IRG/WP/97-20112

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Section 2 Testing

Detection of increased metal cations after wood decay using Chromeazurol-S

by

Frederick Green III, Usha Srinivasan* and Regis B. Miller

Forest Products Laboratory, Madison, WI, U.S.A.

*Wood Science and Technology Center, Fredericton, NB, Canada

Prepared for the 28th Annual Meeting

Vancouver,B.C.

25-30 May 1997

IRG Secretariat
KTH
Brinellvagen 34
S-100 44 Stockholm
Sweden

Detection of Increased Metal Cations After Wood Decay using Chromeazurol-S

Frederick Green III, Usha Srinivaean* and Regis B. Miller
U.S. Department of Agriculture, Forest Service
Forest Products Laboratory, Madison, WI, USA 53705-2398
and
*Wood Science and Technology Center, Fredericton, NB, Canada

SUMMARY

Chromeazurol-S (CAS) is a dye used for the spectrophotometric determination of metals, mainly aluminum (Al), berillium (Be) and copper (Cu). CAS has been widely used for determining the penetration of copper containing preservatives like CCA (AWPA A3-96). Additional uses include: i) detection of utility pole decay (Esyln, 1979), ii) a chemical spot test for aluminum in wood (Kukachka and Miller, 1980) and fungal production of iron III-binding siderophores in agar (Srinivasan et al., 1993, 1994). The objective of this study was to determine the mechanism of color change from pink (negative) to blue (positive) following brown-rot decay (BRD) of Southern pine and white-rot decay, (WRD) of maple blocks using soil-block tests. Changes in wood metals (ppm) were estimated by inductive coupled plasma spectroscopy (ICPS). Although preliminary results suggest that increased translocation of iron into the wood by siderophores during BRD potentiated the CAS color changes, simultaneous translocation of aluminum into the soil blocks by brown-rot fungi (BRF) clearly contributed cumulatively to the positive CAS tests at 12 weeks.

INTRODUCTION

Chromeazurol-S (CAS)is a chromogenic reagent important in the detection of heavy metals. Standard A3 of the American Wood-Preservers Association manual (1996) describes a method for determining the depth of penetration into wood of copper-containing preservatives that requires a 0.5% chrome azurol-S solution. In addition, CAS is used to detect low molecular weight iron binding compounds called siderophores produced by bacteria and fungi in agar media (Fekete et al., 1989; Jellison et al., 1992, 1997; Srinivasan et al., 1993, 1994; Schwyn and Neilands 1987); consecutive determination of iron(III) and aluminum mixtures (Nishida 1969, 1970,); as a chemical spot-test for aluminum for wood identification (Kukachka and Miller; 1980); and to test for decay in utility poles (Eslyn, 1979)

The paper by Eslyn (1979) provided no hypothesis of how or by what mechanism any color change was observed following wood decay. Our objective in this paper was to decay Southern pine and maple wood blocks with brown and white-rot fungi (WRF), respectively, and spray or spot test the blocks with CAS for color changes. Blocks were then analyzed for elemental changes to determine which metals, if any, were the cause of the positive reaction with CAS following decay.

MATERIALS AND METHODS

FUNI : Brown-rot fungi, *Meruliporus incrassata* (Berk. and Curt.) Murr. (MAD-563); *Gloeophyllum trabeum* (Pers. FR.)(Mad 617); *Postia placenta* (FR.)M.Lars.et Lomb. (MAD-698); *Fomitopsis palustris* (TYP-6137) and (L-15755) were maintained on 2% malt agar plates. The white-rot fungus, *Trametes versicolor* (L.:Fr.Pil) (MAD-697) was maintained similarly.

ASTM soil block tests: Weight loss of southern pine or maple blocks (3/4 in square/1.91 cm square) was determined according to ASTM D-2017 (ASTM 1993) after 12 weeks incubation and compared to uninoculated control blocks. Duplicate wood blocks were pretreated with 1% EDTA, 1% iron dextran, or 1% Ruthenium Red (RR) to determine the effects on weight loss during decay.

