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Peroxidases in grass dew derived from guttation: possible role in polymerization of soil organic matter

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Abstract. Peroxidases are enzymes that catalyze the oxidative cross-linking and polymerization of certain organic compounds by hydrogen peroxide and other organic peroxides. This study demonstrates that peroxidases are present in dew (droplets formed as the result of guttation) collected from Bermuda grass hybrids 419 and Tifway 2 [*Cynodon dactylon* (L.) × *Cynodon transvaalensis* Davy], which are warm-season C_4 grasses, and Kentucky bluegrass (*Poa pratensis* L.), which is a cool-season C_3 grass. Peroxidase activity [quantified with horseradish peroxidase (HRP) (activity 152 purpurogallin units/mg) as standard] in guttational fluids collected from grasses during early morning was in the 80 to 120 μg/L range. Isoelectric focusing was used to determine isoelectric points (pI) of the isozymes present in the Bermuda grass dew following dialysis and lyophilization of the collected dew. The pI values ranged from 4.3 to 8.3 with 14 isozymes being detected using guaiacol and hydrogen peroxide as substrates. Peroxidases also were extracted from soil supporting the growth of Bermuda grass. Peroxidases in these soils were most abundant in the top 5 cm layer (activity was in the 6.8 to 16 purpurogallin units/g range). Stability and activity of these peroxidases in the presence of fulvic and humic acids were evaluated. Compared to controls with no added humic substances, peroxidase activity was inhibited by a soil fulvic acid and prolonged by a humic acid. Field measurements indicated that peroxidase activity did not greatly decrease during the winter when the grass was dormant, indicating that the peroxidases released into the soil remain active for a considerable time. Based on results in these studies and previously determined dry and wet deposition of atmospheric peroxides, we estimate that peroxidase-catalyzed reactions in areas planted in these grasses may convert about 8 g C m^{-2} yr^{-1} of labile soil organic compounds to more persistent oligomers and polymers.

Introduction

Peroxides that enter soils through natural processes of dry and wet deposition from the atmosphere (Walcek 1987; Gunz & Hoffmann 1990; Sakugawa et al. 1990; Kleinman 1991; Gao et al. 1993; Watkins et al. 1995a, 1995b) or as part of site remediation efforts (Pardieck et al. 1992) are rapidly decomposed. Peroxidase-catalyzed polymerizations of the organic matter at the soil surface account for part of this decomposition. Peroxidases are enzymes

that, on activation by peroxides, catalyze organic matter oligomerization and polymerization.

Although it is well-known that peroxidases are distributed throughout terrestrial ecosystems, less is known about factors that influence the transport of these enzymes and their role in soil biogeochemistry. The transport of water-soluble substances, including peroxidases, from the root zone up into the shoots of plants can occur via a process called guttation. Water movement during guttation occurs within the vascular cylinder of the root which is surrounded by a boundary, the "endodermis". The waxy layer in the interior walls of the endodermis forces all movement to occur within the endodermal cells. Thus, solutes in the water, such as nutrients, are passed into the vascular cylinder but not back into the cortical area of the root. With the flow of water into the vascular cylinder there is net movement upward through the xylem. The cells which give rise to the xylem become lignified and cellular contents self-destruct, leaving essentially empty cells that are like straws lined end-to-end. These empty cells serve as conduits for the rapid transport of water and solutes upward through the plant. When soil moisture is abundant and near field capacity, water flows into the roots and the plants act as an osmometer with the resulting pressure, i.e., the "root pressure", forcing the contents of the xylem to move upward in the plant. This water is extruded through openings in the leaves called hydathodes. Although previous studies have established that guttational fluids contain water-soluble peroxidases (Ivanoff 1963; Lindner & Brand 1989; Biles & Abeles 1991; Magwa et al. 1993), little is known about the possible role of guttation in transporting plant peroxidases to the soil surface where interactions with peroxides deposited from the atmosphere would be favorable.

