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Research article

Analysis of reduced and oxidized antioxidants in *Hevea brasiliensis* latex reveals new insights into the regulation of antioxidants in response to harvesting stress and tapping panel dryness

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ABSTRACT

Latex diagnosis (LD) is applied to optimize the natural rubber production and prevent tapping panel dryness (TPD), a physiological syndrome affecting latex production in *Hevea brasiliensis*. The reduced thiol content (RSH) is one of the biochemical parameters associated with the risk of TPD. However, RSH is difficult to interpret because of the influence of the environment. In order to better understand the regulation of antioxidants and to better interpret RSH, a key parameter of LD, this study analysed in latex both oxidised and reduced forms of ascorbic acid (AsA) and glutathione, and their cofactors as well as other latex diagnosis parameters in response to harvesting stress (tapping and ethephon stimulation) and TPD occurrence. The content of antioxidants in latex had a high variability among five rubber clones. The concentration in AsA was about ten times higher than GSH in laticifer, GSH accounting for about 50% of RSH. For short-term harvesting stress, RSH increased with tapping frequency and ethephon stimulation. TPD is associated with high latex viscosity and bursting of lysosomal particles called lutoids, as well as for several rubber clones with lower RSH and GSH contents. These results suggest that a high level of RSH shows the capacity of laticifer metabolism to cope with harvesting stress, while a drop in RSH is the sign of long stress related to lower metabolic activity and TPD occurrence. RSH remains an essential physiological parameter to prevent TPD when associated with reference data under low and high harvesting stress. This study paves the way to understand the role of AsA and GSH, and carry out genetic studies of antioxidants.

1. Introduction

Hevea brasiliensis is the main natural rubber-producing plant species. Natural rubber is a polyisoprene compound synthesized in laticifer cells. The cytosol of laticifers, so-called latex, consists of 30–40% natural rubber. It is extracted through bark incision (tapping) of the plant allowing the latex to flow out and be collected. For some rubber clones, tapping is combined with ethephon stimulation to increase latex yield. Several studies revealed that this combination modifies laticifer metabolic activities and latex flow (Silpi et al., 2006; Wei et al., 2015; Sainoi et al., 2017a,b). However, over-tapping and over-stimulation lead to tapping panel dryness (TPD) occurrence, a physiological syndrome hampering latex flow (Bealing and Chua, 1972; Senevirathna and Nugawela, 2009; Putranto et al., 2015; Samuel et al., 2021).

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This physiological disorder draws the attention of rubber scientists due to its detrimental impact on latex yield. Previous studies indicated that the syndrome is induced by multiple factors. Besides over-tapping and over-stimulation, TPD is also determined by genetic, edaphic, and climatic factors (Gohet et al., 1994; Okoma et al., 2011; Chaendaekattu and Mydin, 2014). Tapping panel dryness is induced by over-accumulation of reactive oxygen species (ROS), leading to an oxidative burst and cellular damage. In laticifers, ROS is mainly generated by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, also called respiratory burst oxidase homologs (RBOHs) (Chrestin et al., 1984). NADPH oxidase is localized at the surface of lutoids, which are vacuo-lysosomal particles. Its activity is associated with the production of anion superoxide leading to membrane damages, lutoid bursting and consequent in-situ latex coagulation (Wititsuwannakul et al., 2008). This TPD form is reversible after a tapping rest period. In condition that environmental and harvesting stress are extended, several histological changes occur, such as thylosoid formation, lignified gum, and abnormal division of parenchyma leading to a severe and irreversible form of TPD called brown bast (de Fay and Jacob, 1989; de Fay, 2011; Putranto et al., 2015). In rubber plantations, especially in well-established companies, plant physiological status is monitored through latex diagnosis (LD) allowing yield optimization and TPD prevention (Nguyen et al., 2017; Gohet et al., 2019).

Latex diagnosis assesses four main parameters i.e. total solid content (TSC) indicating latex regeneration between two tappings, sucrose content (Suc) indicating carbohydrate availability, inorganic phosphorus (Pi) reflecting metabolic activity, and thiols content (RSH) for antioxidant capacity and stress level (Jacob et al., 1989). In relation to TPD prevention, RSH behaviour is still not fully understood. In some studies, RSH content was reported to be stable or increase with harvesting stress (Herlinawati and Kuswanhadi, 2012; Rukkhun et al., 2020; Irénée et al., 2020), or decreased (Dian et al., 2016; Gohet et al., 2019). This inconsistency leads to difficulties in interpretating RSH data and calls for a comprehensive study of RSH dynamics in different stress types and levels.

ROS-scavenging systems involve enzymes and antioxidants in laticifers (for review: Zhang et al., 2017). Catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascobate reductase (MDHAR), glutathione synthase (GS), and glutamate-cysteine ligase (GCL) are the main scavenging enzymes identified in *H. brasiliensis* latex. The main antioxidants are ascorbate, glutathione, carotenoid, and vitamin E. ROS-scavenging mechanism mainly relies on the ascorbate-glutathione cycle (Zhang et al., 2017). Two molecules of APX are used to convert H_2O_2 to water, generating two molecules of monodehydroascorbate (MDHA) which are rapidly disproportionated to reduced ascorbate (AsA) and oxidized ascorbate (DHA), catalysed by MDHAR. DHA is recycled by DHAR to AsA using reduced glutathione (GSH) as reducing substrate. The oxidized form of glutathione (GSSG) is generated in this process and recycled to GSH by reduced nicotinamide adenine dinucleotide phosphate (NADPH), catalysed by GR (Noctor and Foyer, 1998; Foyer and Noctor, 2011). In high-stress conditions, ROS accumulation may induce *de novo* biosynthesis of antioxidants (Noctor et al., 2012). Ascorbate is synthesized through the L-galactose pathway, which is converted to L-galactono-1,4-lactone by NAD-dependent L-galactose dehydrogenase (Bulley and Laing, 2016). The latter is oxidized to ascorbate by L-galactono-1,4-lactone dehydrogenase. Other biosynthetic pathways, through galacturonate and glucoronate, have been proposed but the regulation of these pathways is still not deeply known (Smirnoff, 2000; Bulley and Laing, 2016). Glutathione synthesis is commenced by reaction of γ-carboxyl glutamine and α-amino group cysteine to generate γ-glutamylcycteine synthase (γ-ECS) followed by amide bond formation between the α-carboxyl group of cysteine moiety in γ-glutamylcysteine and the α-amino group of glycine in the presence of GS to form GSH (Alscher, 1989; Hasanuzzaman et al., 2017). The increase in the oxidized form of antioxidants indicates active oxidation due to the presence of stress, while the increase of reduced forms indicated *de novo* biosynthesis or activation of the ascorbate-glutathione cycle. Some works in other plant species showed an alteration of reduced and oxidized forms of antioxidants in response to abiotic stress (Marok et al., 2013; Borisova et al., 2016). In *H. brasiliensis*, recent studies on antioxidants and ROS-scavenging enzymes were mainly conducted in response to environmental stress such as salt stress (Yang et al., 2020), drought (Santos et al., 2021), and low-temperature (Ajith et al., 2021). To our knowledge, the study of the alteration of reduced and oxidized forms of antioxidants in latex of *H. brasiliensis* particularly in response to the harvesting stress has not been performed. This approach may give a better explanation about stress regulation in *H. brasiliensis* than assessing RSH solely.

This paper aims to have a better understanding of antioxidant regulation in laticifer in order to better interpret the RSH, as part of LD parameters, and/or identify another potential antioxidant parameter more reliable than RSH. This study is the first report combining agronomical and physiological parameters related to latex production, latex diagnosis, antioxidants and cofactors of *H. brasiliensis* latex in relation to harvesting stress (tapping and stimulation) and TPD occurrence. Observed parameters associated with latex production included latex yield, initial flow (IF), plugging index (PI), and bursting index (BI). For latex diagnosis, TSC, Suc, Pi, and RSH were determined. For antioxidants, the reduced form (AsA and GSH) and oxidized forms (DHA and GSSG) were analysed. Cofactors associated with antioxidants regeneration were assessed including oxidised and reduced nicotinamide adenine dinucleotide (NAD, NADH), and nicotinamide adenine dinucleotide phosphate (NADP, NADPH). The study reveals that AsA is the most concentrated antioxidant in latex and GSH accounts for half of low molecular weight RSH. Only RSH increases significantly with short-term harvesting stress. RSH remains a reasonable antioxidant parameter to characterize latex, yet its interpretation requires information related to harvesting system history, age of the tree, and reference RSH value under no stress and stress conditions.

2. Materials and methods

2.1. Experimental designs and planting materials

The study was carried out at Sembawa Research Centre, Indonesian Rubber Research Institute, Palembang, Indonesia (2°55′38″S, 104°32′19″E). Field experiments were conducted from January to May 2021, with the rainfall ranging from 154.48–435.80 mm/month with rainy days 18–25 days/month. Three field trials were established to study the clonal variation and the evolution of physiological parameters associated with latex production and antioxidants in response to harvesting systems (tapping frequency and ethephon stimulation) and TPD occurrence.

2.1.1. Experiment 1: clonal variation in five recommended rubber clones

The observation was performed in five recommended rubber clones i.e. IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260. IRR 118, BPM 24, and IRR 112 are moderate metabolic clones from Indonesia, derived from cross LCB 1320 x FX 2784, GT 1 x AVROS 1734, and IAN 873 x RRIC 110 respectively (Adou et al., 2017; Darojat and Sayurandi, 2018). Clone RRIC 100 is originated from Srilanka. The moderate metabolic clone came from crossing RRIC 52 x PB 86 (Adou et al., 2017). PB 260 is a high metabolic clone, originated from Malaysia as result of crossing PB 5/51 X PB 49 (Kan Pulchérie et al., 2021).

