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The Role of Cations in the Biodegradation of Wood by the Brown Rot Fungi

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This review describes what is presently known about the role of positively charged ions in the colonization and degradation of wood by brown rot fungi. General patterns of cation accumulation and the roles of iron, manganese, calcium and other cations in the fungal environment are discussed. The physiology of brown rot fungi and mechanisms of wood cell wall breakdown are emphasized. © 1997 Elsevier Science Limited

INTRODUCTION

Positively charged ions (cations) can exist freely in aqueous solution or complexed with anions in natural systems. Metals are a special subset of cations and are often biologically active in low concentrations. Metals vary in their activity or the ease with which they give up electrons to form ions, with metals higher in activity able to oxidize metals lower in activity.

For divalent ions of transition metals, completing ability usually follows the Irving-Williams series (Dawson *et al.*, 1987). Metals of particular interest to this review include iron and manganese along with metals commonly used in wood preservatives such as chromium and copper.

Brown rot fungi are characterized by their ability to degrade rapidly and selectively the hemicellulose and cellulose components of the wood cell wall leaving a residue of modified lignin (Kirk, 1975). Brown rotted wood is customarily brittle with little residual strength, and is characterized by a dark brown color (Eriksson *et al.*, 1990) and by changes in overall cation ratios or concentrations (Jellison *et al.*, 1992). Brown rot organisms produce xylanases, endoglucanases and galactosidases but do not generally appear to produce cellobiohydrolase enzymes, nor have they been shown to produce liginase or manganese peroxidase. In addition, the pattern of brown rot degradation seen within the cell is not one of gradual attack from the lumen in areas adjacent to the hyphae but is a more diffuse depolymerization of the S2 layer, often in the absence of significant initial degradation of the S3 layer (Eriksson *et al.*, 1990). These. facts and the observation that fungal cellulases are too large to penetrate non-modified wood cell walls (Flournoy *et al.*, 1991), suggest that the initial degradative steps in brown rot decay are non-enzymatic in nature. Kirk *et al.* (1991) established that oxidative degradation is involved in the brown rot attack of lignocellulosic materials. Multiple theories have been advanced to explain brown rot degradation of wood, including the involvement of the iron-based Fenton reaction, acid hydrolysis, and the involvement of fungal metabolites ranging from oxalate to biochelators. A more inclusive overview of proposed mechanisms of brown rot degradation is given elsewhere in this volume (Green & Highley, 1997).

Brown rot fungi are the major group of organisms associated with degradation of in-service wood, and they also play an important role in the forest ecosystem and biogeochemical cycling of nutrients (Gilbertson, 1981). Biodegradation of lignocellulose by fungi is of interest both because of the need for decay prevention in wood products and because of its potential biotechnological applications (Goodell, 1989; Paszczynski & Crawford, 1995). This review focuses on characterizing the cation environment associated with decay and on how cation chemistry may influence the ability of brown rot fungi to colonize wood and to degrade wood lignocellulose.

GENERAL TRENDS IN ION ACCUMULATION OR LOSS

Ion concentrations in sound and decayed wood have been examined directly by chemical analysis and indirectly by measurement of wood electrical resistance. Electrical resistance (ER) has been used to detect decay in wood and for locating discolored and decayed wood in living trees (Tattar *et al.*. 1972; Shortle, 1979, 1982). Decreases in wood electrical resistance have been attributed to increases in positively charged ions (Safford *et al.*. 1974; Shortle, 1982). Shortle (1982) found decreased electrical resistance in water extracts of decaying Douglas-fir wood in decay chambers. In wood decayed by the white rot fungus *Trametes versicolor (Coriolus versicolor),* decreased ER was associated with increased potassium ion concentration. In wood decayed by the brown rot fungus *Postia placenta,* decreased ER was associated with increased hydrogen ion concentration (Shortle, 1982; Jellison *et al.,* 1992). Shortle (1990) measured the ER of water extracts of wood from four tree species inoculated with decay fungi. Electrical resistance measured 14 days after inoculation was lowered, indicating that a rapid increase in the ion content of wood is characteristic of the previsual stages of wood decay.

The concentrations of inorganic elements in sound wood, in discolored and decayed wood, and in wood blocks in decay chambers have been determined. The inorganic elements of wood (ash) make up about 0.1-0.5% of the total based on wood dry weight (Ellis, 1965). The bulk of the inorganic elements in wood, accounting for about 70% or more, are the base cations, calcium, potassium, and magnesium. Numerous researchers have analyzed cation concentrations in different species of degraded and non-degraded wood. Young & Guinn (1966) determined the chemical elements in the wood of seven tree species in Maine. Red spruce wood contained 0.082% calcium, 0.020% potassium, 0.007% magnesium, 144 ppm manganese, and 14 ppm iron. Ellis (1965) described the approximate levels of various elements in grand fir wood expressed as ppm dry weight, including: calcium 754, potassium 865, magnesium 171, sodium 23, manganese 19.3, iron 2.6, copper 2.5, zinc, 0.9, aluminum 5.4, and chromium 0.05. Concentrations of calcium, potassium, magnesium, manganese, iron, and aluminum in grand fir decayed by the white rot fungus *Echinodontium tinctorium* were greater than those in non-decayed wood, and calcium accumulation appeared to increase with per cent weight loss in brown rotted otka spruce (Ellis, 1965). Shortle & Bauch (1986) found the concentration of ions in healthy balsam fir sapwood, expressed as μmol g^{-1}, to be: calcium 12.6, potassium 12.7, magnesium 2.5, and manganese 1.0. Discolored and decayed wood in wounded red maple trees were shown to have higher concentrations of potassium and magnesium than sapwood; potassium, calcium, magnesium, and manganese were higher in the column boundary layer than in the sapwood (Safford *et al.,* 1974; Shevenell & Shortle, 1986). Shortle (1982) determined concentrations of potassium, calcium, and magnesium in blocks of Douglas-fir sapwood and heartwood incubated with *P. placenta* and *T. versicolor.* Significant increases in cation concentrations occurred with potassium, calcium, and magnesium in white rotted sapwood; magnesium in brown rotted sapwood; potassium and magnesium in white rotted heartwood; and calcium and magnesium in brown rotted heartwood.

