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Demonstration of a Strict Molecular Oxygen Requirement of Yellow Latex Oxidation in the Central Amazon Canopy Tree Muiratinga (*Maquira sclerophylla* (Ducke) C.C. Berg)

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Abstract

Plant-derived latex is widely used in rubber production and plays important roles in ecological processes in the tropics. Although it is known that latex oxidation from the commercially important tree *Hevea brasiliensis*, results in latex browning, little is known about latex oxidation in highly diverse tropical ecosystems. Here we show that upon physical trunk damage, yellow latex released from the canopy tree Muiratinga (*Maquira sclerophylla* (Ducke) C.C. Berg) is rapidly and extensively oxidized to a black resin in the presence of air within 15-30 min. In a nitrogen atmosphere, latex oxidation was inhibited, but was immediately activated upon exposure to air. The results suggest the occurrence of O_2-dependent oxidative enzymes including polyphenol oxidase (PPO) within the latex of Muiratinga and supports previous findings of a key role of oxidation during latex coagulation.

Keywords: Secondary metabolism; latex oxidation; plant physiology; plant-insect interactions.

1. Introduction

Plants have been interacting with insects for hundreds of millions of years and have evolved numerous chemical and physical means of defense against diverse herbivore feeding strategies.[1] For example, fossil evidence indicates that the emergence of the first lineage of angiosperms occurred during the Cretaceous (Mesozoic Era), about 136- 130 Mya. With this major evolution in plant lineages came the need for the development of increasingly effective defense mechanisms to counter biotic stresses from herbivores and microbes, including secretory structures, classified according to the substance produced.[2] Among the common defense substances secreted in high quantities by many plant species is latex (*cis*-1,4 polyisoprene) (Figure 1), which is commercially exploited as a source of natural rubber.

The first evidence for the occurrence of latex was recorded in an angiosperm fossil from the Eocene epoch (~50 Mya).[3] Latex is stored under pressure within elongate cellular tubes close to the surface in secretory structures known as laticifers, and released into the environment when the laticifers are damaged.[4] Thus, latex functions as an effective physical and chemical defense against biotic stress by traping and/or intoxicating herbivores and microbes and sealing wounds, thereby minimizing the consumption of plant material and the entry and proliferation of herbivores and microbes into plant tissues.[5-7] Globally, an estimated 10 % of angiosperms are thought to produce latex, represented by 20 families.[8] Allthough poorly characterized due to the extremely high biodiversity including an estimated 17,000 tree species, [9] the occurance of latex producing trees is thought to be more common in the tropics than in other biomes[6] , as insect-plant interactions played major roles in the evolution of high tree species biodiversity and habitat specialization.[10] For example, one study estimated that latex production in tropical tree species (14 %) is more common when compared to temperate species (6 %).[11]

Derived from latex, natural rubber is widely used in industrial and medical products as an elastomer with a high stretch ratio and exceptional frictional, strength, durability, and waterproof properties.[12] Following harvesting by making a small incision on the bark and collecting the fluid, latex undergoes commercial processing into rubber. Latex is commercially prepared by the process of vulcanization, invented by Charles Goodyear in 1843,[13] which involves removing the sulfur content and heating in order to produce a polymer that maintains its elastic characteristics in both cold and warm temperatures. While often considered only a modern product associated with fueling the industrial revolution, early inhabitants in the Amazon Forest used latex for numerous purposes before the European arrival in South America.[14] In Brazil with its large population of *Hevea brasiliensis* trees, belonging to the family Euphorbiaceae, native Brazilians supplied the first entry of natural rubber to the international market.[15] However, as latex became an important global commodity, prices rose and production rates increased to meet the growing demand.[15] By 1910, there was an estimated 131,000-149,000 individuals tapping *H. brasiliensis* trees in the Brazilian Amazon, each expected to produce 250-350 kg of rubber every 100 days.[16] Therefore, natural rubber represented an important historical commercial product for Brazil, especially for the city of Manaus, an Amazon city that was considered the rubber capital of the world between 1905 and 1912.[16] During this period, latex was in high demand for the production of rubber tires for automobiles in the United States and Europe. However, seeds of *H. brasiliensis* transported from South America to London were successful introduced to plantations in Asia. By 1915, Asia dominated global supplies of natural rubber prompting a crash in Brazilian natural rubber prices and a collapse of the Brazilian rubber industry.[16]