Peroxidase-catalyzed oxidations are known to have important biological functions in plants (Wellinder et al. 1993), including the polymerization of organic substances to form lignin and other oligomers and polymers (Saunders et al. 1964; Harkin & Obst 1973; Wellinder et al. 1993). Recent studies have shown that reactions involving catalysis by peroxidases account for a major fraction of hydrogen peroxide decomposition in freshwater and lake waters (Cooper & Zepp 1990; Moffett & Zafiriou 1990). Comparatively little is known about the nature, distribution and lifetimes of exocellular peroxidases in litter and soils and their role in peroxide decomposition and organic polymerizations. The abundance of relatively stable peroxidases must have a role in the synthesis of macromolecules such as the humic acids. The polymerization of readily degradable litter, soil organic matter and nitrogen-containing compounds to higher-molecular-weight persistent organic compounds such as humic acids influences the long-term storage of carbon in soils and the biological availability of soil nitrogen (Davidson et al. 1995).

Hydrogen peroxide (H_2O_2) and other peroxides are ubiquitous components of the atmosphere that are strongly affected by various human activities, changes in climate, and changes in solar UV radiation (Heikes et al. 1987; Thompson et al. 1989; Gunz et al. 1990; Sakugawa et al. 1990; Kleinman 1991; Fugelstvedt et al. 1994; Watkins et al. 1995a, b). The formation of gaseous hydrogen peroxide results from free radical reactions that are initiated by photolysis of species such as ozone and formaldehyde, that absorb mainly in the UV-B spectral region (280 to 315 nm). Thus, atmospheric peroxide concentrations tend to increase in response to increased UV exposure. Modeling studies have indicated that stratospheric ozone depletion, which is believed to enhance UV-B irradiance in the troposphere, significantly increases the buildup of hydrogen peroxide (Thompson et al. 1989; Fugelstvedt et al. 1994). Although chemical processes that produce and decompose peroxides in the atmosphere have received considerable attention, comparatively little is known about the fate of the peroxide at the Earth's surface. A variety of studies have shown that hydrogen peroxide is rapidly removed from the gas phase when it is transported to plant and soil surfaces (Heikes et al. 1987; Thompson et al. 1989; Gunz et al. 1990; Sakugawa et al. 1990; Kleinman 1991; Fugelstvedt et al. 1994). Such dry deposition can be a major removal process of hydrogen peroxide from the atmosphere (Kleinman 1991). Potential toxic effects of hydrogen peroxide on vegetation have been considered (Gunz et al. 1990; Sakugawa et al. 1990; Polle & Junkermann 1994).

Here, we present evidence that large quantities of plant peroxidases can be transported from the rhizosphere to surface soil via guttation and that the exocellular peroxidases are long-lived in the soils. Our calculations indicate that peroxidase-catalyzed polymerizations initiated by deposition of atmospheric peroxides may be an important mechanism for the formation of persistent soil organic matter (SOM).

Methods and materials

Guttational fluids (dew) were collected from an athletic practice field and a field within a stadium during the summer and fall of 1993 from the campus of Furman University, Greenville, South Carolina. These fields consisted of a monoculture of Bermuda grass, either the hybrid 419 or the genetic mutant Tifway 2 [*Cynodon dactylon* (L.) × *Cynodon transvaalensis* Davy], Dew also was collected during January of 1994 from a planting of Kentucky bluegrass (*Poa pratensis* L.) located on campus. The dew was collected in the early morning using a specially designed dew harvester that permitted the collection of several liters. The collected dew was either centrifuged for 5 minutes at 3000 g and frozen or dialyzed for 24 h using Spectra/por 10,000 MWCO

membrane tubing and finally lyophilized to a powder. Isoelectric focusing was used to determine isoelectric points (pI) of the isozymes present in the Bermuda grass dew following dialysis and lyophilization of the collected dew.

Soil samples were collected in September 1993 and January 1994 using a 2.54-cm-diameter soil auger. The January samples were separated into 3 components: (A) soil from the surface down to 5 cm, which was rich in organic matter and was very porous; (B) soil from a depth of 5 to 10 cm, which contained less organic matter but was very porous because of its sandy composition; and (C) soil from a depth of 10 to 15 cm which was comprised mostly of clay with little organic matter. All samples were frozen until extraction to determine peroxidase content.