The trees were ten-years-old, planted 6 × 3 m of space, and tapped on the B0-1 panel (5th years of tapping) every three days. The trees were receiving one application of 2.5% ethephon every month since the 2 nd years of tapping (harvesting system noted S/2 d3 ET2.5% 12/y). Three healthy trees, with no dry cut, with homogeneous girth as possible were selected as replications for each clone except for PB 260 which consisted of four replicates. Latex samples collection for physiological and

antioxidant quantification was performed in May 2021, three weeks after stimulation. The result can be accessed in Supplementary data 1.

2.1.2. Experiment 2: effect of harvesting systems

Ten-year-old trees from clones IRR 118 and BPM 24 were used in this experiment. Prior to the experiment, all trees were under the S/2 d3 (without stimulation since open tapping) tapping system in the B0-1 panel. Twenty healthy trees were selected for each clone. Four harvesting systems (S/2 d6, S/2 d3, S/2 d1, and S/2 d3 ET2.5% 12/y) were compared using five trees as replicate for each treatment. Latex samples collection was performed in April 2021, four months after treatments were applied. The data is presented in Supplementary data 2.

2.1.3. Experiment 3: effect of TPD in five recommended rubber clones

Given all trees can have a slight dry cut length, DCL was observed on five recommended clones i.e. IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260 tapped with the harvesting system S/2 d3 ET 2.5% 12/y at B0-1 panel. To assess the TPD severity for subsequent data analysis, trees were divided in two groups, low DCL and high DCL, using the k-means clustering method (Supplementary data 3). Latex samples were collected three weeks after ethephon stimulation to eliminate the stimulation effect. Latex samples collection was performed on May 2021.

2.2. Agronomical and physiological parameters

2.2.1. Collection of latex samples and preparation of latex serum

Latex was collected immediately until 10 min after tapping in a 15 ml plastic tube on ice. After initial latex volume was recorded, 1 ml latex was put in 9 ml of 2.5% TCA and kept on ice for Suc, Pi, and RSH assessment. For AsA, DHA, GSH, GSSG, NAD, and NADP assessment, 100 μL of latex was added to 900 μL of 0.2N HCl in 1 mM EDTA and stored in liquid nitrogen prior to centrifugation. For total NAD and total NADP, 100 μL of latex were added to 900 μL of 0.2 N NaOH in 1 mM EDTA and stored in liquid nitrogen. The rest of the latex was kept on ice for TSC and BI measurement.

Samples in TCA were filtered to obtain clear serum for Suc, Pi and RSH quantifications. For samples in HCl and NaOH, a gradual thawing was applied including 15–20 min on ice and 30–40 min in the refrigerator. The samples were centrifuged using 15,000 rpm at 4 °C for 20 min. The clear serum was transferred into a new 1 ml tube and returned into liquid nitrogen before quantification.

2.2.2. Yield and latex flow assessments

The yield was defined as dry rubber produced per tree per cm of the cut length (g/t/cm) in order to avoid any effect of the tree girth variation between trees. The values were derived from the total weight of fresh latex multiplied by the dry rubber content (DRC) and divided by the cut length of the associated tree. The DRC was estimated at 90% of TSC for these trials. The latex flow parameters assessed include the IF, PI, and BI. The IF was the average latex volume per minute in the first 10 min after tapping. The PI was calculated by dividing the IF with the total latex volume according to Pakianathan et al. (1989).

The BI is the ratio of the free acid phosphatase (FAP) by total acid phosphatase (TAP). The TAP indicated the content of acid phosphatase after lutoid bursting, using Triton X100; while FAP is acid phosphatase in lutoid particles. The FAP substrate was prepared by mixing 50 ml of 0.8 M sodium p-nitrophenyl phosphate (PNPP) at pH 5.0 with 200 ml of 0.6 M mannitol and 145 ml of distilled water. The TAP substrate was prepared by mixing 50 ml of 0.8 M PNPP at pH 5.0 with 100 ml of 0.5% Triton X-100 and 145 ml of distilled water. These substrates were used for the establishment of standards and samples of FAP and TAP. Standard FAP consisted of 2.9 ml of FAP substrate added by 1 ml of 2 N TCA and 0.1 ml of fresh latex of the associated sample, after 10 min of incubation, the solution was filtered. A similar procedure for standard TAP was applied using TAP substrate. For sample FAP and TAP preparations, the latex was put first into the corresponding substrate then TCA was added 10 min later to stop the enzymatic process. For the absorbance measurement, 1 ml of the FAP or TAP solutions were added with 1 ml of 1 N NaOH and made up to 10 ml with distilled water. Absorbance measurement was performed in 96 wells plate using SPECTROstar Nano spectrometer (BMG Labtech, Ortenberg, Germany) at 400 nm with 200 μL of standard/sample per channel. The FAP and TAP are absorbances different between sample and standard for associated sample, while BI is the ratio of FAP to TAP in percentage (Thepchalerm et al., 2015).

The dry cut length (DCL) was observed immediately after tapping. DCL was monitored once a month. The percentage of DCL was calculated through dividing the dry cut length by the total cut length and the final DCL percentage was the average of three consecutive observations. The DCL percentage represents TPD severity.

2.2.3. Determination of latex diagnosis

Four standard LD parameters were assessed i.e., TSC, Suc, Pi, and RSH. For TSC quantification, 5 g of fresh latex put on aluminium disk was oven-dried at a stable temperature of 100 °C for 3 h. The TSC was the result of the dry weight divided by the fresh weight of each sample. The measurement of the Suc protocol was adopted from Dische (1962). The 150 μl of latex serum was added by 2.5% TCA to reach 500 μl of total volume followed by the addition of 3 ml of 5 mM anthrone. Following 15 min of submergence in boiling water, the absorbance was measured at 627 nm wavelength with 200 μL of the solution in each well of the plate.

The procedure for Pi quantification followed Taussky and Shorr (1953). Following four times dilution using 2.5% TCA, 100 μL diluted serum was added by Ferro-sulphate ($FeSO_4$) solution and incubated 5 min at ambient temperature. The absorbance was measured at 750 nm wavelength. The procedure for RSH determination adopted the Ellman assay. The 100 μL sample serum was added by 55 μl of 10 mM 5.5′-dithiobis 2-nitrobenzoic acid (DTNB) and 100 μL of 0.5 M Tris buffer. Following 30 min of incubation at ambient temperature, the absorbance was measured at 412 nm wavelength.

2.2.4. Antioxidant and cofactors determination

For AsA determination, 50 μL HCl serum was pipetted into 96 wells plate added by 50 μL of 200 mM KH_2PO_4 at pH 7.5 and incubated at ambient temperature for 30 min. After 20 μL distilled water addition, the sample was incubated once again at ambient temperature for 5 min then reacted with 40 μL of 10% TCA, 50 μL of 44% H_3PO_4, 40 μL of 65 mM 2,2′-dipyridyl, and 20 μL of 110 mM $FeCl_3$. Before absorbance measurement at 492 nm, the sample was incubated in the oven at 40 °C for 1 h. Total AsA was determined by adding 50 μL HCl serum into 96 wells plate and added by 50 μL of 200 mM KH_2PO_4 at pH 7.5 and 10 μL of 10 mM dithiothreitol (DTT). Following 30 min of incubation at ambient temperature, 10 μL of 0.5% n-ethylmaleimide (NEM) was added and another 5 min incubation at ambient temperature was applied. The sample was then reacted with 40 μL of 10% TCA, 50 μL of 44% H_3PO_4, 40 μL of 65 mM 2,2′-dipyridyl, and 20 μL of 110 mM $FeCl_3$. Absorbance measurement (at 492 nm) was performed after 1-h incubation in the oven at 40 °C. The DHA content was calculated by subtracting the total AsA with AsA.

The total glutathione and GSSG assessment were started with HCl serum neutralization. The 200 μL of HCl serum was reacted with 20 μL of 0.2 M NaH_2PO_4 at pH 5.6 and 200 μL of 0.2 N NaOH then the pH was adjusted to pH 5–6. For the GSSG assay, 280 μL aliquot was reacted with 10 μL of 2-Vinylpyridine (VPD) and incubated 30 min at ambient temperature. The clear supernatant was obtained by 10 min centrifugation at 13,000 rpm. For total glutathione determination, 20 μL of the neutralized sample was reacted with 100 μL buffer A (consisted of 9.5 mg DTNB, 19.6 mL of NaH_2PO_4 at pH 7.0, and 400 μL of 0.5 M EDTA), 10 μL of 20 U GR, and 60 μL distilled water. Following the absorbance measurement at 405 nm, the sample was reacted with 10 μL of 20 mM NADPH then

Table 1. Antioxidants and cofactors content of five recommended rubber clones. For all variables with the same *letter*, the difference between the means is not statistically *significant* according to Tukey Multiple Comparison Method at α = 0.05.