Latex production begins within the cytosol of specialized latex-producing cells [17] where the mevalonate pathway generates monomers (isoprene, C5H8) that are polymerized into polyisoprenes by a rubber cis-prenyltransferase complex on the surface of rubber particles.[18] The water soluble rubber particles are a major component of latex, a complex colloidal suspension which also contain numerous other substances including proteins, alkaloids, carbohydrates, oils, and polyphenols such as tannins that, together with rubber particles, coagulate on exposure to air.[12] However, while primarily studied in the commercially important latex producing species *H. brasiliensis*, little is known about latex coagulation mechanisms among the estimated 20,000 latex-producing plant species.[19]

One key, but highly uncharacterized process, known to be involved in the coagulation of latex, is the oxidation of components including polyphenols and fatty acids. In *H. Brasiliensis*, for example, a discoloration often appears in latex attributed to the action of molecular oxygen-dependent oxidizing enzymes including polyphenol oxidase (PPO) and lipooxygenase (LOX). Early research identified a putative molecular oxygen dependent PPO in latex, which catalyze the oxygen-dependent oxidation of phenols to reactive quinones.[20] In the presence of molecular oxygen (O_2), PPO enzymes are involved in the browning of cut fruits and vegetables, and dry rubber sheets freshly prepared from milky white latex.[21] The browning of latex upon exposure to air, observed in many species, suggests that PPO enzymes are a common component of latex.[6] Moreover, the oxidation products of polyphenols by PPO (α-quinones) and fatty acids by LOX (fatty acid hydroperoxides and their degredation products) are known to covalently bind to amino acids.[6] This suggests that one function of latex oxidation is to decrease the nutritive value of proteins for herbivores. However, RNA-interference studies in transgenic dandelion plants revealed that polyphenol oxidation by PPO plays a critical role in the coagulation process and associated to latex browning.[22] Reducing PPO activity by silencing the PPO gene resulted in dandelion plants that expelled 4-5 times more latex than control plants with residual PPO activity positively correlated to the coagulation rate.

In this study, we evaluated the potential presence of oxygen-dependent oxidizing enzymes within the latex of the canopy tree species Muiratinga (*Maquira sclerophylla*). As a member of the family Moraceae, Muiratinga is a specie reported in the literature to be useful as a timber source and occurs in non-flooded forests throughout Western Amazon.[23] The powdered bark of Muiratinga has been well documented to have powerful hallucinogenic properties, and is consumed by native Brazilians as snuff during annual ceremonies.[24] However, this species was chosen in this study on latex oxidation, as it has been reported in floristic surveys of the central Amazon Basin to possess a latex with the brownish color similar to “coffee with milk”.[25]

2. Experimental Section

The field activities involving latex oxidation occurred in an extensive area of mature tropical forest near the cities of Manaus, Brazil (Figures 2a e 2b). The experiments were carried out in the Experimental Station of Tropical Forestry (E.E.S.T/ZF-2) which contains roughly 22,000 ha adjacent to extensive areas of tropical forest[26]. Roughly 60 Km northwest of Manaus, a single individual of Muiratinga (*Maquira sclerophylla*) was discovered (60° 12’31.4“S 60 12‘34.8“W) with a diameter of 56 cm taken at 4.1 m above the ground (just above the root buttress, see Figure 2c). The tree identification took place in May of 2017, and field experiments on its latex production occurred during May-July 2017. Unlike many other trees nearby, the trunk did not possess visible infections from insects including termites.