Peroxidases were extracted from the soil in the following manner: 10 g of a soil sample were placed in a 15-mL centrifuge tube containing a 0.5 M KCl/20% glycerol solution and mixed gently for 24 h using a Thermolyn Vari-Mix agitator. Two extractions were made using a total volume of 15 mL. Each time, the samples were centrifuged at 3000 g. The supernatant was immediately assayed for its peroxidase activity.

The Contech fulvic acid was obtained commercially from Contech ETC, Ottawa, Canada. Characteristics of this humic substance, which was isolated from Prince Edward Island Podzol soil, have been previously described (Gamble 1970). The standard Suwannee humic acid was obtained from the International Humic Substance Society. Both humic substances were used as received.

Peroxidase activity was determined by using 3,3′,4,4′-tetramethylbenzidine (TMB) (reagent grade, Aldrich Chemical Co.) (1 mg/10 mL) and 30% hydrogen peroxide (Baker Analyzed) (2 μL/10 mL) as the substrates in a 0.05 M phosphate-citrate buffer solution (Liem et al. 1979). The reaction was stopped by adding a 2 M H_2SO_4 solution. All test solutions were placed in ELISA plates and absorbance at 450 nm was determined using a CERES 900 Microplate workstation. Horseradish peroxidase (Sigma, activity 152 purpurogallin units/mg) was used as a standard in all peroxidase determinations. One purpurogallin unit is defined as the amount of peroxidase required to catalyze the formation of 1 mg of purpurogallin from pyrogallol (50 mg/mL) in 20 seconds at pH 6.0 at 20 °C. Soil peroxidase activity was determined by using an “antigen capture” technique where anti-horseradish peroxidase (Sigma) was first attached to the walls of the wells of the ELISA plate prior to the addition of the soil extract solutions. These were incubated in a refrigerator for 24 h before washing the wells with a 10 mM Tris buffer (pH 8). The activity of the attached soil peroxidases was then determined.

Table 1. Properties of dew collected from Bermuda grass hybrids 419 and Tifway 2 [*Cynodon dactylon* (L.) Pers. × *Cynodon transvaalensis Davy*] (C_4 grasses) and Kentucky bluegrass (*Poa pratensis* L.) (C_3 grass). Peroxidase activity based on comparisons to horseradish peroxidase standard with activity of 152 purpurogallin units/mg

	Bermuda grass	Kentucky bluegrass
Peroxidase activity	123 μg/L	83 μg/L
Dissolved organic carbon	89 mg/L	62 mg/L
Ratio of peroxidase activity: organic carbon	1.4 μg/mg C	1.3 μg/mg C
Number of peroxidase isozymes	14	not tested
pH	6.2	6.8
Fe	0.178 mg/l	0.649 mg/l
Cu	below limits of detection	0.047 mg/l
Other enzymes detected	not tested	laccase nitrate reductase dehalogenase nitrilase

The dissolved organic carbon in the collected guttational fluids was determined with a Dorhmann 8C carbon analyzer and the iron and copper concentration by a Perkin-Elmer ICP Plasma II emission spectrophotometer. The expression of the peroxidase isozymes and their isoelectric points was determined by using a Bio-Rad Model 111 Mini IEF cell. The ampholyte had a pH range of 3.3 to 9.3. Peroxidase activity in the presence of either the Contech fulvic acid or the IHSS humic acid also was evaluated.