Decay by *P. placenta* resulted in increased concentrations of calcium, magnesium, iron, and aluminum in spruce wood (Jellison *et al.,* 1992; Ostrofsky *et al.,* 1997). Examination of decay by a variety of fungi indicated that red spruce wood incubated for 8 months with *P. placenta, Trametes versicolor, Phanerochaete chrysosporium, Irpex lacteus, Scytinostroma galactinum,* or *Armillaria sp.* had greater concentrations of calcium and magnesium than the uninoculated, incubated control wood (Ostrofsky *et al.,* 1997). The cation content of decaying conifer wood on the forest floor has been examined by many researchers, including Foster & Lang (1982) who found a net accumulation of calcium and magnesium and loss of potassium with time. Shortle & Bauch (1986) noted that, in contrast to the monovalent cations (hydrogen, potassium, sodium), the divalent cations (calcium, magnesium, manganese) are not readily soluble in water.

Connolly & Jellison (1995) found that *Resinicium bicolor* translocated calcium several centimeters away from red spruce wood blocks and soil in a soil block jar to sites on mycelial cords growing up the inside of the jar. In a similar study in which wood blocks were presoaked in various salt solutions. *Gloeophyllum trabeum* was able to translocate cations into and out of wood blocks (Connolly & Jellison, 1997). McDougall & Blanchette (1996) examined ion adsorption from

soil by pseudoscerotial plates of *Phellinus weirii* and mycelial mats of *Fomitopsis pinicola.* Mycelial mats of *F. pinicola* incubated with soil and water had an increased concentration of manganese, but decreased concentrations of potassium and sodium compared to controls incubated with water. Although the values reported in these studies vary, they confirm that the alkaline earth elements make up the bulk of the inorganic elements in wood, whereas levels of the transition elements, particularly iron, are quite low, especially in non-decayed wood.

Fungal sporocarps may play a role in cation changes in decaying wood. The concentration of calcium in rotting grand fir wood was more than seven times that in sound wood or in fruiting bodies of the white rot fungus *E. tinctorium;* concentrations of potassium and magnesium were greater in fruiting bodies and in decayed wood than in sound wood (Ellis, 1965). Blanchette (1984) compared cation concentrations in sound hemlock wood, in decaying hemlock, and in sporocarps of *Ganoderma tsugae.* Concentrations of potassium were greatest in the sporophores, whereas calcium concentrations were greater in decaying wood than in sound wood or sporophores. Harmon *et al.* (1994) compared cation concentrations in fungal sporocarps growing on conifer logs to original cation concentrations in sound conifer logs. Almost all of the sporocarps examined, including those of *F. pinicola,* had lower calcium concentrations than those in sound wood. Sporocarps of *F. pinicola* had concentrations of potassium, magnesium, and zinc greater than that in the original sound wood of the logs. Concentrations of iron and manganese were also slightly higher in the sporocarps.

Hydrogen ion concentration also changes during the wood biodegradation process. Wood pH usually varies between 3 and 6 (Gray, 1958; Rayner & Boddy, 1988) with the pH values below 5 often being associated with decayed wood (Jellison *et al.,* 1992). The growth optima for wood decay fungi also fall within this range (Rayner & Boddy, 1988; Zabel & Morrell, 1992). Brown rot fungi can decrease the pH of the media in which they grow (Cartwright & Findlay, 1958; Takao, 1965; Jellison *et al.,* 1997). Cowling (1961) found a decrease in the pH of wood extracts from sweetgum wood decayed by the brown rot fungus *Postia placenta (Poria monticola).* Shortle (1990) examined the pH of water extracts of red cedar, red pine, black cherry, and red oak inoculated with a white rot or a brown rot fungus. After 14 days of incubation, the pH of wood inoculated with the brown rot fungus *P. placenta* was lower than that of uninoculated wood or wood inoculated with the white rot fungus. Green *et al.* (1991) used microprobes to measure wood pH in southern pine blocks inoculated with *P. placenta.* The wood pH dropped rapidly to 2.5 and 1.6 during the first week of incubation. The implications of pH changes associated with colonization of wood by brown rot fungi are discussed in more detail later in this review.

IRON IN THE ENVIRONMENT

Iron is the fourth most abundant element by weight in the earth's crust. The bioavailability of iron is limited by its volubility in an oxygenated neutral pH environment because of the formation of insoluble metal oxy(hydr)oxides (Winkelmann & Winge, 1994). In aerobic environments, the most stable form of iron is the ferric state, and little iron is found in the reduced state. In aqueous environments, relatively insoluble oxy(hydr)oxide forms of iron predominate (Cotton & Wilkinson, 1976; Winterbourn, 1991). Total iron levels in the wood are usually significantly lower than those found in the soil and are often in the 0-2-µmol range in non-degraded wood (Jellison *et al.,* 1992, 1993).