The experiment on latex oxidation used combined methods. The first method consisted on making a small incision of approximately 10 cm with a knife at 1 m distance from the base of the tree and recording images of the wound site every minute between 0-60 minutes with a small camera (GoPro HERO 4 Silver Edition camera, GoPro, Inc., San Mateo, California). The second method involved quickly placing the removed piece of the bark with underlying wood attached (phloem and xylem structure) into a 2.0 L dynamic flow through glass chamber with nitrogen flowing through at 3.0 L min^{-1} with images recorded every minute for 60 min with another small camera. Following this period, the nitrogen flow was switched off and the lid was immediately removed to allow the ambient air to interact with the wood/latex sample with images continuing to be collected every minute for an additional 30 min.

3. Results

The Moraceae family of trees, which consist of over 38 genera and 1180 species are widespread in tropical and subtropical regions. Moreover, latex production by trees of the Moraceae family is considered common with latex from several species including *Maquira coriacea* ((H.Karst.) C.C.Berg) and *Maquira sclerophylla* said to be a deadly poison.[27] In this study, we analyzed an individual of *Maquira sclerophylla* for the potential presence of molecular oxygendependent latex oxidizing enzymes. This species is distributed from the Amazon Basin to Suriname in non-flooded forests (e.g. plateaus).

Upon initial removal of a small section of bark with phloem and sap wood from the Muiratinga individual at roughly 1 m height, yellow latex flowed out from the wound site (Figures 3 and 4). However, within 1 minute following the damage, considerable browning of the latex could be observed with a full brown color occuring by 10 min. As the darkening process of the latex continued, within 25 minutes the latex was completely black (Figures 4, but also see Figure 3). Moreover, the resulting darkened latex no longer appeared as a flowing liquid colloidial suspension, but as a hardened resin. Therefore, it was observed that extreme and rapid browning of the latex co-occurred with latex coagulation.

In order to evaluate the posibility that oxygen-dependent enzymes like PPO are responsible for both rapid and extensive browning, and play key roles in the latex coagulation process, the removed bark and attached phloem and sapwood from Figure 4 were quickly placed in a glass flowthrough chamber under a nitrogen atmopshere (Figure 5). Despite the rapid browning of the yellow latex at the wound site which showed significant changes after only 1 min, the yellow latex present in the removed bark with phloem and sapwood under the nitrogen atmosphere remained as a yellow suspension for the entire duration that the nitrogen was flowing through the chamber (30 min). However, upon switching off the nitrogen flow and opening the lid of the chamber to allow ambient air to enter and interact with the detached bark with phloem and sapwood, significant browning could be observed after 1 min under ambient air with a complete browning and coagulation of the yellow latex present after 25 min (Figure 6).

4. Discussion

Plant-derived latex is widely used in natural rubber production and plays important roles in ecological processes in the tropics including the defense against herbivores and microbes. In Amazon Basin, although latex from the commercially important tree *Hevea brasiliensis* is known to poses a redox system regulating latex oxidation,[28] resulting in spontaneous discoloration, little is known about latex oxidation in highly diverse tropical ecosystems. In this study, we show that the wound-induced relase of latex from the stem of

Muiratinga (*Maquira sclerophylla*), is rapidly and extensively oxidized to a black resin in the presence of air within 30 min. The results demonstrate an extreme browning of yellow latex in Muiratinga to a black resin, far beyond what has been observed in other species to date. Moreover, a strict molecular oxygen dependence of the yellow latex browning was found by removing molecular oxygen from the headspace atmosphere of the damaged bark and phloem and sapwood.