Clone	RSH (mM)	AsA (mM)	DHA (mM)	GSH (mM)	GSSG (mM)	NADH (μM)	NAD (μM)	NADPH (μM)	NADP (μM)
IRR 118	0.39 ± 0.03 b	3.25 ± 1.01 a	0.12 ± 0.19 a	0.21 ± 0.04 b	0.005 ± 0.007 b	0.63 ± 0.34 a	0.50 ± 0.14 a	0.54 ± 0.14 a	0.003 ± 0.002 a
BPM 24	0.70 ± 0.03 a	2.56 ± 1.01 a	0.43 ± 0.19 a	0.34 ± 0.04 ab	0.059 ± 0.007 a	0.57 ± 0.34 a	0.37 ± 0.14 a	0.51 ± 0.14 a	0.003 ± 0.002 a
IRR 112	0.27 ± 0.03 b	3.52 ± 1.01 a	0.40 ± 0.19 a	0.13 ± 0.04 b	0.020 ± 0.007 ab	0.81 ± 0.34 a	0.26 ± 0.14 a	0.60 ± 0.14 a	0.004 ± 0.002 a
RRIC 100	0.21 ± 0.03 b	5.11 ± 1.01 a	0.07 ± 0.19 a	0.14 ± 0.04 b	0.022 ± 0.007 ab	0.46 ± 0.34 a	0.58 ± 0.14 a	0.57 ± 0.14 a	0.002 ± 0.002 a
PB 260	0.73 ± 0.03 a	3.20 ± 0.87 a	0.42 ± 0.19 a	0.47 ± 0.04 a	0.040 ± 0.006 ab	0.66 ± 0.29 a	0.51 ± 0.12 a	0.57 ± 0.12 a	0.003 ± 0.00a

absorbance measurement at 405 nm was performed five times with 30 s intervals. For GSSG determination, 50 μL of the sample was reacted with 100 μL buffer A, 10 μL GR (20 U), and 30 μL distilled water then absorbance measurement was performed at 405 nm. Following a reaction with 10 μL of 20 mM NADPH the absorbance measurement at 405 nm was performed five times with 30 s intervals. The total glutathione and GSSG contents of samples were determined through the calculation of the coefficient of the slope carried out with the standard curve. The GSH was derived by subtracting the total glutathione with GSSG (total glutathione = GSH +2 GSSG).

The quantification procedure for NAD, NADH, NADP, and NADPH was adopted from Queval and Noctor (2007). Sample preparation for NAD determination was carried out by incubating 200 μL of the HCl serum in the boiling water for 2 min followed by rapid cooling and addition of 20 μL of 0.2 M NaH_2PO_4 at pH 5.6. The pH was adjusted with 0.5 M NaOH to 5–6. For total NAD determination, 200 μL of NaOH serum was incubated in the boiling water for 2 min followed by rapid cooling and the addition of 20 μL of 0.2 M NaH_2PO_4 at pH 5.6. The pH was adjusted with 0.5 M HCl to 7–8. Each sample (20 μL in volume) was reacted with 100 μL of 10 mM 4-1-piperazineethanesulfonic acid (HEPES) in 2 mM EDTA solution, 20 μL of 1.2 mM 2,6-dichlorophenolindophenol (DCPIP), 10 μL of 10 mM phenazine methosulfate (PMS), 25 μL distilled water, and 10 μL of 2500 U/ml alcohol dehydrogenase (ADH). The reaction was started by 15 μL of absolute ethanol addition. The absorbance was measured at 600 nm five times with 30sec intervals. The NAD and total NAD contents were determined through the calculation of the absorbance slope. The NADH was derived by subtracting the total NAD value with the NAD value.

The neutralization procedure for NADP and total NADP quantification was the same as NAD and total NAD quantification protocol. For absorbance measurement, 30 μL sample was reacted with 100 μL of 10 mM HEPES and 2 mM EDTA solution, 20 μL of 1.2 mM DCPIP, 10 μL of 10 mM PMS, 30 μL distilled water, and 10 μL of 10 mM glucose 6-phosphate. The reaction was started by 10 μL of glucose 6-phosphate (G6PDH at 200 U/ml). The absorbance was measured five times with 30sec intervals at 600 nm wavelength. The NADP and total NADP contents were determined through the calculation of the absorbance slope. The NADPH was derived by subtracting the total NADP value with the NADP value.

2.3. Data analysis

Statistical analysis was carried out using the XLSTAT program (Addinsoft Inc., New York, USA). Interaction between Clones and Harvesting system (2nd experiment) was analysed through 3-ways or 2-ways analysis of variance (ANOVA). For these analyses, pairwise comparison of harvesting systems for each clone was performed by Bonferroni method with α = 0.05/(n ((n-1)/2)), n being the number of treatments. To study the effect of clonal variation on the antioxidants and the effect of tapping frequency, ethephon stimulation, and DCL severity (1st and 3rd experiment), a one-way ANOVA followed by Tukey HSD test with α = 0.05 was implemented. A supervised Linear Discriminant Analysis with DCL severity as dependent categorical variable was carried out involving yield, latex flow, latex diagnosis, antioxidants, and cofactors parameters to find combinations of these parameters that could explain occurrence of TPD. Finally, to predict linearly the DCL percentage, a multiple stepwise regression analysis using the Akaike Information Criterion (AIC) was performed.

3. Results

3.1. Clonal variability of antioxidants in latex

The content of antioxidants was analysed in latex from five recommended rubber clones of a 5-year-old polyclonal trial. It showed a significant clonal variation in RSH, GSH and GSSG, while AsA, DHA, NADH, NAD, NADPH, and NADP were not significant (Table 1). Clones BPM 24 and PB 260 had higher RSH (0.70 mM and 0.73 mM, respectively) compared to other three clones i.e., IRR 118 (0.39 mM), IRR 112 (0.27 mM) and RRIC 100 (0.21 mM). These two clones also had higher GSH, 0.34 mM for BPM 24 and 0.47 mM for PB 260, than the others (ranging from 0.13 mM to 0.21 mM). Among five rubber clones observed, the highest GSSG was BPM 24 (0.059 mM) followed by PB 260 (0.040 mM), RRIC 100 (0.022 mM), and IRR 112 (0.020 mM). A significantly lower GSSG was found on IRR 118 (0.005 mM).

The AsA and DHA were not significantly different among clones. The AsA ranged from 2.56 to 5.11 mM, while DHA from 0.07 to 0.43 mM. The cofactors' determination resulted in no significant differences among clones. The NADH content ranged from 0.46 to 0.81 μM and NAD ranged

Table 2. Interaction analysis between the effect of clone, tapping, ethephon stimulation and the combination of factors at 4 months after treatments application. Numbers in bold indicate significant according to Bonferroni test at α = 0.017.

Source		Yield	IF	PI	TSC	Suc	Pi	RSH	AsA	DHA	GSH	GSSG	NAD	NADH
Clone	F	6.259	**10.108**	0.817	2.262	**16.718**	**71.640**	**23.357**	**6.960**	0.208	2.982	0.968	0.124	0.075
	Pr > F	0.018	**0.003**	0.373	0.142	**0.000**	**< 0.0001**	**< 0.0001**	**0.013**	0.651	0.094	0.333	0.727	0.786
Tapping	F	0.726	0.230	1.564	**30.844**	**7.145**	**40.342**	**10.577**	0.003	0.448	1.470	1.567	2.859	**7.018**
	Pr > F	0.492	0.796	0.225	**< 0.0001**	**0.003**	**< 0.0001**	**0.000**	0.997	0.643	0.245	0.224	0.072	**0.003**
Ethephon	F	**20.596**	1.857	**33.164**	**45.748**	0.037	**66.409**	**7.049**	0.199	0.219	0.351	0.051	2.049	1.165
	Pr > F	**< 0.0001**	0.183	**< 0.0001**	**< 0.0001**	0.849	**< 0.0001**	**0.012**	0.658	0.643	0.557	0.823	0.162	0.289
Clone x Tapping	F	0.074	0.536	0.052	3.453	**8.845**	**7.702**	1.087	0.272	0.462	0.223	0.408	1.332	0.320
	Pr > F	0.929	0.590	0.950	0.044	**0.001**	**0.002**	0.349	0.763	0.634	0.801	0.668	0.278	0.728
Clone x Ethephon	F	5.107	0.179	0.342	1.205	**7.260**	**18.368**	**11.174**	4.882	0.395	0.242	0.981	0.097	0.029
	Pr > F	0.031	0.675	0.563	0.281	**0.011**	**0.000**	**0.002**	0.034	0.534	0.626	0.329	0.758	0.866