Results

Current research has demonstrated the presence of peroxidases in dew (droplets formed as the result of guttation) collected from Bermuda grass hybrids 419 and Tifway 2 [*Cynodon dactylon* (L.) Pers. × *Cynodon transvaalensis Davy*], which are warm-season C_4 grasses, and Kentucky bluegrass (*Poa pratensis* L.), which is a cool-season C_3 grass. The guttational fluids of both grasses were shown to contain peroxidases. A number of peroxidase isozymes were observed in the collected dew samples as illustrated by the data for the Bermuda grass dew (Table 1). Isoelectric focusing patterns and corresponding pI values of peroxidase enzymes in the dew were determined following dialysis and lyophilization. The pI values ranged from 4.3 to

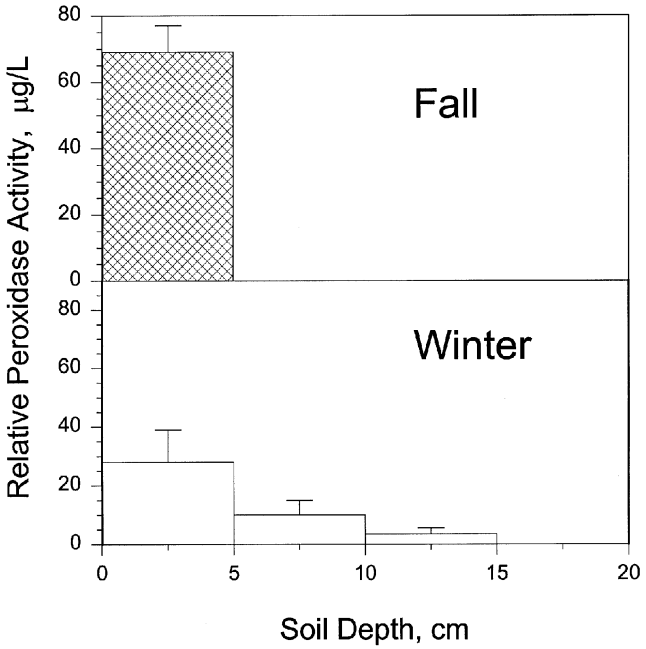

Figure 1. Relative Bermuda grass peroxidase activity found in the soil using horseradish peroxidase activity as a standard. The horseradish peroxidase standard had an activity of 152 purpurogallin units/mg. The fall soil sample was taken from the top 5 cm on 11 October 1993 and winter samples were taken from the surface to a depth of 15 cm on 24 January 1994. The latter were separated into sequential 5 cm. soil samples, ± SD.

8.3 with 14 isozymes being detected using guaiacol and hydrogen peroxide as indicating substrates.

Peroxidases also were extracted from the soil supporting the growth of Bermuda grass during the various seasons. The peroxidase activity in soils of the warm-season grass (Bermuda grass) were high during both late summer and winter (Figure 1). Since this grass is dormant during the winter, the minimal decrease in peroxidase activity during winter months indicates that peroxidases remain active in the soil for a considerable period of time. Results of the winter sampling demonstrated that peroxidase activity decreased with increasing depth in the soil (Figure 1).

Properties of the two types of guttational fluids are compared in Table 1. Peroxidase activity, which was determined by comparison to a standard solution of horseradish peroxidase, was found to be very similar when the activity was normalized to the organic carbon content of the fluid. The fluids also contained iron, which likely was in colloidal form given the extremely low solubility of iron in circumneutral water, and copper. Given the fact that both