As wound-induced browning of latex has been described as a common phenomenon in numerous latex producing species, and attributed to the activity of molecular oxygen-dependent PPO enzymes,[22] our results suggest that PPO activity in the yellow latex of Muiratinga is extremely high. However, the results also suggest that while stored in the laticifers, PPO remains inactive due to low O_2 concentrations and/or the requirement of proteolytic cleavage of a single precursor (pre-PPO) in order to generate the active PPO enzyme.[22] Thus, PPO activity in Muiratinga is high only when the yellow latex is exposed to ambient air following rupture of the laticifers, and/or prePPO is cleaved to PPO by a protease. PPO activity has been linked with latex coagulation and hypothesized to play a critical role in wound sealing and influence the quantity of latex released from wounds.[22]

The rapid and extensive latex browning observed in Muiratinga upon exposure to air is consistent with previous studies on polyphenol oxidation with molecular oxygen catalyzed by PPO enzymes that generate light absorbing pigments including α-quinones and diquinones. [29,30] Therefore, the view of a central role of polyphenol oxidation as a central mechanism in latex coagulation is supported. However, as other oxidative enzymes may be involved including peroxidases and lipooxygenases, additional mechanistic studies are needed to evaluate this possibility. Nonetheless, given the extreme browning and rapid coagulation of yellow latex from the damaged Muiratinga stem under ambient air, the results suggest that this species may be an ideal candidate for detailed mechanistic studies on the relationship between latex browning, the activity of oxidative enzymes, and the coagulation process. Therefore, the results of this study have important implications for understanding the mechanisms of latex coagulation and its ecological role(s) including wound sealing and defense against herbivores.

Moreover, it has been noted that the Moraceae family, with 1180 species registered,[31] has as hallmark the presence of laticifers and a milky latex.[32] It is well documented that in the many tree species making up this family, the bark, leaves and also likely the inflorescences are lactiferous.[27] The latex has been described as white or yellow and often turns brownish or reddish upon exposure to air [27], suggesting that the oxidation of latex is widespread in this family. However, a survey across the Moraceae family is needed to evaluate this possibility and the speed and extent of latex browning as a consequence of PPO and other oxygendependent enzyme activities. By observing variations in latex browning and coagulation rates among the 1180 tree species, this would present a unique opportunity to evaluate quantitative relationships between latex browning, the activity of oxidative enzymes, and the coagulation process.

5. Conclusion

The extreme browning and rapid coagulation of yellow latex from the damaged Muiratinga stem under ambient air suggest that this species may be an ideal candidate for detailed mechanistic studies and quantitative relationships between latex browning, the activity of oxidative enzymes and the coagulation process. In addition to contributing to an improved understanding of plant physiological ecology of latex in the tropics, these and other mechanistic studies on latex oxidation processes will hopefully lead to a more sustainable commercial exploitation of arboreal latex in diverse tropical ecosystems, as well as contribute to their environmental and ecological conservation.

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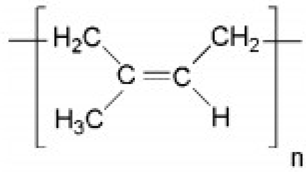

Figure 1. Chemical structure of latex (*cis*-1,4 polyisoprene)