Iron is essential for life as we know it. It plays a central role in general metabolic processes, such as electron transfer in the respiratory chain, redox reactions with inorganic substances, acid–base reactions, and DNA synthesis and cleavage (Matzanke, 1994). Iron is a component of extracellular enzymes responsible for lignin degradation in white rot fungi and is a cofactor of many enzymes involved with oxidation/reduction reactions. Additional specific enzymes dependent upon transition metals include: superoxide dismutase; peroxidases, which act as NAD(P)H oxidases that form and scavenge active oxygen species; and catalase, which converts H_2O_2 to H_2O and O_2. Although iron is an essential element, it can also pose a significant hazard within the cell and is carefully regulated. Iron has the ability to catalyze the formation of toxic oxygen free radicals, such as the highly reactive hydroxyl radical via the Fenton reaction ($H_2O_2 + Fe^{2-} Fe^{3+} + OH^- + OH$). Hydroxyl radicals also can be generated in the presence of metals by the related Haber-Weiss reaction (Haber & Weiss, 1934). Active oxygen species derived from molecular oxygen and interconversion pathways are reviewed by Baker & Orlandi (1995).

Research by Koenigs (1974, 1975) and others, concerning the action of Fenton reagents in the presence of cellulose and wood cell components, suggests that iron and hydrogen peroxide are involved in the production of highly reactive hydroxyl radicals, which could initiate the depolymerization of cellulose in wood. Research by Highley (1980) and Schmidt *et al.* (1981) showed that wood degraded by reactive products of the Fenton reaction displayed chemical and physical features characteristic of brown rotted wood. Several more recent hypotheses, involving both iron and oxalic acid, have been proposed, and it has been suggested that oxalate may function to reduce iron III to iron II, which then reacts with H_2O_2 to yield OH. Hyde and Wood (1995) and others (Zepp *et al.,* 1992; Sedlak & Hoigné, 1993; Sulzberger & Laubscher, 1995), however, have observed that oxalate does not reduce ferric iron, except as a light-dependent reaction. Therefore, oxalate could not function as a direct catalyst of Fenton type chemical reactions in wood. Other mechanisms must exist for iron reduction (Goodell *et al.,* 1997).

Other recent hypotheses concerning mechanisms related to the role of iron in fungal wood degradation include those involving fungal glycopeptide compounds (Enoki *et al.,* 1989; Hirano *et al.,* 1995). These compounds have been reported to bind reduced iron and to both reduce and oxidize the metal. Wood (1994) has investigated pathways for the production of Fenton reagent in fungi, and Hyde & Wood (1995) have proposed a mechanism for brown rot degradation, based on Fenton chemistry and the enzymatic generation of reduced iron species.

To solubilize and sequester ferric iron, many organisms have developed efficient high affinity iron acquisition systems (Neilands, 1974; Neilands *et al.,* 1987), which allow the organism to compete successfully for limited metals in the environment. These high-affinity, low-molecular-weight iron chelators (siderophores) are used by microorganisms to assimilate metals for use in the organism's metabolic functions. Ferrated siderophores can be taken up by specific microbial transport systems (Neilands *et al.,* 1987; Barton & Hemming, 1993). Production of siderophore-like compounds by the decay fungi, including many of the brown rot fungi, was demonstrated by Fekete *et al.* (1989). Biochelators from the brown rot fungus *Gloeophyllum trabeum* were partially purified and localized within degrading wood by immunoelectron microscopy (Jellison *et al.,* 1991). These compounds have subsequently been characterized as phenolates, including dihydroxybenzene derivatives (Goodell *et al.,* 1997). In addition, these compounds have been shown to be active in one-electron oxidation reactions and are capable of hydroxyl radical generation (Chandhoke *et al.,* 1992; Goodell *et al.,* 1995, 1997). Production of both the chelators and the fungal sheath can be influenced by metal concentrations in culture and repressed at high iron concentrations (Jellison *et al.,* 1997). Electron paramagnetic resonance has been used to confirm the ability of the chelator isolated from *G. trabeum* to chelate iron III, and the ability of these compounds to reduce iron has been documented using ferrozine assays (Goodell *et al.,* 1997). Reduction of metals by dihydroxybenzoic acid derivatives has previously been shown (Xu and Jordan, 1988). Superoxide reduction of iron in the presence of redox active low-molecular-weight chelators had been shown by Chen *et al.* (1995). Reduction of cellulose chain length in the presence of iron, chelator and hydrogen peroxide has been demonstrated by gel permeation chromatography (Goodell *et al.,* 1997). Recent work on chelators produced by the brown. rot fungus *G. trabeum* and a role for their participation in the redox cycling of iron and the process of wood degradation by the brown rot fungi have been recently reviewed (Goodell *et al.,* 1997).

THE ROLE OF MANGANESE, ZINC AND COPPER

The trace elements, manganese, copper, and zinc are required for life in virtually all organisms. However, none of these metals exists at high levels in the earth's crust. Because of their limited volubility in aerobic, aqueous environments, these metals are even less available to fungi. Therefore, all fungi have mechanisms for the specific transport of these nutrients to fulfil their physiological requirements (Winkelmann & Winge, 1994). The uptake systems for manganese, copper and zinc ions are energy-dependent, specific, and of high affinity in yeasts and other fungi (Kosman, 1994).