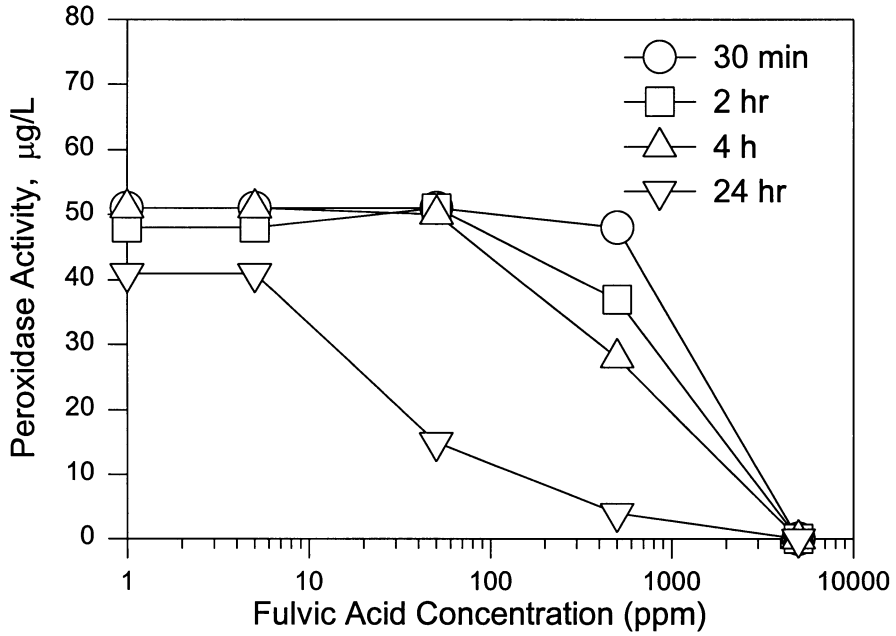

Figure 2. The inhibitory effect of fulvic acid (Contech ETC) upon the guttational fluids (dew) released by Bermuda grass (a C4-type plant) upon peroxidase activity.

iron and copper are known to catalyze organic oxidations by peroxides via Fenton reactions (e.g., see Blough & Zepp 1995), control experiments were conducted to determine the effect of Fe(III) and Cu(II) on the oxidation of the acceptor, TMB, at neutral pH in aqueous solutions of hydrogen peroxide present at the same initial concentration used in the peroxidase assays. The controls indicated that little product formed with absorbance in the visible spectral region used in the assay and hence that the iron and copper caused little ($< 5\%$) positive interference with the peroxidase assay. These results, however, did not rule out the possibility of a negative interference caused by competitive decomposition of the peroxide by the iron or copper.

Other control studies showed that the guttational fluid from the bluegrass site contained small amounts of laccase, another enzyme that catalyzes the oxidation of TMB in the absence of added peroxide (Table 1). Comparisons of TMB oxidation rates with and without added peroxide indicated, however, that laccase activity accounted for $< 10\%$ of the observed oxidation of TMB during the assay. Other enzymes, including nitrate reductase, dehalogenase, and nitrilase were also found in the bluegrass dew.

Prompted by previous studies of peroxidase interactions with humic substances (e.g., Serban & Nissenbaum 1986; Zepp et al. 1988), we examined

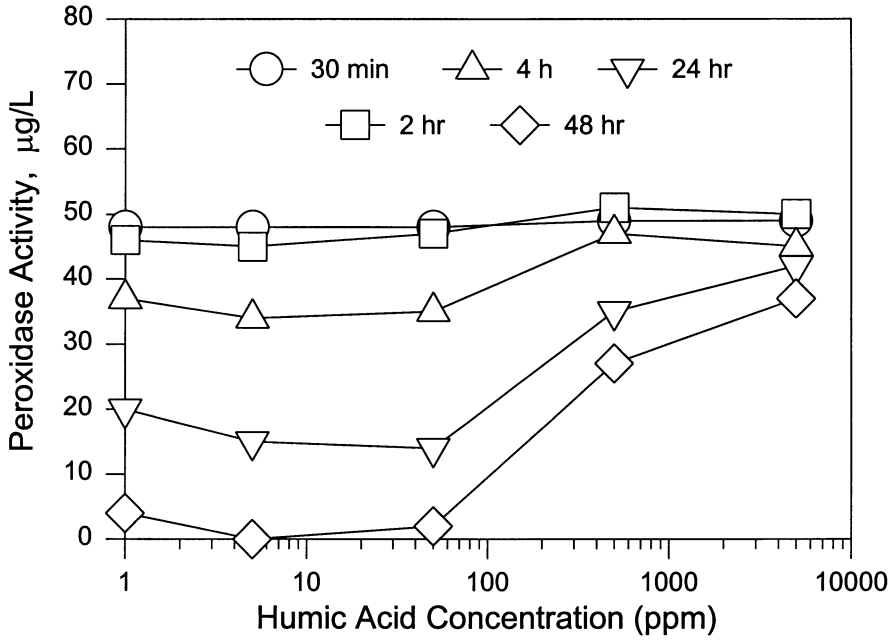

Figure 3. The interaction between humic acid (IHSS Suwanee River humic acid) and guttational fluids (dew) released by Bermuda grass (a C_4-type plant) upon peroxidase activity.

the effects of added fulvic and humic acids on the peroxidase-catalyzed oxidation of TMB in the guttation fluids. A commercial fulvic acid isolated from a Canadian soil reduced peroxidase activity in the Bermuda grass guttation fluid. The degree of reduction in activity increased with increasing time and fulvic acid concentration (Figure 2). A humic acid standard obtained from the IHSS had complex effects on peroxidase activity, reducing activity at lower concentrations, but actually prolonging activity at high concentrations (Figure 3).