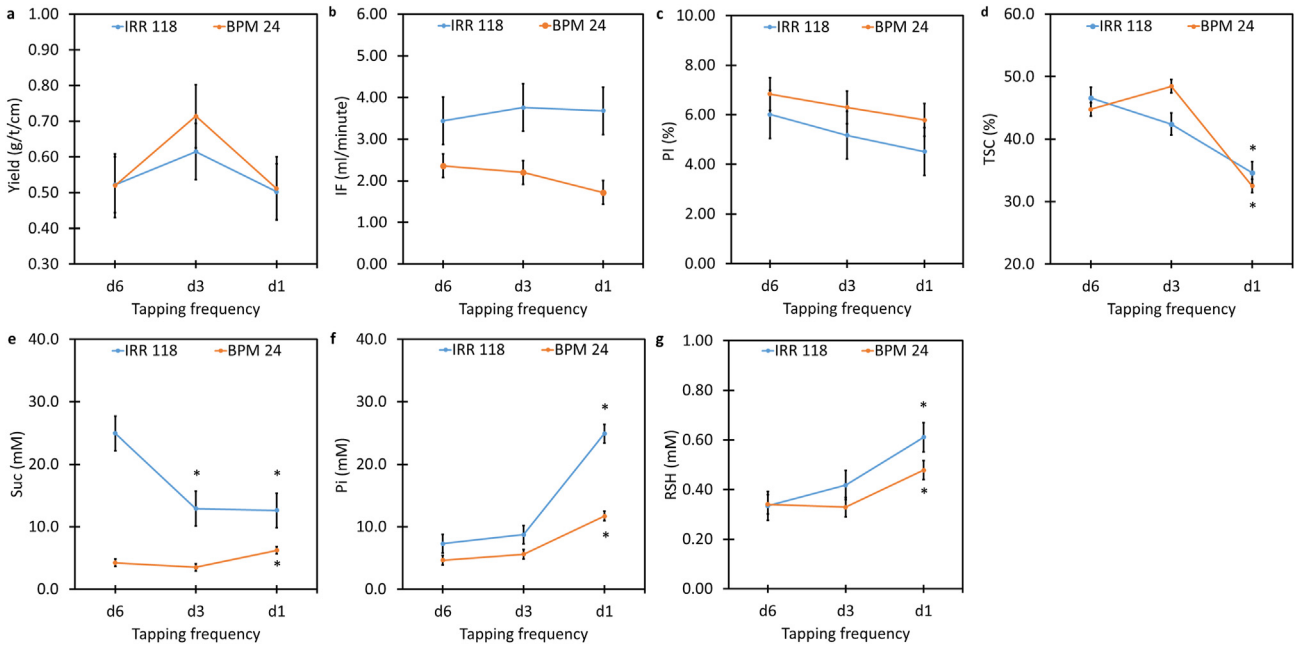

Figure 1. Effect of tapping frequency on yield (a), initial latex flow (b), plugging index (c), total solid content (d), sucrose (e), inorganic phosphorus (f), and thiols (g). The effect of tapping was analysed separately by clone. Data are least-squares means ± standard error. *indicated significant different from other tapping frequencies in the same clone according to Tukey Multiple Comparison Method at α = 0.05.

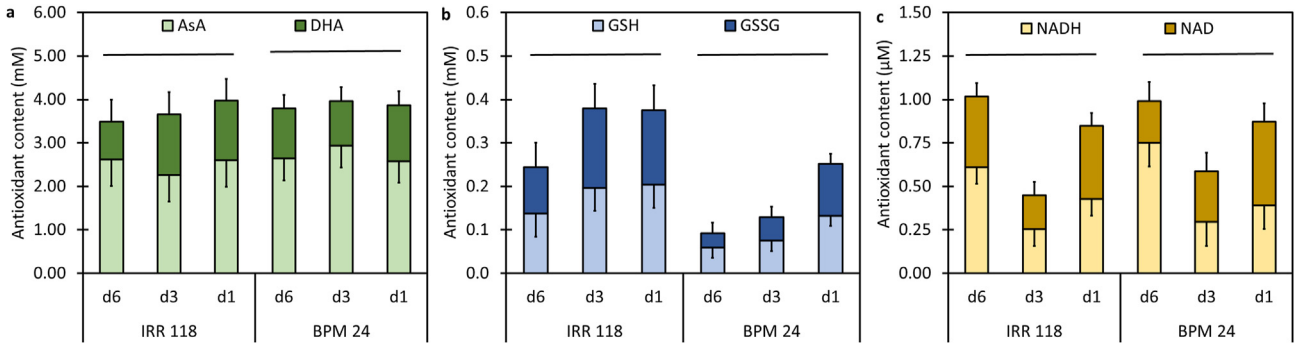

Figure 2. Effect of tapping frequency on reduced and oxidized forms of ascorbate (a), glutathione (b) and nicotinamide adenine dinucleotide (c) contents. The effect of tapping was analysed separately by clone. Data are least-squares means ± standard error.

from 0.26 to 0.58 µM. The NADPH ranged from 0.51 to 0.60 µM, while NADP from 2 to 4 nM. This result showed that the concentration in ascorbate was higher than thiols and glutathione. Cofactor contents were much lower than antioxidants.

3.2. Effect of tapping frequency and ethephon stimulation

The two rubber clones IRR 118 and BPM 24 were selected from another 10-year-old trial for further studies for their contrasting value in RSH content and level of susceptibility to TPD, clone IRR 118 being tolerant to TPD. The effect of tapping frequency (d1, d3 and d6) and ethephon stimulation (d3 ET2.5% 12/y) was analysed 4 months after application of the treatments. Interaction analysis showed that the clone factor had a strong effect on IF, Suc, Pi, RSH, and AsA (Table 2). The effect of tapping was significant on TSC, Suc, Pi, RSH, and NADH, while the effect of stimulation was significant on yield, PI, TSC, Pi, and RSH. The interaction of clone x tapping was significant on Suc and Pi only, while the interaction of clone x stimulation was significant on Suc, Pi, and RSH. According to this analysis, Pi was significantly affected by all factors. Given that ethephon stimulation had a significant effect on several important parameters, the factors (tapping and stimulation) were analysed separately using a two-way clone x treatment ANOVA. Results of these analyses are summarized by Interaction Plots shown in Figures 1, 2, 3, and 4. On these figures, error bars for each mean value correspond to standard errors of means.

Tapping frequency significantly affected TSC, Suc, Pi and RSH but not on yield, IF and PI parameters (Figure 1). High tapping frequency (d1) significantly decreased TSC in clones IRR 118 and BPM 24, while d6 and d3 were comparable. In IIR 118, TSC decreased from 46.55% to 34.63% and in BPM 24 from 44.76% to 32.50%. A decrease was also found on Suc for IRR 118 in d6 (24.95 mM) to d3 (12.92 mM) and then stable in d1 (12.62 mM). In BPM 24, the Suc increased in d1 (6.24 mM) compared to d6 (4.24 mM) and d3 (3.50 mM). The Pi in d6 and d3 were not significant, yet it increased significantly in d1 for both clones. The Pi in d6, d3, and d1 were 7.30 mM, 8.74 mM, and 24.90 mM, respectively for IRR 118, and 4.63 mM, 5.61 mM, and 11.71 mM for BPM 24. The RSH of clone IRR 118 increased significantly in d1 (0.61 mM) compared to d6 (0.33 mM), while in d3 was intermediate (0.42 mM). For clone BPM 24, RSH in d6 was not significantly different with d1 (0.34 mM and 0.46 mM respectively); while d1 was significantly higher than d3 (0.33 mM).

The effect of tapping frequency on reduced and oxidized forms of antioxidants was not significant for both rubber clones (Figure 2). The

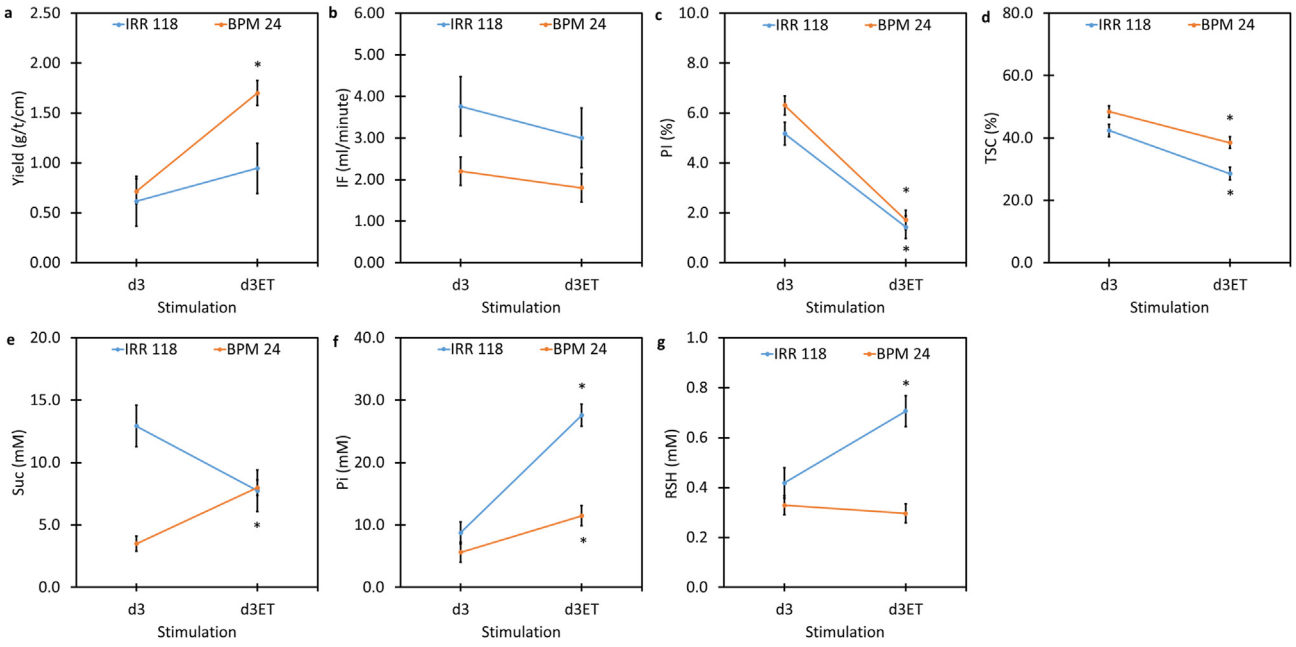

Figure 3. Effect of ethephon stimulation on yield (a), initial latex flow (b), plugging index (c), total solid content (d), sucrose (e), inorganic phosphorus (f), and thiols (g). The effect of stimulation was analysed separately by clone. Data are least-squares means ± standard error. *indicated significant different from non-stimulated treatment in the same clone according to Tukey Multiple Comparison Method at α = 0.05.