Copper, an essential micronutrient for fungal growth, functions as a metal activator of several fungal enzymes, e.g. oxidases, and in the synthesis of pigments. However, fungal growth is strongly inhibited by high copper concentrations.

Copper is an active component in many fungicides where it reacts with thiols and other functional groups of fungal enzymes. Consequently, the level of copper in the fungal environment is critical not only for proper maintenance of cellular metabolism, but also for the survival of the organism (Garraway & Evans, 1984). Copper at high environmental levels is highly toxic and exerts its effects initially at the cell surface. Growth inhibition is believed to be at the site of the cell membrane, where copper interferes with solute transport and other membrane phenomena (Ross, 1975). A number of reports provide evidence that copper can cause genetic perturbations in fungi. Copper can cause mutagenic effects (Von Rosen, 1964) and has been shown to become localized in the nucleoli and chromosomes in yeast (Lundegren *et al.,* 1972). Antoine (1965) has suggested that the presence of copper can induce increased resistance to copper in yeasts. Fungi vary considerably in their response to copper as a function of environmental pH (Garraway & Evans, 1984). Whereas copper is stimulatory for the growth of *Penicillium notatum* at pH 7, it is inhibitory at pH 2. For most fungal species examined, growth inhibition by copper increases with decreasing culture medium pH. Environmental pH may affect binding affinity as well as the volubility of copper (Ross, 1975).

Manganese is a micronutrient required as a cofactor in many enzyme systems, e.g. in the TCA cycle and in nucleic acid synthesis (Griffin, 1994). Manganese can replace magnesium and bind to ATP to function as the required cation in phosphate transfer reactions. Manganese activates other enzymes, such as superoxide dismutase, protein kinase, adenyl cyclase, and RNA polymerase (Garraway & Evans, 1984). The physiological effects that manganese exerts on fungi vary considerably (Garraway & Evans, 1984; Auling, 1994). For example, in *A. niger,* manganese deficiency results in the reduction of the levels of lipid, protein, and nucleic acids (Kubicek *et al.,* 1979; Orthofer *et al.,* 1979). In the same organism, it was also found that ammonium ions and amino acids accumulate intracellularly, accompanied by an efflux of high levels of amino acids (Habison *et al.,* 1979). Zonneveld (1975) found that manganese controls sexual differentiation in *Aspergillus nidulans.* Manganese deficient cells of *A. nidulans* failed to form cleistothecia. This has been shown to be related to the absence of β 1,3-glucan (Zonneveld, 1975). In two species of *Penicillium.* Tinnel *et al.* (1974) found that conidiation was stimulated by the addition of manganese in the culture medium.

In the white rot wood decay basidiomycete, *Phanerochaete chrysosporium,* manganese functions in the regulatory control of various proteins during secondary metabolism (Boominathan & Reddy, 1992). At intermediate levels of the metal (11 ppm), lignin peroxidase and manganese peroxidase are formed. Lignin peroxidase is formed almost exclusively at low manganese concentrations (1.6-0.3 ppm) and repressed at high concentrations (40-199 ppm). High manganese concentrations induce production of manganese peroxidase (Bonnarme & Jeffries, 1990). The manganese-requiring enzyme from lignin-degrading *P. chrysosporium* oxidizes manganese II to manganese III, which is subsequently chelated by organic acids such as oxalic acid (Wariishi *et al.,* 1989, 1992; Paice *et al.,* 1993). The manganese III complex oxidizes lignin and phenolic lignin model compounds (Glenn *et al.,* 1986). The manganese peroxidase supports the hydrogen peroxide-independent lipid peroxidation of unsaturated fatty acids and the oxidation of toxic polycyclic aromatic hydrocarbons (Moen & Hammel, 1994). This manganese-dependent oxidation of polycyclic aromatic hydrocarbon organopollutants is the basis for *P. chrysosporium* tolerance to creosote wood preservatives (Moen & Hammel, 1994). Manganese has also been shown to enhance atrazine transformation by the white rot fungus *Pleurotus pulmonarius* (Masaphy *et al.,* 1996).

The role of manganese in the biodegradation of wood by brown rot wood decay fungi is less clear. During degradation of wood, divalent manganese is mobilized by *P. placenta* and *G. trabeum* (Illman *et al.,* 1988, 1989). Manganese appears to be released from low-molecular-weight ligands in susceptible wood species. The resultant accumulation of magnetic resonance detectable manganese is not totally due to oxalic acid or water produced by the fungi (Illman *et al.,* 1989; Highley & Illman, 1991; Illman, 1991). Because of the redox potential of manganese and the ease of manganese substitution for iron, several researchers have predicted a role for manganese in the non-enzymatic mechanisms involved in wood biodegradation (Illman & Highley, 1989; Jellison *et al..* 1992).