Chromeazurol-S: CAS was prepared as a 0.5% aqueous solution plus 5.0% sodium acetate and sprayed onto test blocks following 12 weeks decay.

Elemental analysis: Inductive coupled plasma spectroscopy was performed at the UW Extension Soil and Plant Analyisis Lab in Madison, WI on duplicate samples. (P, K, Ca, Mg, S, Zn, B, Mn, Fe, Cu, Al and Na contents were estimated in ppm)

RESULTS

The decay results of brown-rotted pine blocks and white-rotted maple blocks are shown in Table 1. The weight loss of brown-rotted blocks ranged from 16-60%. All brown-rotted decay blocks turned blue when sprayed with CAS except for blocks 9 and 10. Blocks 9 and 10 were treated with 1% Ruthenium Red and showed only 1% weight loss. The weight loss of white-rotted maple blocks ranged from 43-54%. EDTA treated maple blocks retained pink color while iron treated maple blocks turned blue to CAS.

Elemental analysis of control and decayed wood blocks are shown in Table 2. The largest changes occurred in the brown rotted blocks as indicated by increases in Fe>Al>Mg>Mn>Cu. The largest increases are shown in Fe, over 100 fold for *F . palustris.*

White-rot decay was characterized by little or no translocation of elemental metals into the wood even though the decay weight losses averaged near 50% for *T . versicolor*. Only magnesium increased about 2-fold. Treatment with EDTA appeared to prevent the pink to intermediate purple CAS color change observed in control maple and 1% iron dextran contributed to the color change to blue.

Table 1. - 12 Week Soil Block Test of Southern Pine and Maple Blocks Sprayed with CAS (N=2)

Brown-Rot/ Southern Pine	Weight Loss (%)	CAS Color Test
C1 /C2: Control	N/A	Pink
T3 /T4: M. incrassata (MAD 563)	36/60	Blue
T5 /T6: G. trabeum (MAD 617)	16/17	Blue
T7 /T8: P. placenta (MAD 698)	47/46	Blue
T9 /T10: P. placenta (1.0% R Red)	1/1	Pink
T11/T12: F. palustrus (TYP 6137)	29/29	Blue
T13/T14: F. palustrus (L 15755)	39/41	Blue

White-Rot/Maple	Weight Loss (%)	CAS Color Test
C1/C2: Control	N/A	Pink
T3/T4: T. versicolor (MAD 697)	51/52	Purple
T5/T6: T. versicolor (1% EDTA)	54/54	Pink
T7/T8: T. versicolor (1% iron dextran)	43/43	Blue

Table 2. Elemental Analyses of Control and Decayed Wood Blocks by ICP Spectroscopy (ppm based upon original wood weight)

Brown-Rot Test (Wt. loss/Fungus)		....Ca	....Mg	....Mn	....Fe	....Cu	....Al
Control Pine	C1	768	166	32	3	9	38
	C2	1732	500	172	28	22	68
T3	(52% M. incrassata)	843	310	164	210	5	142
T4		1840	687	456	561	11	354
T5	(17% G. trabeum)	1647	459	238	147	18	157
T6		847	313	136	83	19	117
T7	(50% P. placenta)	2402	1787	1520	1127	77	3291
T8		1029	797	603	549	36	1467
T11	(29% F. palustris)	1094	866	662	1566	32	2306
T12		1544	1042	760	2254	43	2728
T13	(40% F. palustris)	1522	948	571	536	19	1120
T14		947	692	296	256	16	569
White-Rot		....Ca	....Mg	....Mn	....Fe	....Cu	....Al
Control Maple	C1	1253	184	35	8	4	53
	C2	1399	181	34	8	4	54
T3	(50% T. versicolor)	994	382	22	5	3	32
T4		1123	360	17	2	3	30
T5	(55% T. versicolor)	944	381	20	3	3	25
T6		969	403	26	2	2	27
T7	(43% T. versicolor)	948	373	16	910	3	30
T8		939	382	15	914	3	27