Discussion

Peroxidases are widely distributed in plants and microbiota (Saunders et al. 1964; Wellinder et al. 1993). The present study indicates that peroxidases are present at significant concentrations in the dew collected from both C_3 and C_4 grasses commonly found in North America (Table 1). The dew was derived from guttation fluids in the grass shoots that were present following precipitation or watering. As observed in previous studies of guttation fluids and xylem sap of plants (Ivanoff 1963; Lindner & Brand 1989; Biles & Abeles

1991; Magwa et al. 1993), the peroxidases in guttation fluids from the grasses were shown to exist in a number of isozymic forms. The previous research has focused on assigning peroxidase isozymes to specific sites and functions within plants and plant cells (Wellinder et al. 1993). Based on previous studies, it is likely that the dew peroxidases originated mainly in the grass roots. Evidence was presented by Magwa et al. (1993) that the peroxidases in guttational fluids of *Helianthus annus* (sunflowers) were derived primarily from the roots.

Our results indicate that peroxidases are present in the soils of the grass-covered sites with the highest concentrations near the soil surface (top 5 cm) (Figure 1). We assume that the high concentrations near the soil surface reflect in part the pumping of peroxidases from the root zone up into the grass shoots via their guttation fluids. The drying of peroxidase-laden dew on the tips of the plants leads to accumulation of peroxidase on the shoot surface. Eventually, the shoot dies and ends up as part of the surface litter on the soil. The higher concentrations also possibly reflect the release of peroxidases on decomposition of root litter, which is more abundant in the upper part of the root zone.

Our results further demonstrate that humic substances in the soils may have important effects on peroxidase activity. The results that we observed with inhibition by a soil fulvic acid are consistent with previous studies that likewise demonstrated that fulvic acids inhibit the activity of horseradish peroxidase (Zepp et al. 1988). Such inhibition indicates that the fulvic acid may be competing with the acceptor, in this case the TMB, for the active site or that it may be inactivating the peroxidase (Figure 2). On the other hand, we also found that a standard humic acid enhanced the stability of the grass-derived peroxidases (Figure 3). Other studies have noted that humic acids have a strong tendency to associate with peroxidase enzymes (Serban & Nissenbaum 1986). Thus, these peroxidases are stable for some time as free enzymes but their binding to humic acids may further enhance their stability. Additional research is required to better define the complex, competing effects of humic substances on peroxidase-catalyzed oxidations.

Our field studies provide evidence of the stability of exocellular peroxidases in soils. The longevity of the peroxidases was demonstrated by our observations that the soil supporting the growth of a warm-season grass, such as Bermuda grass, has detectable peroxidase activity during the time the grass is dormant (Figure 1). Significant peroxidase concentrations were observed near the soil surface several months into the dormant season of the Bermuda grasses, indicating that the peroxidases are stable in the soils under these field conditions. The high concentrations of peroxidases that we observe at the land-atmosphere interface (on the plant shoots and soil surface) appear

to favor reactions between the peroxidases and peroxides deposited from the atmosphere although other pathways for peroxide decomposition, such as catalase-catalyzed decomposition, are also likely to be competitive.