Zinc, an essential nutrient for fungal growth, participates in many diverse cellular processes (Ross, 1994). For example, zinc functions as a cofactor and in the structure of many enzymes

involved in intermediary metabolism. It also functions in nucleic acid metabolism, and in the cell division cycle (Failla, 1977). Zinc is a cofactor of numerous fungal enzymes, including aldolase, glyceraldehyde-3-phosphate dehydrogenase, NADH-dependent lactate dehydrogenase, pyruvate decarboxylase, and superoxide dismutase (Garraway & Evans, 1984). In fungal secondary metabolism, zinc is required in the syntheses of antibiotics, ergot alkaloids, and polyketides (Weinberg, 1977; Failla & Niehaus, 1986), and in the formation of the scleroglucan hyphal sheath in *Sclerotium rolfsii* (Pilz *et al.,* 1991). Zinc-deficient fungal cultures exhibit marked physiological perturbations (Garraway & Evans, 1984). In the yeast, *Rhodotorula gracilis,* zinc-starved cells showed lower levels of RNA, reduced protein synthesis, and lower numbers of mitochondria (Cocucci & Rossi, 1972). Zinc only becomes toxic at relatively high concentrations. For example, *Neocosmospora vasinfecta* grows optimally at zinc concentrations of 25 and 100 µM. However, when this fungus is subjected to 100 mM zinc, a 90% reduction of growth occurs (Paton & Budd, 1972). Adiga *et al.* (1961) suggest that toxicity resulting from high zinc concentrations may be due to an apparent magnesium nutritional deficiency of the fungus. Data from Budd (1989) revealed that when *N. vasinfecta* is grown under low magnesium and normal zinc culture conditions, mycelial uptake of zinc is enhanced approximately 300 times and suggested that the enhancement of zinc uptake is a function of an induced and activated magnesium transport system.

THE ROLE OF CALCIUM, MAGNESIUM AND POTASSIUM

Base cations are crucial for the proper function of proteins, nucleic acids, and phospholipid molecules. Calcium is essential for stabilizing membranes, magnesium is required as a cofactor for many enzymes and for ATP utilization, and potassium is presumed to be the dominant electrolyte in the cytosol of all fungi.

Calcium is probably required for many of the same cellular functions in fungi as it is for other eukaryotes, but our understanding lags behind that of plant and animal systems (Warwar & Dickman, 1996). Consistent with other eukaryotic systems, cytosolic divalent calcium in fungi is tightly controlled and maintained at a concentration of approximately 0.1 µM (Belde *et al.,* 1993; Bush, 1993). Also, in a fashion similar to other eukaryotes, calcium in the cells of fungi is sequestered at higher concentrations within certain membrane bound compartments. For example, in *Saccharomyces cerevisiae,* the vacuole concentration has been measured at about 1.3 mM (Belde *et al.,* 1993).

Despite these observations, the necessity of calcium for the traditional functions that have been ascribed to it in plants and animals has not been uniformly demonstrated in the fungi (Griffin, 1994; Warwar & Dickman, 1996). The mechanism by which calcium concentrations are maintained in the fungi also may be different than in plants and animals. Calcium-dependent ATPases and sodium/calcium exchangers are found on the plasma membranes of animal cells and plant cells (Belde, 1993) and the P-type Ca^{2+} ATPases found in the plasma membranes of plant cells are thought to be particularly important in maintaining the low concentration of calcium in the plant cell cytosol (Bush, 1993). In fungi, however, Ca^{2+}/H^{+} antiporters have been thought to be the dominant mechanism of calcium extrusion from the cell (Belde, 1993). **A** clear understanding of how baseline levels of calcium are maintained in fungi has still not yet been established.

Despite these uncertainties, second messenger pathways are so universal and so highly conserved that it is very likely that changes in calcium concentration in the cytosol of fungi are critical in intracellular communication and functional activation. The archetypal signal transduction cascade begins with a cell receptor binding to a ligand at the plasma membrane. This activates G-proteins and releases phosphoinositides. This then results in release of calcium from intracellular stores. The subsequent increase in the cytosolic calcium concentration can activate protein kinases or calmodulin binding, which then leads to effecter protein action in the cells. In brown rot fungi, secretion of cellulolytic enzyme systems requires calmodulin activation (Highley, 1989). What type of effecter protein becomes involved may depend upon the magnitude of calcium cation concentration change, the duration of that change, or both. Spiking of the cytosolic calcium cations can result from the release of intracellular stores of calcium in the fungal vacuole and perhaps the endoplasmic reticulum (Belde *et al.,* 1993; Knight *et al.,* 1993).

Calcium is also likely to be important in fungal morphogenesis (exclusive of signal transduction

effects), although its role in this regard is less certain than is its role as a second messenger. Griffin (1966) showed an important role for calcium in the branching of fungi, and calcium has been shown to be important in the tip growth of some fungi (Robson *et al.*, 1991; Garrill *et al.*, 1992; Jackson & Heath, 1993). However, the importance of calcium in hyphal tip growth of basidiomycete fungi has yet to be confirmed.

Calcium is a critical micronutrient needed for signal transduction and membrane integrity. It is controlled at very low concentrations in the cytosol, but is sequestered within the fungal vacuoles and perhaps the endoplasmic reticulum at concentrations three to four orders of magnitude greater than the cytosol. Although it is a micronutrient, there are reports of calcium accumulation by wood decay fungi (Cromack *et al.*, 1975; Jellison *et al.*, 1992; Ostrofsky *et al.*, 1997). Calcium accumulation does not appear to be determined by brown rot or white rot effects since some species of both will accumulate calcium. Patterns of accumulation can depend upon the species and isolate of fungus, the type of wood being decayed, the degree of soil contact and the composition of the soil.