DISCUSSION

Metals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation. Many metals are essential, e.g., K, Na, Mg, Ca, Mn, Fe, Co, Ni, Cu, Zn, Mo, whereas others have no known essential biological function(s), e.g., Al, Ag, Cg, Sn, Au, Sr, Hg, Ti, Pb. All these elements can interact with microbial cells and be accumulated as a result of physico-chemical mechanisms and transport systems of varying specificity, independent of, or directly and indirectly dependent on, metabolism (Gadd, 1993).

In the context of this paper, the brown-rot fungi (BRF) and white-rot fungi (WRF) after decay of the respective wood species seem to have increased the levels of certain metal ions, which must have been sequestered from the soil (Dragun, 1988). Although these experiments were limited in scope to five BRD fungi and one WRD fungus, certain important points emerged.

All fungi sequester the essential metals from their surroundings through many different mechanisms. Mostly when the metal ions of interest are plentiful, they are able to freely diffuse through their cell membrane. In situations where the metal ions are low in concentration the fungi produce specific ion chelating low molecular weight protein based compounds, e.g., "siderophores" which are specific to iron (III) chelation.

Mechanisms of iron transport are well studied (Fekete et al., 1989; Goodell et al,. 1997; Jellison et al ., 1991, 1992). Siderophores are compounds produced by bacteria and fungi for scavenging iron (III) from the environment for transport across membranes. Since 1970, the number of well characterized siderophores has risen to over 40 (Hider, 1984). Certain siderophores also possess a high affinity for Al^{+3} and Cu^{+3}. Aluminum forms a trivalent cation with high affinity for siderophores, and as such, aluminum in the soil may compete for translocation extracellularly with iron (III).

In the results obtained, it is evident that BRF in general increased the concentration of 5 of the 12 elements tested and changed the CAS test from pink to blue. It is also evident that the total concentration of the six metals listed in Table 1, varied with each fungal species to some extent. All the metal ions listed are essential ions that may play an important role in the physiological functioning such as production of metalloenzymes of the BRF. Of the six metals listed, iron is of great interest as it is part of the Fenton reaction that is proposed as a decay mechanism for BRF. However, the elemental analysis of the BR decayed blocks not only showed high levels of Fe but also of aluminum, nearly 50 times more than the control blocks. Native soil concentrations of iron and aluminum are 7,000 - 550,000 and 10,000 - 300,000 respectively (Dragun, 1988). Is it possible that aluminum may play some role in the decay mechanism interchangeably with Fe or on its own. Jellison et al., (1992) also showed a progressive pattern of aluminum accumulation similar to iron during brown-rot but not white-rot decay. However, there is a concern whether the aluminum may be available for use. Aluminum binds as tightly to siderophores as does Fe (III), however it's relaese from the siderophore, unlike iron, would not be facilitated by a reduction step. Thus, absorbed aluminum

would tend to remain bound to the siderophore and not be available for transport into the cell.

One of the key differences between brown-rot decay fungi and white-rot decay fungi is the accumulation of oxalic acid (Shimazono, 1951; Green et al., 1991; Dutton and Evans, 1996). Due to production of oxalate decarboxylase, most WRF degrade oxalic acid while BRF accumulate this organic acid driving the pH of the microenvironment below 2.0. Small amounts of oxalate in solution increase the effective volubility of aluminum and iron in soil increasing transport of Al^{+3} and Fe^{+3} ions into wood during brown-rot decay (Griffiths et al., 1994). This is reflected in the elemental analysis by ICP spectroscopy in this study. However, if oxalic acid reduces available Fe^{+3} to Fe^{+2} then this would also limit availability of iron (III) to complex with CAS, and make Al more critical to positive CAS color change. Also, the oxalic acid may help in the reduction of aluminum oxide bound to the siderophores, as reported by Franz et al., (1991) where citric acid production by Pencillium spp. can solubilize zinc from zinc oxide.

