table 2 are either eddy correlation, profile gradient, or chamber.

The ozone flux data presented here are the first data to suggest that the 'counter-gradients' measured by others may not have been counter-gradient (i.e., associated ozone fluxes could have been upward as the measured gradient would suggest and not downward and 'counter-gradient' as assumed). The explanation for these upward ozone fluxes is unknown. Upward

fluxes suggest surface or canopy emissions of ozone. Atmospheric boundary layer ozone usually requires nitric oxide (NO), nonmethane hydrocarbons, and solar ultraviolet energy to drive its photochemical production (Logan 1983). Forests are sources of natural nonmethane hydrocarbons, which are known precursors for ozone production and a possible cause of higher rural ozone concentrations. Concentrations of nitrous oxide (N_2O) above typical ambient levels have been measured under and above the snowcover at GLEES (Sommerfeld and others 1993). Since the same microorganisms that generate N_2O also generate NO (Davidson 1991, Hutchinson and Davidson 1993), the possibility of an NO source during winter months exists. The concentration of NO at GLEES is likely very small.[3]

Table 2.--*Summary of recent ozone flux experiments*

Authors (s)	Technique*	Forest type	Flux	Mean concentration	Deposition velocity	Time/ location
Enders (1992)	EC	Spruce	-	5 to 50 (ppb)	-1.0 to 1.8 (cm s^{-1})	June 1989 Germany
Enders (1989)	EC	Spruce	-	10 to 40	-8.9 to 1.8	June 1987 Germany
Fontan and others (1992)	PR	Pine	-0.11 max	18 to 38	0.04 to 0.8	September 1985 France
Matt and Womak (1989)	EC	Spruce-fir	0 to -0.4 ($\mu g\ m^{-1}\ s^{-1}$)	5 to 50	0.1 to 0.8	September 1987 Maine
Lopez and others (1993)	PR	Pine	-	20	0.2 to 0.5	September 1984 France
Padro (1993)	EC	Deciduous	-	25 to 100	0.3 to 1.0 0.1 to 0.3	August 1988 April 1990 Canada
Rondon and others (1993)	CH	Spruce-pine	0 to -0.2	5 to 70	0 to 0.5†	June 1990 Sweden
Wesely (1983)		Deciduous	-	- (no snow impact)	0.01 to 0.37	January 1981 North Carolina
Zeller present	EC	Spruce-fir	-0.6 to 1.0	23 to 70	-1.5 to 0.4	January study September 1992 Wyoming

* EC: Eddy Correlation; CH: Chamber; PR: Gradient Profile.
† Per projected needle area.

Several hypotheses for upward ozone flux are possible:

(1) Static electric discharge is a potential phenomenon to explain the direct production of NO and O_3. Some authors describe static electric effects of surface snow: Scott and Levin 1970, Wishart 1968.

(2) Since ozone does not readily deposit or react with snow,[4] a possible explanation for the measured upward ozone fluxes is the wintertime absence of ozone sinks (Zeller 1992) coupled with local turbulent wind-flow patterns giving an apparent upward flux at the specific site where the measurements were made.

[3] Mosier, Arvin. 1993. Personal communication with the author. On file with: U.S. Department of Agriculture, Forest Service, 240 W. Prospect, Fort Collins, CO 80526.

[4] Stocker, D.; Zeller, K. [In preparation]. Oxidant fluxes over snow measured by eddy correlation.

(3) If upwind sources of nitrogen oxides were transported into the Snowy Range area, it is possible that they could scavenge ozone above the forest canopy, thus creating a local vertical ozone gradient that decreases with height and transports ozone upward.

(4) The snow/air interface is known to contain concentrations of hydrogen peroxide (H_2O_2). It is possible that some chemistry at the snow surface is generating ozone.

The delineating factor for upward versus downward fluxes appears to be either snowcover or (to a lesser extent) ambient temperature. Both are environmental situations that potentially affect stomatal function. Note that the measured fluxes are usually diurnal in nature reflecting daytime vertical ozone mass transfer. Some of the measurements show continued upward ozone fluxes during the night; however, nighttime fluxes are more often weaker than daytime fluxes.

CONCLUSIONS

The ozone flux data measured by eddy correlation at the GLEES Brooklyn tower, Snowy Range, Wyoming, show reasonable summer growing season deposition (fluxes of -0.5 $\mu g\ m^{-2}\ s^{-1}$) and deposition velocity (values of 0.4 $cm\ s^{-1}$). During the winter and nongrowing seasons, upward ozone fluxes were measured. The late winter upward fluxes are the same magnitude as the summer downward fluxes, and V_d's frequently approached -0.9 $cm\ s^{-1}$. As ozone does not readily deposit on snow, the measured rate of ozone deposition is expected to decrease during the winter. The upward ozone flux measurements presented here suggest either: (1) Some unknown source of ozone below the 23-m measurement height; or (2) some other mechanism effecting local ozone fluxes. For example, a possible mechanism might be the continued uptake of ozone by the forest canopy particularly in daylight hours during the winter months. This mechanism could create a local situation where ozone is scavenged within the canopy height layer, then the unscavenged ozone below and adjacent to the snow would be available to move upward to replace the scavenged ozone.

In order to investigate these unusual results, further ozone flux measurements and detailed turbulence measurements must be made at several heights within and above the forest canopy along with vertical ozone and meteorological profiles. The addition of nitrogen oxide data would be necessary to explain or reject potential ozone sources.

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The featured paper on magnetic resonance imaging and an invited one regarding chemical scientists' responsibilities to our environment were supplemented by 26 additional manuscripts and posters in such areas as: polyphenol, polyamine, sucrose, isoprenoid, stilbenol, lignin, fiber, smoke, ozone, and inorganic ion chemistry; tree seed, tree root, tree bark, woody plant tissue, seedling, fungal, beetle, and termite biology; representing biochemistry, physiology, pathology, morphology, entomology, mycology, silviculture, and horticulture.

CAUTION: Pesticides can be injurious to humans, domestic animals, desirable plants, and fish or other wildlife--if they are not handled or applied properly. Use all pesticides selectively and carefully. Follow recommended practices for the disposal of surplus pesticides and pesticide containers.

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