The calcium concentration within the fungal thallus may be the result of vacuolar sequestration, fungal cell wall binding, or binding within the extracellular matrix. McDougall & Blanchette (1996) found that melanized structures produced by *P. weirii* were able to bind many cations efficiently. The extracellular matrix is thought to be polyanionic in nature, and could be a considerable sink for binding calcium. Another possible mechanism, for the brown rot fungi in particular, is the production of calcium oxalate crystals. Brown rot fungi are capable of producing a substantial quantity of calcium oxalate crystals. However, the relationship between calcium oxalate crystal production and calcium accumulation is uncertain (Cromack *et al.*, 1975; Aho *et al.*, 1979; Connolly & Jellison, unpublished results). Despite the fact that calcium accumulation in brown rot fungi has not been clearly related to calcium oxalate crystal production, observations of calcium oxalate crystals are extremely common. Calcium oxalate forms three types of associations with hyphae and can be categorized as encrusting crystals, adhering crystals, or free crystals (Connolly *et al.*, 1996).

The function of calcium oxalate crystal production in the brown rot fungi is unclear. It is likely that the precipitation of oxalic acid in calcium oxalate crystals serves multiple functions (Micales, 1995). Calcium oxalate crystals may be metabolic waste products. Another possible function is suggested by the fact that the calcium found in some woods is essentially 100% exchangeable (Connolly & Jellison, 1997). Given the relatively large amount of calcium present in the wood, this exchangeable calcium could interfere with the ability of the brown rot fungi to lower the pH of the microenvironment by binding protons and releasing calcium cations (Connolly & Jellison, 1994). Precipitation of the calcium might prevent the calcium from moving back to the exchangeable pool within the wood, thus contributing to the acidification of the microenvironment. Precipitation of calcium also may prevent calcium that has been released from the wood walls from interfering with the normal metabolic functions of the fungus (Whitney & Arnott, 1987).

Colonization of the wood by brown rot fungi in some cases can be facilitated by the destruction of the wood pit membranes and ray parenchyma cell walls. Green *et al.* (1995) present evidence for a coordinated attack upon pits based upon oxalic acid production and endopolygalacturonase activity. It is possible that this process is more complete and efficient when calcium is not only removed from the pit membranes into the soluble pool, but also removed from soluble pool into relatively insoluble calcium oxalate crystals. Ghio *et al.* (1992) found that calcium oxalate crystals produced by *Aspergillus niger* bound iron extremely efficiently and offered evidence that the surface of these crystals was the site of damaging radical generation.

Magnesium is considered to be a macronutrient for fungi (Griffin, 1994). It is an essential cofactor for cell wall synthesis, ATP utilization, DNA repair, and many other metabolical reactions. As an electrolyte, magnesium is probably of secondary importance to potassium (Griffin, 1994). Patterns of magnesium and potassium accumulation by brown rot fungi have been observed, but the results have not been consistent (Tyler, 1982a). Cromack *et al.* (1975) found potassium levels above that found in the wood, whereas McDougall & Blanchette (1996) found potassium diminution in the presence of soil. The patterns are difficult to discern. and the mechanism by which accumulations or loss might be achieved has not been identified.

In forest floor wood, it is not uncommon to observe depletion of potassium from decaying wood (Gosz *et al.*, 1973; Lambert *et al.*, 1980;

Connolly, 1996). This can be explained by the leachability of potassium in the natural condition where percolating water from rains might deplete potassium. However, this does not explain the paradox of why magnesium and calcium are often retained when these nutrients are supposedly required in less concentration in the mycelium than is potassium. Given the frequent observation of significant loss of potassium from wood during decay, it could be possible that potassium is not the only major electrolyte in the cytosol. How these fungi continue to grow in a habitat that is often losing significant amounts of potassium is an adaptation worth investigating.

HYDROGEN ION CONCENTRATIONS

The internal pH environment of the fungal cytosol and the membrane bound organelles must be maintained for the proper function of enzyme systems. Maintenance of internal pH is around neutrality. However, in a manner similar to calcium ion changes, positive and negative hydrogen ion spikes are essential for cell funtion and response (Busa & Nuccitelli, 1984; Holyoak *et al.*, 1996). Rates of DNA and RNA synthesis often follow pH changes, with increases in the rate of DNA and RNA synthesis occurring during increases in pH (Madshus, 1988). Very small changes in pH may result in important changes in cAMP and calmodulin–Ca^{2+} interactions (Busa & Nuccitelli, 1984). Protein and enzyme conformations are very responsive to pH, and normal binding phenomena depend not just on pH, but also on changes in pH.

The pH is also critical for the functioning of organelles. Within organelles such as the fungal vacuole and mitochondria, pH and proton partitioning essentially determine organelle function. ATP is generated within the mitochondria from proton gradients, and the vacuoles of fungi are thought to be important in the modulation of intracellular pH. This modulation is achieved in great measure by virtue of the acidic nature of the vacuoles (Klionsky *et al.*, 1990).

The extracellular microenvironment of the brown rot fungal hypha is an acidic one (Green *et al.*, 1991; Hyde & Wood, 1995; Palfreyman *et al.*, 1996). The adaptive nature of this microenvironment, and the mechanism by which it is achieved, is an area of active investigation. There is evidence to support the very important role that oxalic acid plays in producing this acidic environment (Takao. 1965). Oxalic acid is a small organic acid with two low pKa values. In addition to this strong acidic character, oxalic acid is often produced in great quantities by brown rot fungi. These factors make it a very strong candidate for playing a central role in producing the acidic environment associated with brown rot colonization of wood. Mechanistic hypotheses for the role of oxalate in the regulation of pH gradients required for brown rot degradation also have been proposed (Hyde & Wood, 1995; Goodell *et al.*, 1997). However, there are other factors that deserve consideration along with oxalic acid.