The need for improved estimates of the role of soil organic matter polymerization (humus formation) in providing a sink for atmospheric carbon dioxide has been discussed in several recent publications (e.g., Alperin et al. 1995; Davidson et al. 1995; Melillo et al. 1995; Post et al. 1995). The potential role of peroxidase-catalyzed reactions in the polymerization of soil organic matter by deposition of atmospheric hydrogen peroxide can be estimated using results of other studies that were discussed earlier. Hydrogen peroxide removal from the gas phase at the Earth's surface, which may initially involve irreversible partitioning into condensation on plant and soil surfaces, is considerably more rapid than its transport to the surface. Thus, dry deposition is transport-limited and its rate of loss from the atmosphere is insensitive to the mechanism(s) of peroxide decomposition in plants and soils. Likewise, wet deposition of peroxide is controlled by precipitation events. Studies of dry deposition velocities indicate that they fall in the range of 1 to 5 cm s^{-1} in temperate and high-latitude (boreal) biomes, about the same as those observed for other hydrophilic species such as nitric acid (Walcek 1987; Gao et al. 1993; Hall & Claiborn 1997). Assuming a mid-range value of 3 cm s^{-1} for temperate regions, and atmospheric peroxide concentrations in the 0.1 (winter) to 1.0 ppbv (summer) range (Heikes et al. 1987; Gunz et al. 1990; Sakugawa et al. 1990; Kleinman 1991; Watkins et al. 1995a, b), the computed dry deposition flux is 1×10^{-14} to 10×10^{-14} moles cm^{-2} s^{-1}. This flux is higher than that derived from wet deposition, at least in temperate regions. The computed mean flux from wet deposition is at the lower end of the range of dry deposition fluxes (2×10^{-14} moles cm^{-2} s^{-1}) even assuming a mean peroxide concentration in precipitation of 10 M and annual precipitation of 65 cm, both of which are at the upper end of the range observed in mid-latitudes. These fluxes represent the maximum peroxide available for reactions within litter and soils, because a portion of the peroxide reaching the Earth's surface is decomposed on plant surfaces. The overall stoichiometry of a peroxidase-catalyzed oxidation is:

$$AH_2 + H_2O_2 \rightarrow A + 2H_2O$$

where A represents some oligomeric product. If it is further assumed that about 50% of the peroxide that enters the soil surface activates a peroxidase enzyme, as observed in aquatic environments (Cooper & Zepp 1990; Moffett & Zafiriou 1990), and that a typical monomer has about 10 carbon atoms, then hydrogen peroxide deposition could potentially polymerize up to 0.002 to 0.02 g C m^{-2} d^{-1} with the highest polymerization rates occurring during

the summer months when atmospheric peroxide concentrations are highest and the lowest rates during winter. By comparison, recent computations indicate that the mid-and high-latitude terrestrial ecosystems in the Northern Hemisphere are acting as a sink for atmospheric CO_2 that is approximately 1 to 2 Pg C yr^{-1} (1 Pg = 1×10^{15} g) (Ciais et al. 1995) which corresponds to about 0.06 to 0.12 g C m^{-2} d^{-1}, averaged over vegetated land in these regions.

Conclusions

A study has been undertaken on the peroxidases in the dew and soils of two types of grasses that are commonly found in the southern part of North America. The results suggest that large amounts of peroxidases are pumped from the root zone to the land surface via guttation in the grasses. Peroxidase concentrations in the dew of both Bermuda and blue grasses were found to be in the 80 to 120 μg/L range, based on comparisons to a peroxidase standard with activity of 152 purpurogallin units/mg. This peroxidase transport process contributed to the observed high concentrations of peroxidases in the surface litter and soils of the grass-covered sites. The peroxidases are relatively stable enzymes with significant concentrations present during the winter months during periods of grass dormancy. Humic substances are shown to have important effects on the activity of the dew peroxidases, with a fulvic acid reducing activity but a humic acid preserving activity at higher concentrations.

The high concentrations of peroxidases that we observed at the land-atmosphere interface (in the dew and on the plant shoots and soil surface) favor exocellular reactions between the peroxidases and peroxides deposited from the atmosphere. Peroxidases are known to be involved in reactions which result in the polymerization of organic substances, increasing their environmental persistence. The significance of these reactions may not only be related to the accumulation of complex organic molecules but also the “trapping” of nitrogen-containing compounds which then results in the reduction of available nitrogen for the growth of plants.

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