CAS and rubeanic acid have approximately the same sensitivity to detect copper at ca. 25 ppm. However, the AWPA method for copper determination typically detects more than 2000 ppm Cu at a standard 0.4 psf retention. Therefore, relatively small increases in copper are not likely to be the main contributor to the dark blue color observed in BRD with only two fungi exceeding 25 ppm. (Table 2). The higher concentrations of Al > Mg > Mn observed in BRD would be likely candidates to activate the CAS, however, Mg and Mn are not reported to actuate CAS. Kukachka and Miller (1980) observed that certain tropical woods containing high Fe were negative for CAS but that high Al was uniformly positive for CAS. It is possible that in certain instances, iron is chelated or sequestered and not available to bind to CAS (Nishida, 1969). Because BRF translocated higher levels of Al then Fe, we conclude that the either the aluminum alone or a combination of Al and *Fe* caused the blue CAS reaction.

With the white-rot decay blocks, the results were less dramatic than with the BRF. The maple wood blocks decayed by T . versicolor did not undergo a dramatic change from pink to blue as evidenced by a light purple (intermediate) color in the untreated, yet heavily decayed maple blocks. Pretreatment with EDTA slightly increased weight loss by chelating transition metals and blocking CAS color change even at 55% weight loss. Iron dextran pretreatment resulted in 900 ppm iron in the blocks, thus demonstrating that iron content can change the spray test from pink --> blue. There is no clearcut reason for T . versicolor blocks 3 & 4 to turn intermediate purple. Jellison et al., (1992) reported 2-fold increases in Mn during white-rot decay of poplar chips. However, it is interesting that the concentration of Mn is depleted in the WRF decay blocks (Table 2). It is possible the Mn is being used by the WRF for the Mn dependent lignin peroxidase activity.

Although the limited sample numbers of this study do not give a conclusive answer as to why certain metal ion concentrations increase or decrease during decay, it definitely sheds light on the importance and role of various metal ions in the physiological functioning of the decay fungi and the severity of ground contact decay. In addition it gives an insight to the variability in the detection of metal ions by the CAS reagent.

REFERENCES

ASTM
1993. D 1413. Standard test method for wood preservatives by laboratory soil-block cultures. The ASTM Book of Standards. ASTM, Philadelphia, PA.

AWPA.
1996. Method for determining penetration of copper-containing preservatives, American Wood-Preservers' Association Standard A3-96, p. 150-152.

Bailey, J.H.E. and D.W. Reeve.
1994. Spatial distribution of trace elements in black spruce by imaging microprobe secondary ion mass spectrometry, Journal of Pulp and Paper Science: 20(3):J83-J86.

Benderliev, K.M. and N.I. Ivanova.
1996. Determination of available iron in mixtures of organic chelators secreted by scenedesmus incrassatulus, Biotech. Tech. 10(7):513-518.

Blamey, F.P.C., C.J. Asher, G.L. Kerven and D.G. Edwards.
1993. Factors affecting aluminium sorption by calcium pectate, Plant and Soil, 149:87-94.

Borggaard, Ole K.
1992. Dissolution of poorly crystalline iron oxides in soils by EDTA and oxalate, Z. Pflanzenernahr. Bodenk., 155:431-436.

Cromack, Kermit Jr., Phillip Sollins, William C. Graustein, Karen Speidel, Allen W. Todd, Gody Spycher, Ching Y. Li and Robert L. Todd.
1979. Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus Hysterangium crassum, Soil Biol. Biotechm. 11:463-468.

Dutton, M.V. and C.S. Evans.
1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment, Can. J. Microbiol 42:881-895.

Dragun, James.
1988. Element fixation in soil, In: The Soil Chemistry of Hazardous Materials, pp. 76-77.