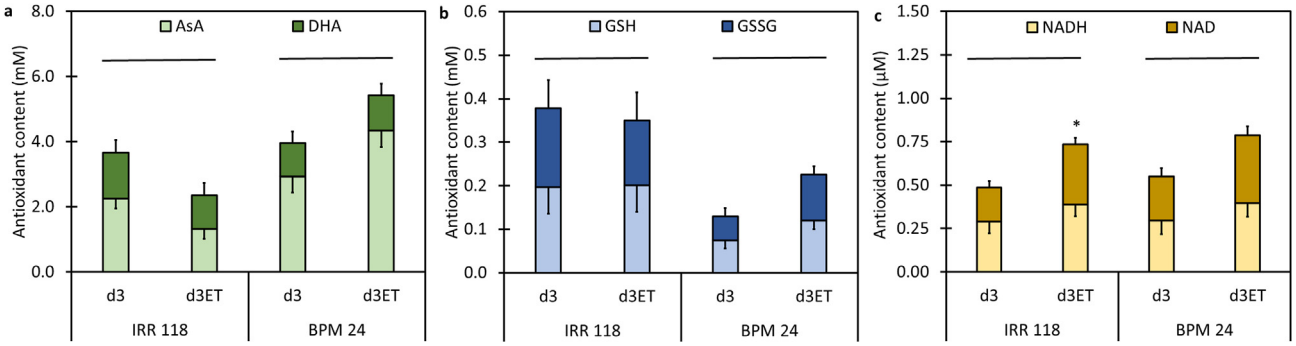

Figure 4. Effect of ethephon stimulation on reduced and oxidized forms of ascorbate (a), glutathione (b) and nicotinamide adenine dinucleotide (c) contents. The effect of stimulation was analysed separately by clone. Data are least-squares means ± standard error. *indicated significant different from non-stimulated treatment in the same clone according to Tukey Multiple Comparison Method at α = 0.05.

AsA of clone IRR 118 ranged from 2.26 mM to 2.62 mM and BPM 24 from 2.58 mM to 2.93 mM. The DHA ranged from 0.87 mM to 1.40 mM in IRR 118 and from 1.03 mM to 1.29 mM for BPM 24. The GSH ranged from 0.14 mM to 0.20 mM for IRR 118 and 0.06 mM–0.13 mM for BPM 24. The GSSG for IRR 118 ranged from 0.11 mM to 0.18 mM and 0.03 mM to 0.12 for BPM 24. The cofactor content was also not affected significantly by tapping frequency. NADH for IRR 118 ranged from 0.25 µM to 0.61 µM and for BPM 24 from 0.30 µM to 0.75 µM. The NAD for IRR 118 was 0.20 µM–0.42 µM and for BPM 24 from 0.24 µM to 0.48 µM.

The calculation of ratios between reduced by oxidized forms of antioxidants did not reveal oxidation (Supplementary data 5). Ratio AsA/DHA for clone IRR 118 in d6, d3 and d1 was 2.63, 1.22 and 1.92 respectively, while for clone BPM 24 was 2.31, 2.17, and 2.64. The ratio of GSH/GSSG for IRR 118 clone in d6, d3 and d1 was 1.11, 1.24 and 1.37 respectively, while for BPM 24 was 1.80, 2.55, and 1.18. A significant decrease was only encountered in NADH/NAD for BPM 24, from 5.27 in d6 to 0.68 in d1 while in d3 was intermediate (1.10). For IRR 118, the ratio of NADH/NAD was not different among tapping frequency i.e. 1.66 in d6, 1.30 in d3, and 1.08 in d1.

The ethephon stimulation had significant effect on yield, latex flow, and physiological parameters except IF (Figure 3). Ethephon stimulation significantly increased the yield for clone BPM 24 from 0.71 g/t/cm to 1.70 g/t/cm, while for clone IRR 118 was no significant different between stimulation treatment (0.95 g/t/cm) and without stimulation (0.62 g/t/cm). The PI decreased dramatically with the ethephon stimulation in both clones. PI decreased from 5.17% to 1.43% and 6.30%–1.72% for clones IRR 118 and BPM 24, respectively. TSC dropped from 42.4% to 28.6% for clone IRR 118 and from 48.5% to 38.5% for clone BPM 24. A significant increase in Suc was found for stimulated trees (7.99 mM) compared with non-stimulation (3.50 mM) in clone BPM 24; conversely, a decrease in values for IRR 118 was observed (from 12.92 mM to 7.74 mM), yet was not statistically significant. Metabolic activity, reflected by the Pi, increased significantly by ethephon stimulation in both clones. In IRR 118, Pi increased from 8.74 mM to 27.57 mM and in BPM 24 from 5.61 mM to 11.46 mM. The RSH increased with stimulation for IRR 118 (from 0.42 mM to 0.71 mM), while for BPM 24 was stable (from 0.33 mM to 0.30 mM).

The reduced and oxidized forms of antioxidants and cofactors were not significantly affected by ethephon stimulation except for an increase

Table 3. Interaction analysis between the effect of clone, DCL severity and the combination of factors. Numbers in bold indicate significant according to Bonferroni test at $\alpha = 0.05$.

Source		Yield	IF	PI	BI	TSC	Suc	Pi	RSH	AsA	DHA	GSH	GSSG	NADH	NAD	NADPH	NADP
Clone	F	**9.599**	1.010	**12.311**	**5.083**	0.740	**2.612**	**13.384**	**46.404**	0.726	1.011	**18.313**	2.550	1.701	2.179	1.054	0.043
	Pr > F	**< 0.0001**	0.414	**< 0.0001**	**0.002**	0.570	**0.050**	**< 0.0001**	**< 0.0001**	0.580	0.413	**< 0.0001**	0.054	0.169	0.089	0.392	0.996
DCL severity	F	**55.635**	**14.982**	**40.966**	**11.156**	1.805	**15.172**	**56.789**	**28.963**	0.927	**7.680**	**11.466**	1.037	3.474	0.953	1.948	1.840
	Pr > F	**< 0.0001**	**0.000**	**< 0.0001**	**0.002**	0.187	**0.000**	**< 0.0001**	**< 0.0001**	0.341	**0.008**	**0.002**	0.315	0.070	0.335	0.170	0.183
Clone x DCL severity	F	**10.311**	2.184	1.017	**4.156**	0.425	**3.486**	**14.780**	**5.899**	1.741	0.969	**3.986**	0.868	2.312	0.318	1.811	1.046
	Pr > F	**< 0.0001**	0.088	0.410	**0.007**	0.790	**0.016**	**< 0.0001**	**0.001**	0.160	0.435	**0.008**	0.491	0.074	0.864	0.146	0.396

in NAD for clone IRR 118 (Figure 4). The AsA of non-stimulated and stimulated trees were 2.26 mM and 1.33 mM for clone IRR 118, while for BPM 24 was and 2.93 mM and 4.34 mM. The DHA for IRR 118 was 1.40 mM (without stimulation) and 1.03 mM (with stimulation); for BPM 24, DHA was 1.03 mM (without stimulation) and 1.09 mM (with stimulation). Although GSH tended to increase for clone BPM 24, it did not show significant difference between non-stimulated and stimulated trees (0.20 mM and 0.20 mM for IRR 118, 0.08 mM and 0.12 mM for BPM 24, respectively). The GSSG for IRR 118 was 0.18 mM in non-stimulated trees and 0.15 mM in stimulated trees; for BPM 24, the GSSG was 0.05 mM in non-stimulated trees and 0.11 mM in stimulated trees. The NADH concentration in latex increased from 0.20 μM to 0.39 μM in clone IRR 118 and from 0.30 μM to 0.40 μM for clone BPM 24, in non-stimulated and stimulated treatments, respectively. Similarly, the NAD concentration was higher in stimulated treatment compared to non-stimulated treatment: 0.35 μM instead of 0.20 μM for IRR 118 and 0.39 μM instead of 0.25 μM for BPM 24.

There was no significant effect of ethephon stimulation on the ratio between the reduced and oxidized forms of ascorbate and glutathione (Supplementary data 5). The AsA/DHA ratio of non-stimulated and stimulated trees was 1.22 and 1.09 for clone IRR 118, and 3.36 and 4.24 for clone BPM 24 respectively. The GSH/GSSG ratio for IRR 118 was 1.24 (non-stimulation) and 2.65 (ethephon stimulation), for BPM 24 was 2.55 (non-stimulation) and 1.15 (ethephon stimulation). The NADH/NAD ratio for IRR 118 was 1.30 (non-stimulation) and 1.17 (ethephon stimulation), for BPM 24 was 1.10 (non-stimulation) and 1.00 (ethephon stimulation).

3.3. Effect of TPD occurrence

This experiment was conducted on two groups of trees called low DCL and high DCL, these later trees being associated with TPD. Interaction analysis showed that the effect of clone was significant on yield, PI, BI, Suc, Pi, RSH, and GSH, while the effect of DCL severity was significant on yield, IF, PI, BI, Suc, Pi, RSH, DHA, and GSH (Table 3). The clone x DCL severity interaction was significant on yield, BI, Suc, Pi, RSH, and GSH. In this analysis, yield, BI, Suc, Pi, RSH, and GSH were significantly affected by all factors. Given that clone had a significant effect on several important parameters, the factor DCL severity was analysed separately for each clone using a two-way clone x treatment ANOVA. Error bars for each mean value of in Table 5 and Figure 6 correspond to standard errors of means.

