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Insight into prevention of enzymatic browning and increase of antioxidant potential of crepe rubber with ethephon stimulation

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Abstract
The effect of different ethephon concentrations 1%-5% (w/v) on colour of crepe rubber and plasticity retention index (PRI) was studied. The study was conducted with the RRISL 121 clone using S/2, d3 harvesting system. The results revealed that ethephon stimulation affects strongly the antioxidant and polyphenol oxidase (PPO) inhibitory potential. Further, thiol content and PRI values were increased while giving low Lovibond colour index upto 3% ethephon concentration and the properties were found to be inferior above 3% concentration. A probable reaction mechanism is proposed for colour improvement with optimum concentration of ethephon.

Key words: Ethephon stimulation, Lovibond colour index, plasticity retention index Polyphenol oxidase, Thiol content

Introduction

Latex crepe is the purest form of natural rubber available in the market and Sri Lanka is the world's leading producer of pale crepe and sole crepe for the export market (Seneviratne *et al.*, 2003). Latex crepe is widely used in rubber teats, balloons, condoms, infant items and toys. However, dark colour developed in dry rubber is a major issue in some industries which require a pale colour raw material. Therefore, colour is one of the most important raw rubber properties in latex crepe. The colour of the rubber depends on non-rubber constituents of latex. Raw natural rubber contain about 94% hydrocarbon (cis 1- 4, Poly isoprene) and 6% non rubber constituents such as proteins, lipids, carbohydrate (Allen, 1963). It has been reported that carotenoids, tocotrienols, polyphenol oxidase, fatty alcohol esters, unsaturated fatty acids, fatty alcohols, di-glyceride and mono - glyceride in cause the discoloration natural rubber (NR) (Sakdapipanich, *et al.*, 2007). The concentration of polyphenols, proteins, carotenoids are 2 X 10^{-2}, 1 and 3 X 10^{-5} (w/w) respectively and their contribution to the total absorptivity in the yellow brown region are 2.3, 0.1 and 0.01% respectively. Out of the total absorbance 90% is attributed to the polyisoprene in rubber molecule (Yusree *et al.*, 2012). The enzymatic discoloration is caused by the naturally occurring phenols and amino phenols in latex (Coupe *et al.*, 1972). Discoloration due to ethephon stimulation has been reported (Yapa, 1976), however there is no evidence for the effect of ethephon concentration on colour of crepe rubber. Polyphenol oxidases are the key enzymes in the natural coagulation and darkning of latex (Brozozowska-Hanower, 1978) and they are located inside the frey-wyssling particles. Frey-wyssling particles are mainly lipid in character and are not present in ammonia preserved concentrated latex. Apparently, they are either eliminated during centrifuging or they have got dissolved in the serum when the latex is ammoniated (d'Auzac, 1989). The polyphenol oxidase is responsible for the darkening of coagulated rubber on exposure to air or oxygen (Wititsuwannakul *et al.*, 2002). With the presence of polyphenol oxidase, phenols and amino phenols combine with oxygen from air to form orthoquinones. These orthoquinones react with naturally occurring amino acids and proteins in latex, giving coloured compounds resembling melanin (Mason, 1955). Both polyphenol oxidase (PPO) and phenolic substances are present in latex but do not react with each other intact laticifers. However, in the process of fractionation and coagulation, the Frey-Wyssling particles are disrupted and the enzyme released into the latex serum. This subsequently leads to discolouration of latex (Schadel and Walter, 1981). Stimulation by ethephon greatly reduces latex polyphenol oxidase activity which is known to be correlated positively with plugging index, which results in increase of phenolic substances in stimulated latex. Significant increase in phenolic content reported (after first & second tapping) after ethephon treatment is due to decrease in phenol oxidase activity (Cretin, 1978). Brozozowska-Hanower *et al., 1978* showed a definite lowering of o-diphenol oxidase activity in lattices at the first tapping after stimulation. Phenolic compounds have been reported to react, after enzymatic oxidation with thiols such as cysteine (CSH) and glutathione (GSH) to form colorless product (Mason, 1955). The presence of thiols in high concentration in latex act as an antioxidant hence, it can be used to improve colour (Yapa, 1976).

Glutathione is an endogenous antioxidant that plays a major role in reducing reactive oxygen species (ROS) formed during cellular metabolism (https://en.wikipedia.org/wiki/Glutathione). Therefore a clone with high phenolic content can still yield a colorless or light colour rubber, provided that it contains sufficient thiols. Glutathione is known to play a major role in reducing ROS formed during cellular metabolism. Therefore, the objective of the present study was to investigate the effect of ethephon concentration on latex discolouration and underpinning reasons.

Materials and Methods

Experimental design and location of the study

Six experimental blocks comprising 75 healthy trees from mono clonal (RRISL 121) mature rubber trees were selected from the Galewatta Division, Dartonfield Estate, Agalawatta, Sri Lanka for the experiment. Each block was divided into three replicates comprising 25 trees. Trees in each block were stimulated with 1.6g of 1%, 2%, 3%, 4% and 5% (w/v) ethephon applied on the tapping panel using an ¼" paint brush and tapped with half spiral based once in three days harvesting system by keeping one block without stimulation as the control. All the experimental tapping blocks were tapped by the same tapper in order to avoid tapper variability.