Acidification by catabolism is a common presumption, but this is often unsupported by the stoichiometry (Buss & Nuccitelli, 1984). Some eukaryotic cells accumulate lactic acid, and are noted to be more acidic than other cells. In this case, the protons are not derived from the organic acid at all, but from ATP hydrolyses (Buss & Nuccitelli, 1984). This suggests that the presence of an accumulated organic acid such as oxalic acid may not be the only factor associated with a reduced pH. In addition, there is currently a lack of experimental agreement on oxalate quantification. Several methods are available for oxalate analysis, but when more than one method is used within the same study, there is disagreement (Jordan *et al.*, 1994, 1996). Without reliable and cross-referential methods of oxalate quantification, it is difficult to attribute pH levels confidently to oxalate alone.

There are many H^+ATPases in the plasma membranes of fungi. In *Saccharomyces cerevisiae*, H^+ATPases constitute 20% of the membrane protein, and 40%-60% of the ATP consumed by the plasma membrane can be attributed to H^+ATPase activity (Holyoak *et al.*, 1996). Such ATPases could be at work in the brown rot fungi, contributing to the very low pH values observed in many studies. Lastly, these fungi produce and secrete many acidic group-carrying compounds such as phenolic acids and other simple organic acids generated during metabolism. The role of oxalic acid in the generation of the observed low pHs is also complicated by the fact that this acid readily precipitates with calcium, a divalent cation found in relative abundance in wood. Precipitation chemistry is highly dependent upon thermodynamic factors, but the precipitation itself also influences the thermodynamics of the microenvironment. In preliminary work involving organic acid-fed cultures (Connolly & Jellison,

1995), it was found that pH did not correlate well with total oxalate or with soluble oxalate. The most likely case is that many factors result in pH values between *ca* 3.5 and 5, but that it is oxalate that drives the pH to extremely low levels.

The adaptive nature of the acidic external environment of brown rot fungi is also a topic of considerable speculation. Although peptides can move within the wood cell wall, cellulolytic enzymes cannot. It is thought that the acidic environment created by the brown rot fungi may contribute to the decay process by loosening and softening the wall to allow other fungal metabolites access to the cell wall matrix. The low pH also may saturate the available exchange sites within the wood wall with protons and thus extract cations that are useful for fungal metabolism or are structurally important to the wood walls. Pectic substances seem to have the ability to induce some brown rot fungi to produce oxalic acid (Green *et al.*, 1994), and it has been shown that this also might be tied to polygalacturonase activity (Green *et al.*, 1995). This would be a very potent combination in destroying pit membranes in wood and would thus give the fungi greater access to the wood.

The pH also has an extremely important role in influencing the redox potential. Although the details remain to be elucidated, iron clearly plays an important role in brown rot decay. One mechanism by which this may be achieved is by the generation of radicals via Fenton chemistry. An acidic environment assists in the solubilization of iron, pushing the equilibrium towards the ferrous state. Oxalic acid may be particularly important in this, and in solubilizing the dilute amounts of iron oxy(hydr)oxides within the wood (Goodell *et al.*, 1996). The low pH would then also influence what types of compounds could stabilize iron in a form useful to the fungus.

As already mentioned in the context of intracellular enzymes, pH significantly influences enzymatic activity. Ultimately, if the fungus is to obtain energy from the wood, it must digest the wood extracellularly to produce diffusible sugars and short-chain polysaccharides. The low pH environment is optimal for some of the enzyme systems that the fungus possesses. Green *et al.* (1995) found that extracellular polygalacturonase activity of a brown rot fungus was optimal at low pHs between 2.8 and 4.5. Purified fractions of endoglucanases from a white rot organism exhibited optimal enzymatic activity between 3.5 and 5 (Eriksson & Pettersson, 1975). However, Mischak *et al.* (1989) found that the activity of a cellulase system in the non-wood decay fungus *Trichoderma* sp. dropped in an acidic environment, and it was in this environment that proteinases became more active. Wood *et al.* (1988) also claim that the majority of cellulase optima are in the region of 6.0. Hence, there is still a lack of detail regarding the pH optima of the critical enzymatic players involved in wood biodegradation.

METAL TOXICITY

Certain heavy metals are required by the fungi for metabolism, whereas others have no known biological role and can be toxic. The fungi must be able to sequester essential trace metals from wood and soil. These metals may be present in concentrations ranging from suboptimal to toxic levels. Toxic levels of metals often occur in wood or soils as a result of industrial pollution or via the impregnation of wood with inorganic wood preservatives such as chromated copper arsenate, ammoniacal copper arsenate, or ammoniacal copper zinc arsenate. Whereas fungi have metabolic requirements for trace metals, the same metals are often toxic at concentrations only a few times greater than those required (Hughes & Poole, 1991). The metals required by fungi include copper, iron, manganese, molybdenum, zinc, and nickel. Non-essential metals commonly encountered include chromium, cadmium, lead, mercury, and silver (Gadd, 1993). Since metals can be potent growth inhibitors of microorganisms, it is not surprising that the fungal diversity of metal contaminated sites is reduced (Zibilske & Wagner, 1982; Babich & Stotzky, 1985). Metal tolerant fungal species, however, are present in both metal and non-metal contaminated sites (Arnebrant *et al.*, 1987), and 'tolerance' for high concentrations of metals often can be induced by repeated culturing in high metal medium (Macara, 1978). Several brown rot fungi are metal-tolerant and have some capacity to degrade wood treated with copper-based preservatives (Murphy & Levy, 1983; Leithoff *et al.*, 1995; Illman & Highley, 1996a). Although copper-tolerant fungi have been isolated from sporadic wood failures in temperate zones, most degradation by copper tolerant fungi has been found in warm climates (Morrell, 1989). Degradation of CCA-treated wood has raised questions about resulting oxidation states of arsenic and chromium.