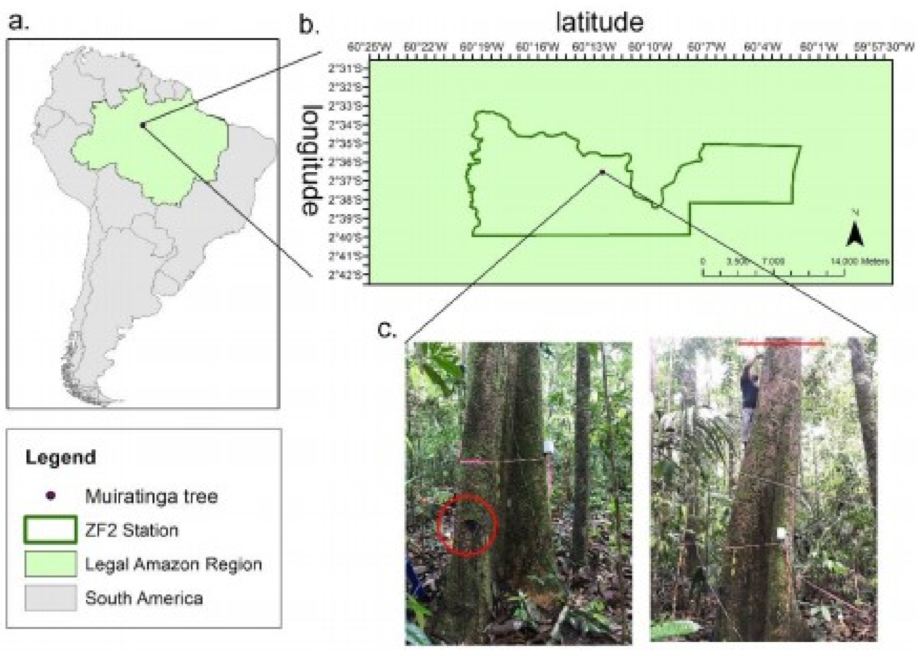

Figure2. Location of field study on latex oxidation on a Muiratinga (*Maquira sclerophylla*) individual discovered in the ZF2 tropical forest reserve in the central Amazon rainforest (map based on Datum SIRGAS2000, 20S Zone). (a) Map showing the legal Amazon Forest region in South America, (b) the ZF2 tropical forest reserve field station, and (c) the Muiratinga individual (60° 12'31.4"S 60 12' 34.8"W) studied for latex oxidation. Note the red circle in Figure 2c highlights the wound with the black oxidized latex formed after the experiment shown in Figures 4-6. Also shown in Figure2c is a red horizontal line indicating the height at which tree diameter measurements were taken

Figure 3. Example of yellow latex oxidation upon physical trunk damage of the canopy tree Muiratinga (*Maquira sclerophylla* (Ducke) C.C. Berg). Exposed yellow latex is rapidly and extensively oxidized to a black resin in the presence of air within 30 min

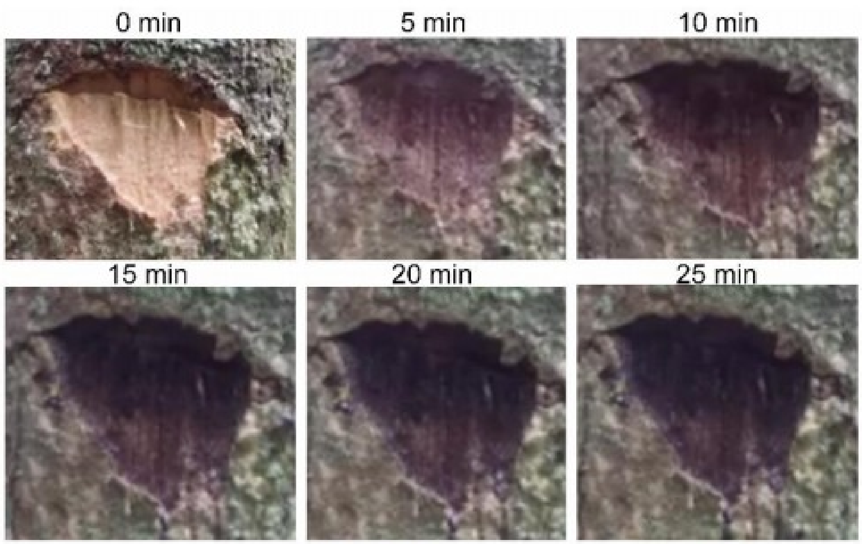

Figure 4. Time series of images of yellow latex oxidation on the stem of the Muiratinga individual, following the removal of a small piece of bark together with phloem and sapwood. See Figure 3 for zoomed out image of damaged area on the tree, and Figures 5 and 6 for time series images of the removed bark in nitrogen followed by air, respectively