Freshly tapped latex collected into vessels, immersed in ice and then send to the laboratory immediately for the analysis of sucrose and thiol content without preservation and the other fraction was processed into un-fractioned un-bleached crepe rubber (UFUB).

Determination of thiol content in natural rubber latex

Analysis of thiol content was carried out by using colorimetric method developed by Boyne and Ellman, 1972 with three replicates. Serum was extracted by coagulating 1g of latex with 2.5% tri-chloro acetic acid (TCA) and volumed up to 25mL. To 2mL of extract, 0.1mL of dithio- bis -nitrobenzoic acid solution (10 mM) and 2mL tris (0.05M) was added and the optical density was measured at 412nm within 30min using a Jenway 6405 UV/Vis spectrophoto-meter. The amount of thiol was estimated against a standard curve prepared using reduced glutathione.

Determination of Lovibond Colour Index

The test was carried out according to ISO 4660 (2011) with three replicates. Three test pieces were cut from a homogenized UFUB sample using test piece punch and pressed them together (3.2-3.6 mm thick). The test pieces were then laminated and pressed in a mould between two sheets of transparent polyester film, with mould covers superimposed at a pressure of not less than 3.5 MPa for 5min at 150 ^{0}C±3^{0}C. The 3 test pieces (1.6 mm thick) were retained in the mould and it was compared with standard Lovibond colour glasses (4/19A) under diffuse daylight against a matt white background.

Determination of plasticity retention index

The test was carried out according to ISO 2930(2009) with three replicates. The test pellets were cut from a homogenized sample of UFUB in order to get sample thickness of 3.4 mm. The rapid plasticity number of unaged test pieces and test pieces aged at 140 ± 0.2ºC for 30 min were measured using parallel plate plastimeter.

Infra-red Spectroscopy

The rubber samples were extracted with acetone for 16 hours and acetone extract was sandwiched between two NaCl cells and Fourier transform infrared spectroscopy (FTIR) spectra were recorded on a Nicolet 380 spectrophotometer with 32 scans to improve signal to noise ratio.

Data analysis

The experimental design was complete randomized design and data was analyzed using Minitab 16 statistical software. One-way ANOVA was performed for basic parameters together with mean separation by Tukey method.

Results and Discussion

With s/2 d/3tapping system, the thiol content (antioxidant) and plasticity retention index increases up to 3% and above that concentration the properties were drastically reduced with increasing ethephon concentration (Table 1). It is evident from the results that highest antioxidant activity showed at 3%, hence maximum PRI value also obtained at 3% stimulant level. Above 3%, the thiol content decreased. This may be due to enhanced in vitro synthesis of reactive oxygen species (ROS). These ROS groups are oxidized glutathione (GSH), leading to decrease of their content in latex.

Table 1. *Variation of Thiol content, Plasticity Retention Index and Lovibond colour Index for raw rubber samples treated with 1%-5% ethephon*

Ethephon concentration (%)	Thiol content (mmol/g)	Plasticity Retention Index	Lovibond Colour Index
0	0.67^{e}	59.59^{d}	1.5
1	0.78^{bc}	69.06^{c}	1.0
2	0.81^{b}	76.6^{b}	1.0
3	0.88^{a}	83.13^{a}	1.0
4	0.73^{cd}	71.74^{bc}	1.5
5	0.71^{de}	70.43^{c}	1.5

*Means that do not share same letter are significantly different P<0.05

According to Lovibond colour index 1%-3% ethephon treated samples showed lighter colour than the control sample. 4% and 5% samples are very bright yellow colour with comparison to other samples (Fig. 1). It is evident from the results that with increasing ethephon concentration up to 3%, the Lovibond colour index is reduced, with simultaneous increase in thiol concentration. The lighter colour observed in rubber with ethephon treatment may be due to prevention of phenolic discolouration with thiol compounds. Therefore thiol concentration above a critical level allow a permanent protection against enzymatic browning (Richard *et al.* 1991). However, this level may often be dependent on the physiological condition of the tree. This is in agreement with the explanation given by Mason and Pterson, 1965 who explain that "in a system containing o-diphenol and glutathione, the total oxygen consumption increases sharply with increasing thiol content and this sharp break is accompanied by the marked difference in the colour of the final product".

The probable reaction mechanism of catechol with glutathione is given below (Fig. 2).

The o-quinone which leads to phenolic discolouration is blocked by glutathione (GSH) and it is reduced o-quinone back to catechol. If GSH is not present O-quinone can react with other phenolic compounds or amino acids (in protein) or it can self- polymerize into melanin like brown pigment (Gacche *et al.* 2006). The proposed mechanism is confirmed by FTIR spectrum of acetone extract of rubber sample.