Linear discriminant analysis of high and low DCL severity for five recommended rubber clones showed several subgroups more or less clustered (Figure 5). Subgroup 1 consisted of IRR 112 and IRR 118 with low DCL. Subgroup 2 consisted of BPM 24 and PB 260 with low DCL and PB 260 with high DCL. Trees with low DCL from susceptible clones (PB 260, BPM 24) were associated with high GSH and RSH, to a lower extent also with GSSG. Subgroup 3 consisted of RRIC 100 with low DCL. This later was close to the subgroup comprising all clones except PB 260 with high DCL. In high DCL conditions, all clones were in one cluster, suggesting a similar response to over-accumulation of the stress, except for PB 260, which was close to the low DCL of TPD-susceptible clones. Trees with high DCL were associated with high PI and BI, two markers of latex coagulation. The values of the yield, latex flow, antioxidants, and co-factors for low and high DCL trees are shown in Supplementary Data 3 and 4.

The DHA was higher in high DCL trees for clones IRR 118 (0.68 mM) and RRIC 100 (0.42 mM) than that of the low DCL trees (0.13 mM and 0.10 mM respectively), other clones showed no significant differences (Figure 6). The GSH was only significantly different between low and high DCL for clone PB 260, a significant decrease was found (0.43 mM and 0.23 mM), while other clones remained stable. In IRR 118, GSSG doubled in high DCL trees (0.02 mM) compared to low DCL (0.01 mM) and NADH dropped significantly from 1.77 μM to 1.02 μM. A significant drop in NADP was also encountered for clone RRIC 100 from 0.44 μM in

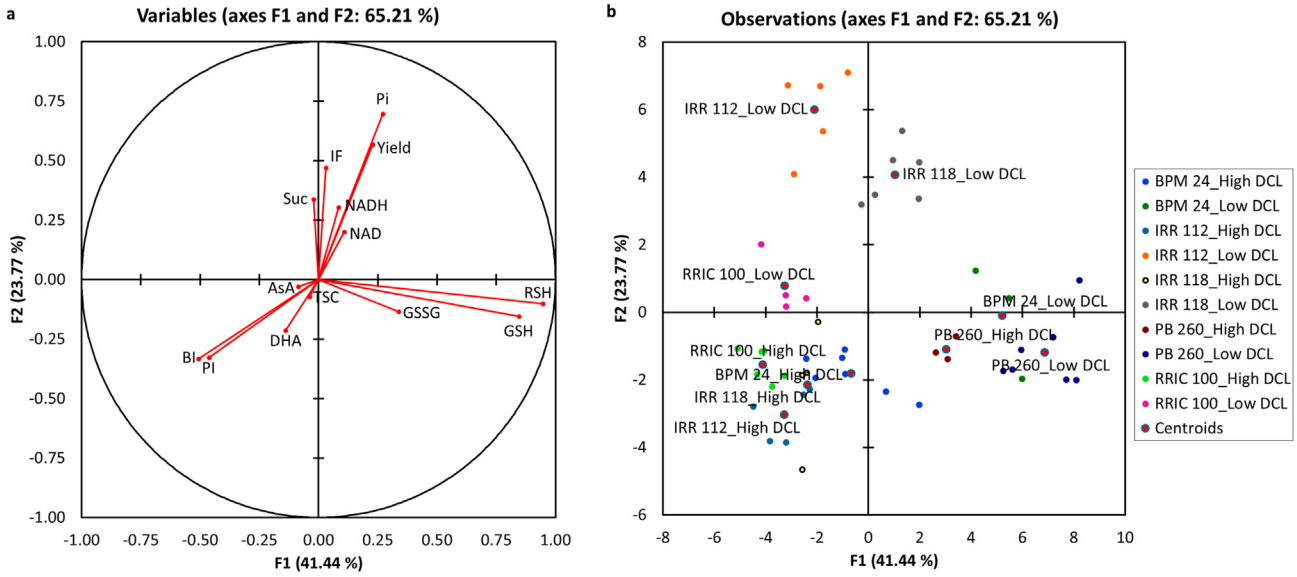

Figure 5. Discriminant analysis of different DCL severity on five recommended rubber clones (correlation chart (a), observations chart (b)).

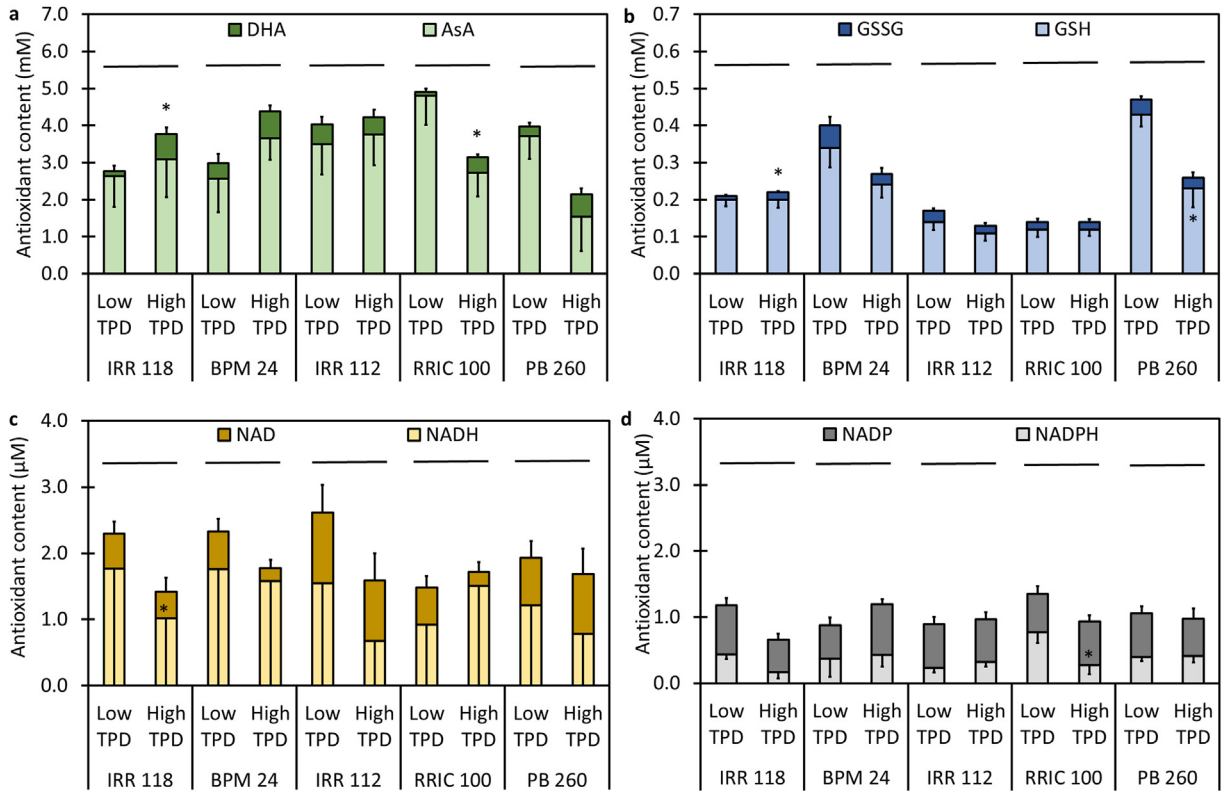

Figure 6. Effect of DCL severity on reduced and oxidized forms of ascorbate (a), glutathione (b), nicotinamide adenine dinucleotide (c), nicotinamide adenine dinucleotide phosphate (d). The effect DCL severity was analysed separately by clone. Data are least-squares means ± standard error. *indicated significant different from low DCL in the same clone according to Tukey Multiple Comparison Method at α = 0.05.

low DCL to 0.17 µM in high DCL. There were no significant difference between low and high DCL trees on AsA, NAD and NADP for all five observed clones.

A multiple stepwise regression analysis was performed to predict the DCL percentage. According to the Akaike Information Criterion (AIC), the best models included seven independent variables: yield, Suc, DHA, GSH, GSSG, NADH, and NAD with the AIC values 280.341 and R^2 = 0.744 (Table 4). The best model follows the equation:

DCL = 91.11–(0.49 x Yield)–(2.36 x Suc) + (25.17 x DHA)–(81.44 x GSH) + (272.06 x GSSG)–(0.08 x NADH)–(0.05 x NAD).

3.4. Change in the proportion of glutathione in the reduced thiol content

The ratio of GSH/RSH varied among clones, stress level and the TPD severity (Table 5). In IRR 118 clone, GSH/RSH ratio was stable in d6 and d3 (0.47 and 0.43 respectively) then dropped in d1 (0.32) and in

Table 4. Model selection for TPD percentage based on AIC method. The best model for the selected selection criterion is displayed in blue.