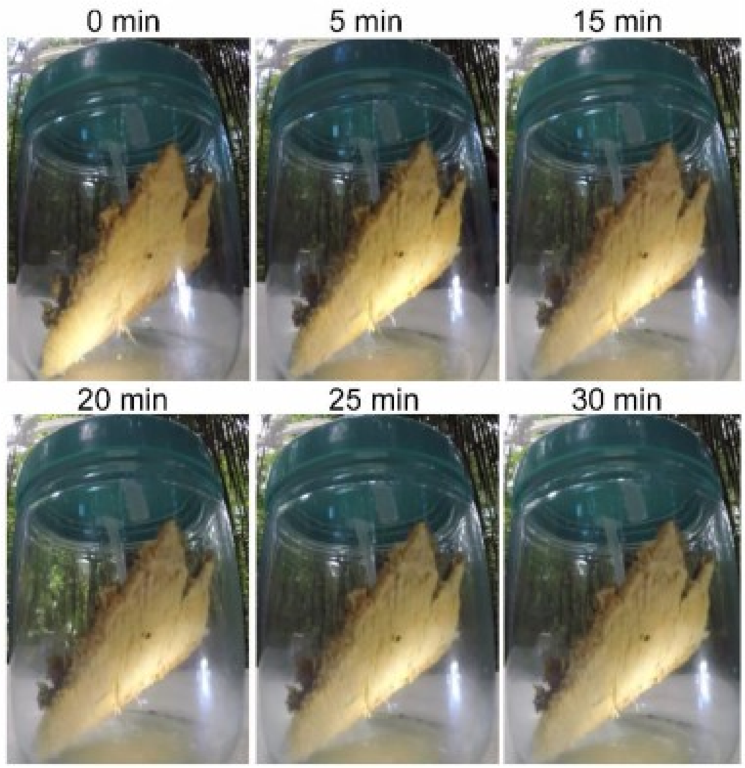

Figure 5. Time series of images demonstrating the inhibition of yellow latex oxidation under a nitrogen atmosphere. The small piece of bark together with phloem and sapwood that was removed from the stem in Figure 4 was rapidly placed in a glass chamber with nitrogen flowing through

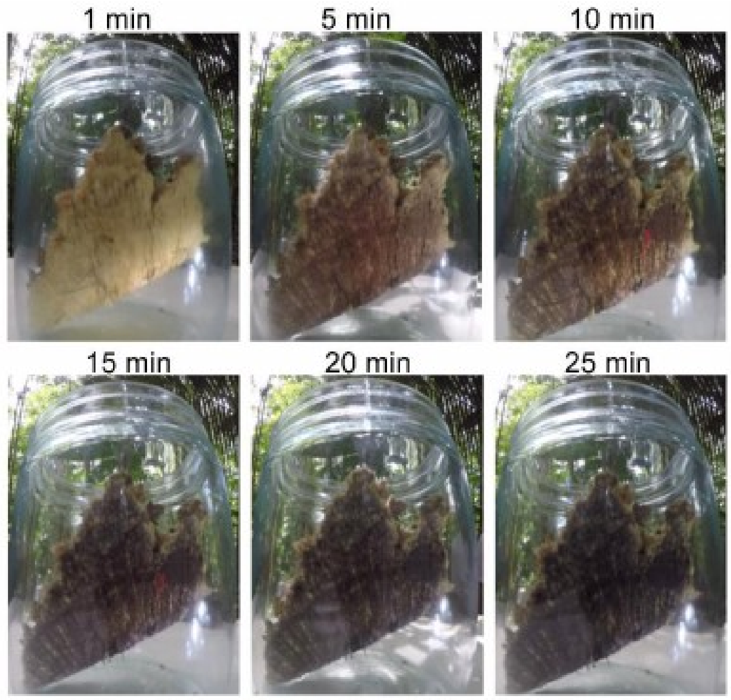

Figure 6. Following 30 minutes under a nitrogen atmosphere from Figure 4, yellow latex oxidation in the small piece of bark that was removed from the stem together with phloem and sapwood, was rapidly oxidized when exposed to ambient air