Illman and Highley, 1996b) developed an X-ray fluorescent technique to detect chromium and arsenic oxidation states in wood and have confirmed that chromium in wood is primarily in the less toxic, more stable Cr^{3+} valence state. Electron paramagnetic resonance (EPR) also has been used to detect oxidation states of chromium and copper in CCA-treated wood (Hughes *et al.*, 1994; Ruddick *et al.*, 1994).

Metals bind to a wide range of ligands, including those presented by the structures of biological organisms. Fungal cell walls are composed primarily of polysaccharide, but also contain some proteins and lipids. Functional groups available for complexation with metals include phosphate, hydroxyl, carboxyl, sulfhydryl, and amine groups. The toxicity of metals results primarily from direct contact and their ability to bind to cells or cellular metabolizes (Gadd & White, 1989). This binding may block functional groups on enzymes, denature enzymes by inducing confirmational changes, displace essential metals, or interfere with transport processes (Ross, 1975; Gadd & White, 1989; Gadd, 1990). The release of fungal degradative enzymes into the surrounding environment renders the enzymes particularly susceptible to denaturation by metals.

Fungi have evolved several mechanisms to prevent cellular contact with metals but most can be characterized as either extracellular avoidance or intracellular sequestration. The hyphal sheath contains many functional groups capable of binding metals and precluding their contact with the fungal cell wall (Sutter *et al.*, 1983). Another method of extracellular complexation lies in the ability of the fungi to produce organic acids such as oxalic acid and citric acid. The resistance of some species of decay fungi to copper has been linked to their production of copious amounts of oxalic acid (Murphy & Levy, 1983). Extracellular binding of metals also may be effected by the fungal pigments known as melanins (Daniel & Nilsson, 1989). These molecules contain phenolic, carbonyl and hydroxyl groups and are capable of binding metals (Gadd & de Rome, 1988; Senesi *et al.*, 1987; McDougall & Blanchette, 1996). Melanins may be located within the cell wall, on its surface or even a distance away from the fungal hyphae. It has been postulated that, aside from affording the fungus protection against metals by immobilizing them, the bound metals also may protect the fungus against other mycophagous pathogens or less metal-tolerant species competing for the same substrate (Rizzo *et al.*, 1992).

Fungi are able to sequester high concentrations of metals within the hyphae (Littke, 1982). They are able to store metals within vacuoles and possibly also within the cytoplasm bound to certain proteins that complex the metals and reduce their reactivity within the cell. The fungal vacuole serves to store ions and participates in the maintenance of cytosolic homeostasis (Garrill, 1995). Metal concentrations are also regulated by storage in and release from fungal vacuoles. In yeasts, it has been shown that the majority of internalized cobalt, manganese, magnesium, zinc, and potassium is found in the fungal vacuole either in an ionic state or bound to orthophosphate (White & Gadd, 1986). Polyphosphates are the only inorganic molecule found within vacuoles, and when bound to metals, appear in micrographs as visible polyphosphate granules (Doonan *et al.*, 1979).

Fungi have been shown to accumulate metals in their fruiting bodies and can concentrate metals to higher concentrations than are present in the surrounding environment (Bertrand & Bertrand, 1947; Cromack *et al.*, 1975). Zabowski *et al.* (1990) demonstrated that fungal sporocarps collected from a site treated with metal-rich municipal sludge contained higher amounts of metal than did those from a non-sludge treated site. Although differences exist in the relative abilities of different species to accumulate metals, it has been shown that fungal sporocarps representing numerous species obtained from an urban area contain higher amounts of metals than do those obtained from rural areas, presumably as a result of greater metal concentrations in the soil (McCreight & Schroeder, 1977; Laaksovirta & Alakuijala, 1978). Decay fungi have been investigated for use as bioindicators of general levels of metal pollution in the environment. Whereas this can be loosely indicative, too many physico-chemical environmental factors can affect the uptake and concentrations of metals within the sporocarps to make this a reliable tool (Tyler, 1982b).

SUMMARY

This review has examined the role that cations play in the colonization and biodegradation of wood by fungi. Wood-inhabiting fungi require cations internally for key metabolic pathways controlling growth, development, cell function and reproduc-

tion. In addition, they also require the presence of cations in the environment as essential components of the degradative system, which enables them to obtain nutrients through the breakdown of the wood cell wall. Selected cations, such as copper and chromium, and, at high concentrations, iron and manganese, also can be highly toxic to the fungi. Understanding cation requirements and toxicity levels for the brown rot fungi will help in developing an enhanced understanding of how these fungi are able to colonize and degrade the wood cell matrix.

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