Eslyn, W.E.
1979. Utility pole decay Part 3: detection in pine by color indicators. Wood Sci. Technol. 13:117-126.

Fekete, F.A., V. Chandhoke and J. Jellison.
1989. Iron-binding compounds produced by wood-decaying basidiomycetes, Appl. Environ. Microbiol. 55(10):2720-2722.

Franz, A., W. Burgstaller and F. Schinner.
1991. Leaching with Penicillium simplicissimum: Influence of metals and buffers on proton extrusion and citric acid production. Appl. Environ. Microbiol., 57(3):769-774.

Gadd, G.M.
1993. Interactions of fungi with toxic metals, New Phytology, 124, 25-60.

Garcic, A.
1979. A highly sensitive, simple determination of serum iron using chromazurol B, Cinica Chimica. Acta. 94:115-119.

Goodell, B., J. Jellison, J. Liu, G. Daniel, A. Paszczynski, F. Fekete, S. Krishnamurthy, L. Jun and G. Xu.
1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood, J. Biotech. (in press)

Green, F., III, M.J. Larsen, J.E. Winandy and T.L. Highley.
1991. Role of oxalic acid in incipient brown-rot decay. Mater Org. 26(3):191-213.

Griffiths, R.P., J.E. Baham and B.A. Caldwell.
1994. Soil solution chemistry of ectomycorrhizal mats in forest soil, Soil. Biol. Biochem. 26(3):331-337.

Hider, R.C.
1984. Siderophore mediated absorption of iron in structure and bonding II, Springer-Verlag, Heidelberg, pp. 25-81.

Jellison, J., V. Chandhoke, B. Goodell and F.A. Fekete.
1991. The isolation and immunolocalization of iron-binding compounds produced by Gloeophyllum trabeum, App. Microbiol. Biotechnol. 35:805-809.

Jellison, J., K.C. Smith and W.T. Shortle.
1992. Cation analysis of wood degraded by white- and brown-rot fungi, IRG/WP/1552-92, 16 p.

Jellison, J., J. Connolly, B. Goodell, G. Doyle, B. Illman, F. Fekete and A. Ostrofsky.
1997. The role of cations in the biodegradation of wood by the brown-rot fungi. Inter Biodeter. Biodegra. (in press)

Kukachka, B.F. and R.B. Miller.
1980. A chemical spot-test for aluminum and its value in wood identification, IAWA Bulletin n.s., Vol. 1(3)104:109.

Nishida, H.
1969. Consecutive determination of iron (III) and aluminum in their mixture using EDTA titration with together spectrophotometric method with chromazurol S, Japan Analyst, 18: 1451-1456.

Nishida, H.
1970. A study on the spectrophotometric determination of iron (III) with chromazurol S, Japan Analyst, 19: 221-226.

Schwyn, B. and J.B. Neilands.
1987. Universal chemical assay for the detection and determination of siderophores, Anal. Biochem. 160:47-56.

Shimazono, H.
1951. The biochemistry of wood-rotting fungi: the accumulation of oxalic acid. J. Jpn. For. Soc. 33(2):393-397.

Shiso, Y. and T. Takeuchi
1968. Spectrophotometric determination of iron (III) with chromazurol S and cetyl trimethylammonium chloride, Japan Analyst, 17, 1519-1523.

Srinivasan, U., T.L. Highley and A. Bruce.
1993. The role of siderophore production in the biological control of wood decay fungi by Trichoderma spp. International Biodeterioration and Biodegradation Symposium. Leeds UK. (Proceedings in press).

Srinivasan, U., A. Bruce and T. Highley.
1994. Siderophore production by Trichoderma spp. and its importance in the biological control of wood decay fungi, IRG/WP/94-10070, Bali, Indonesia, 33 p.

Young, H.E. and V.P. Guinn.
1966. Chemical elements in complete mature trees of seven species in Main, Tappi, 49(5):190-194