Number of variables	Variables	MSE	R^2	Adjusted R^2	Mallows' Cp	Akaike's AIC	Schwarz's SBC	Amemiya's PC
1	Yield	583.403	0.276	0.261	58.907	320.403	324.227	0.753
2	Yield/Pi	425.311	0.483	0.461	30.886	305.547	311.283	0.559
3	Yield/Suc/DHA	344.983	0.590	0.563	17.450	296.006	303.654	0.462
4	Yield/Suc/DHA/GSH	302.402	0.648	0.617	10.979	290.320	299.880	0.412
5	Yield/Suc/DHA/GSH/GSSG	267.970	0.695	0.660	6.171	285.152	296.624	0.371
6	Yield/Suc/DHA/GSH/GSSG/NADH	242.734	0.730	0.692	3.102	281.057	294.441	0.342
7	Yield/Suc/DHA/GSH/GSSG/NADH/NAD	235.375	0.744	0.702	3.034	**280.341**	295.637	0.337
8	Yield/Suc/DHA/GSH/GSSG/NADH/NAD/NADPH	233.408	0.753	0.704	3.850	280.717	297.925	0.340
9	Yield/IF/Suc/DHA/GSH/GSSG/NADH/NAD/NADPH	234.474	0.757	0.703	5.136	281.710	300.830	0.346
10	IF/PI/Suc/Pi/AsA/DHA/GSH/GSSG/NADH/NAD	234.698	0.763	0.703	6.290	282.492	303.524	0.352
11	IF/PI/Suc/Pi/AsA/DHA/GSH/GSSG/NADH/NAD/NADPH	238.618	0.766	0.698	7.969	284.021	306.966	0.363
12	IF/PI/BI/Suc/Pi/AsA/DHA/GSH/GSSG/NADH/NAD/ NADPH	241.417	0.769	0.694	9.463	285.271	310.127	0.373
13	Yield/IF/PI/BI/Suc/Pi/AsA/DHA/GSH/GSSG/NADH/NAD/ NADPH	245.101	0.772	0.689	11.055	286.658	313.427	0.384
14	Yield/IF/PI/BI/TSC/Suc/Pi/AsA/DHA/GSH/GSSG/NADH/ NAD/NADPH	251.828	0.772	0.681	13.019	288.604	317.284	0.400
15	Yield/IF/PI/BI/TSC/Suc/Pi/AsA/DHA/GSH/GSSG/NADH/ NAD/NADPH/NADP	259.087	0.772	0.672	15.000	290.575	321.167	0.418
16	Yield/IF/PI/BI/TSC/Suc/Pi/RSH/AsA/DHA/GSH/GSSG/ NADH/NAD/NADPH/NADP	266.935	0.772	0.662	17.000	292.574	325.079	0.436

Table 5. Ratio of reduced glutathione content (GSH) to reduced thiol content (RSH) in five recommended rubber clones. Nd = not determined.

Clone	d6	d3	d1	d3ET	Low TPD	High TPD
IRR 118	0.47 ± 0.08	0.43 ± 0.08	0.32 ± 0.08	0.28 ± 0.08	0.55 ± 0.08	0.52 ± 0.08
BPM 24	0.19 ± 0.08	0.24 ± 0.08	0.27 ± 0.08	0.41 ± 0.08	0.48 ± 0.11	0.63 ± 0.07
IRR 112	Nd	Nd	Nd	Nd	0.55 ± 0.08	0.52 ± 0.08
RRIC 100	Nd	Nd	Nd	Nd	0.59 ± 0.09	0.64 ± 0.08
PB 260	Nd	Nd	Nd	Nd	0.59 ± 0.07	0.45 ± 0.11

ethephon stimulation (0.28). In BPM 24, the ratio increased from 0.19 in d6 to 0.24 in d3 and remained stable in d1 (0.27) then increased with ethephon stimulation (0.41). Observation in five recommended rubber clones under different DCL groups showed that the GSH/RSH ratio was comparable for low DCL, ranging from 0.48 to 0.59, while it was more varied for high DCL, ranging from 0.45 to 0.64, for high DCL.

4. Discussion

4.1. Analysis of oxidised and reduced antioxidants improved our knowledge on antioxidant regulation in latex but requires further studies with higher and longer harvesting stress

Latex is supposed to have three major antioxidants, namely thiol, ascorbate and tocotrienol (Zhang et al., 2017). This study showed that AsA had a much higher concentration than GSH in *H. brasiliensis* latex. It suggested an important role of AsA in the oxidative stress scavenging process and calls for further investigation. Indeed, the scavenging mechanism of H_2O_2 mainly involves the ascorbate–glutathione cycle. Ascorbate peroxidase (APX) neutralizes H_2O_2 into water (H_2O) through electron transfer from AsA. The oxidized form of ascorbate (DHA) is re-generated back to AsA using GSH as the electron donor, and the GSSG is transformed back to GSH through NADPH-dependent GR as electron donor (Foyer and Halliwell, 1976; Noctor and Foyer, 1998).

In 1960, McMullen quantified thiols of low molecular weight in latex (McMullen, 1960). He found that at least 93% of the total –SH in latex is in the form of glutathione and cysteine in the average molar ratio of 1.6. Although the ratio GSH/RSH varies from 0.19 to 0.64 according to the clone and the harvesting system, most of the data presented in this work ranged about 50%.

An alteration of the reduced and oxidized form of antioxidants in response to abiotic stress was reported in other species. In *Zea mays*, a study by Xie et al. (2018) showed the ratio of AsA/DHA ranged from around 2 to more than 10 and GSH/GSSG from 1.5 to 3.5 in response to drought induction. In another study in *Spinacia oleracea*, water deficit treatments altered AsA/DHA ratio from 0.32 to 16.67 and GSH/GSSG from 0.08 to 0.51 (Hodges and Forney, 2000). In the present study, the ratio of AsA/DHA and GSH/GSSG in response to harvesting stress ranged from 1.09 to 2.64 and from 1.11 to 2.65 respectively. The result did not provide significant data to describe the oxidation, regeneration, and biosynthesis level of antioxidants under harvesting stress. It might be due to the level and duration of the stress, which was not strong enough to induce significant alteration of ascorbate and glutathione metabolism. It leads to an assumption that laticifers, which is naturally part of plant defence against herbivory and pathogens (Konno, 2011; Ramos et al., 2019; Gracz-Bernaciak et al., 2021), are equipped with powerful scavenging system that can neutralize stress in a certain level and duration. Deng et al. (2015) reported that the *GR2* gene was more expressed in bark than in latex. In addition, Tistama et al. (2019) and Fipriani et al. (2019) reported that RSH content in TPD-affected trees was higher in bark than in latex. These raise question about antioxidant regulation in non-latex producing cells. In this study, AsA and GSH contents did not significantly change in response to tapping, ethephon, and upon TPD. This calls for further investigations with a larger number of replicates to determine the effect of the tree phenology and time of application of harvesting stress.

4.2. RSH level is prone to genetic and environmental factors but RSH remains a good physiological marker of latex metabolism if supported by reference data

RSH is one of the LD parameters defined as the total sulfhydryl compounds in latex. RSH can protect subcellular organelles by trapping

Table 6. Yield, latex flow, antioxidants, and cofactors dynamic in different type and duration of the stress.

Parameter	Low stress	Temporary stress		Long stress		Cellular damage
	d3, d6 (this study)	d1 (this study)	d3ET (this study)	HTF (from literature)	HSF (from literature)	High TPD (this study)
Yield (g/t/cm)	-	-	↗	↘	↘	↘
IF (ml/minute)	-	-	-	Nd	Nd	↘
PI (%)	-	-	↘	↗	Nd	↗
BI (%)	Nd	Nd	Nd	Nd	Nd	↗
TSC (%)	-	↘	↘	↘	↘	↗
Suc (mM)	-	↘	↗	↘	↘	↘
Pi (mM)	-	↗	↗	↘	↘	↘
RSH (mM)	-	↗	↗	↘	↘	↘
AsA (mM)	-	-	-	Nd	Nd	-
DHA (mM)	-	-	-	Nd	Nd	-
GSH (mM)	-	-	-	Nd	Nd	-
GSSG (mM)	-	-	-	Nd	Nd	-
NADH (pmol/200 μL)	-	-	-	Nd	Nd	-
NAD (pmol/200 μL)	-	-	-	Nd	Nd	-
NADPH (pmol/200 μL)	Nd	Nd	Nd	Nd	Nd	-
NADP (pmol/200 μL)	Nd	Nd	Nd	Nd	Nd	-

- = stable; ↘ = decrease; ↗ = increase; HTF = high tapping frequency; HSF = high ethephon stimulation frequency; Nd = not determined

toxic oxygen molecules (Herve Chrestin, 1985). This parameter is used to estimate the antioxidant capability to overcome oxidative stress due to the harvesting system in latex. The RSH content ranges from 0.5 to 0.9 mM in latex (Jacob et al., 1984) and can reach up to 2.2 mM, according to Chrestin (1985). In this study, the observation of five recommended rubber clones (IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260) showed a clonal variation with the value ranging from 0.21 mM to 0.73 mM. In wheat, the inherent genetic variation in endogenous antioxidants content is suggested due to the variation in biosynthesis and utilization pathways (Roy et al., 2017). The genotypes that have higher endogenous antioxidants have more capability to withstand oxidative stress. The endogenous antioxidant content and anti-oxidative enzymatic activities could be biomarkers for oxidative stress (Fatima et al., 2019; Sanoubar et al., 2016).

Although RSH increased in response to high tapping frequency whatever the tested clones, a clonal variation was noticed in response to the ethephon stimulation. Clone IRR 118 is more tolerant to TPD than clone BPM 24. These clones showed different latex physiology status and exhibited a specific clonal response to ethephon stimulation with a significant increase in RSH for clone IRR 118 while it was stable for clone BPM 24. The clonal characteristic in response to harvesting systems are essential parameters to optimize the yield and prevent TPD. Gohet and collaborators developed a clonal latex metabolic typology based on latex diagnosis (Gohet et al., 2019). This tool could be tested to better understand the context of evolution of RSH content.