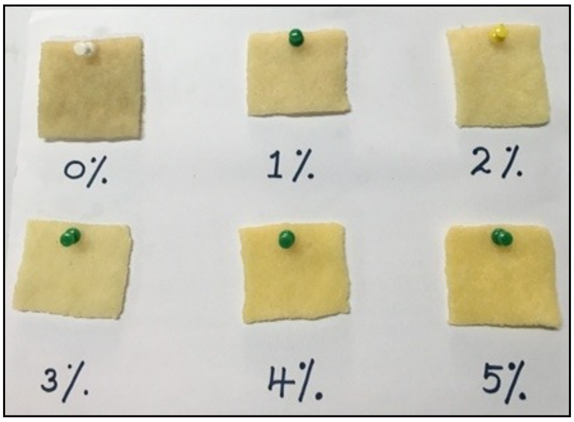

Fig. 1. Visual appearance of rubber samples from the trees treated with 1%-5% concentrations of ethephon

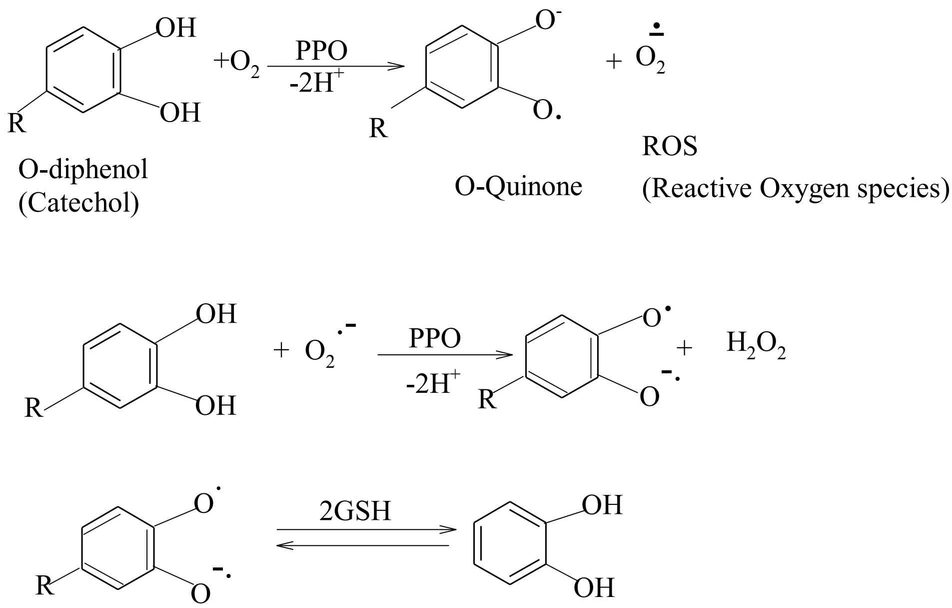

Fig. 2. The probable reaction mechanism for oxidation of O-diphenol in the presence of glutathione

Characteristic semi-quinone C----O stretching band appear around 1510-1475cm^{-1} (Berthomieu *et al.,* 1990). In 0%, 4% and 5% ethephon treated samples showed prominent semi-quinone peak, while 3% and 2% shows very weak band and they disappear in 1% ethephon treated sample (Fig. 3). Therefore it can be assumed that most of the orthoquinones are reduced by glutathione (GSH) and converted to catechol in 1%, 2% and 3% ethephon treated samples.

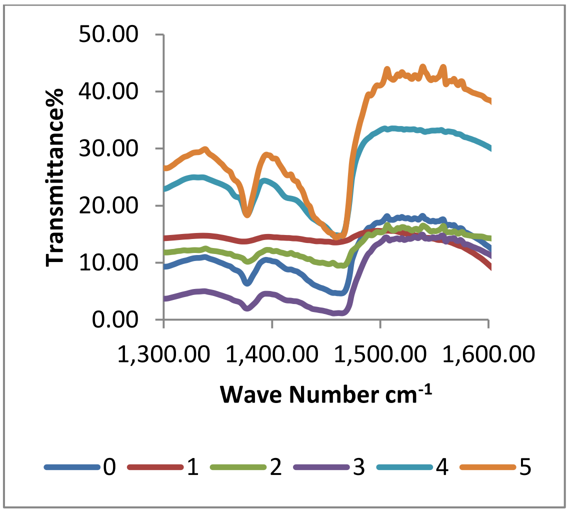

Fig. 3. FTIR spectrum of acetone extract of rubber obtained from the trees treated with different concentrations of ethephon

Conclusions

With S/2, d3 low frequency harvesting system, increase in ethephon concentration resulted in an increase of thiol content and PRI value up to 3% ethephon. Above that level PRI value decreased as a result of decrease in thiol content. Ethephon treated 1%-3% samples showed significantly low Lovibond colour index (lighter colour) and transient increase in colour observed for 0%, 4% and 5% samples. This is a biologically controlled method that can be used to improve the colour of crepe rubber with low frequency harvesting systems.

It is evident from the results that ethephon concentration directly affects the raw rubber properties. To achieve best raw rubber properties, application of recommended dosage is important as over dosage may lead to discolouration. This method can be used to achieve lighter colour rubber by adding glutathione in latex stage as an alternative to apparently toxic, bleaching agent.

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