In this present study, both studied clones showed a significant increase in RSH in response to high tapping frequency (d1). In response to ethephon stimulation, RSH significantly increased for clone IRR 118 while it remained stable for clone BPM 24. These data were collected four months after the application of harvesting system treatments, which can be considered as a short-term or temporary stress. The increase in RSH was associated with greater latex metabolic activity associated with high Pi. Chrestin (1985) suggested that a high RSH concentration might result from more active glutathione synthesis as a compensation of the oxidative processes driven by tapping and stimulation. Although no significant alteration in GSH was noted in this study, the tendency to increase GSH in response to tapping for both clones and to the ethephon stimulation for clone BPM 24 tend to confirm the hypothesis of Chrestin. In addition, other low molecular weight non-protein components of RSH not detected by Ellman reagent might play a crucial role in response to harvesting systems. In temporary stress, active biosynthesis of RSH was allowed due to sufficient energy availability, which was provided by carbohydrate substrate. These showed the relation between RSH, Suc, and Pi.

Interestingly, several studies showed a drop in RSH after long-term effect of high ethephon stimulation frequency (HSF) (Lacote et al., 2010; Traore et al., 2011; Dian et al., 2016a; Dian et al., 2017) and high tapping frequency (HTF) (Obouayeba et al. 2009), although in other study was stable (Sainoi et al., 2017a,b). In long-term observation, RSH was generally lower for high-stress treatment than lower stress counterpart and was accompanied by low Suc and Pi. This condition showed a low ability for RSH biosynthesis due to a lack of energy substrate. The discrepancy of RSH value in temporary stress and long stress suggested that RSH is influenced by stress duration.

In high TPD-affected trees, RSH dropped along with other physiological parameters. Our result was in line with Putranto et al. (2015) and Guo et al. (2016), who reported a significant lower RSH content in latex of TPD-affected trees. This suggested that the metabolic activity of laticifers had already collapsed. The low energy reflected by low Pi might lead to a limited biosynthesis of antioxidants including RSH. This study showed that RSH is influenced by clone, harvesting stress, and duration. It might explain why RSH is difficult to interpret by knowing only the value in a certain condition. The knowledge of the RSH dynamic in both non-stressing and stressing conditions for each clone may help to determine the current stress level of a given plant.

For that reason, RSH remains a good physiological marker of latex metabolism if supported by reference data. In this study, RSH was the only antioxidant factor that significantly changed according to the level

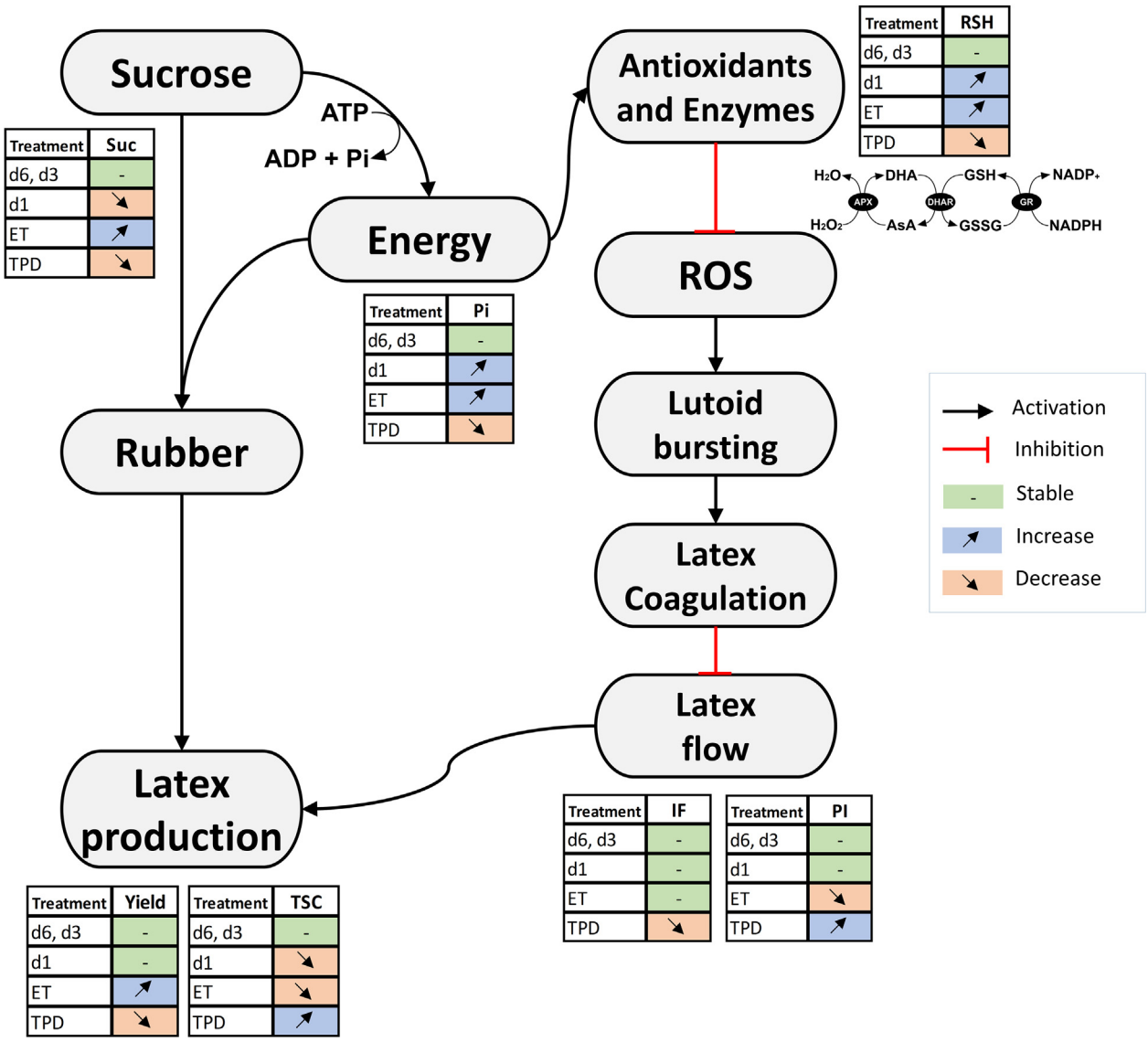

Figure 7. Working model of physiological and antioxidant dynamics in response to harvesting stress and TPD occurrence.

of the stress in laticifer. The RSH increased in temporary stress then decreased in long-term stress and high TPD-affected trees (Table 6). There was no significant alteration of other antioxidant forms (AsA, DHA, GSH, and GSSG) as well as the cofactors (NADH, NAD, NADPH, and NADP). It suggested that RSH remained the appropriate antioxidant parameter to characterize latex. However, several studies showed that RSH was also determined by plant age (Nguyen et al., 2016), canopy condition (Chen and Cao, 2008), temperature (Alam et al., 2003; Sreelatha et al., 2011), and rainfall (Sayurandi et al., 2017). Therefore, its interpretation requires information related to harvesting system history, plant age, TPD percentage, and reference of maximum RSH value under no stress and minimum value in high-stress conditions as well as other LD parameters data (TSC, Suc, and Pi). By combining these factors, it can be determined whether the plant is under temporary stress, long-stress accumulation, or even already in an over-stress condition.

4.3. RSH relationship with other physiological parameters in response to harvesting stress

Tapping frequency and ethephon stimulation affected yield, latex flow, and physiological parameters (Figure 7). In healthy trees, Suc significantly decreased for high tapping frequency due to a rapid sucrose consumption as a consequence of active rubber biosynthesis to regenerate the expelled latex. Our result was partially in line with Chantuma et al. (2011) and Rukkhun et al. (2020), which showed that Suc was relatively stable for high tapping frequency treatments. High Pi indicated a high rate of metabolic activities in laticifers, allowing an increase in energy, which is available for rubber and antioxidants biosynthesis, in particular RSH. However, the effect of RSH on lutoid stability was insignificant as the IF and PI remained stable. The decrease in TSC suggested an active water influx into laticifers leading to a dilution effect of rubber content. It also suggested a limited rubber regeneration between two tappings leading to an insignificant change in the yield.

Ethephon stimulation increased yield significantly through carbohydrate catabolism enhancement, rubber biosynthesis intensification, and inhibition of proteins related to rubber particle aggregation (Wang et al., 2015). In this study, ethephon stimulation significantly lowered PI, expressing a long latex flow. It might also result from high RSH content providing adequate protection of lutoid from harmful free radicals. The high available energy reflected by high Pi enables to maintain a high level of RSH biosynthesis. The increase in Suc indicated an active sucrose importation into laticifers as compensation for the high rubber

biosynthesis rate. The dilution effect of rubber content was also observed in ethephon stimulation in accordance with Tungngoen et al. (2011) and An et al. (2015).

In high TPD-affected trees, the yield dramatically dropped. The logical explanation is the reduction of metabolic activity in laticifers. In that condition, the availabilities of carbohydrate and energy were already low leading to limited Suc and water importations, reflected by low Suc and high TSC in accordance with Tistama et al. (2019). RSH biosynthesis was also disrupted due to lack of energy leading to lutoid bursting and latex coagulation, which was confirmed by a high PI. This phenomenon was also reported by Guo and collaborators (Guo et al., 2016).

5. Conclusions

This paper set out that the RSH content in latex can be a marker of the stress status of laticifers and lead to adjust the harvesting system to prevent TPD if data are interpreted with regard to the history of harvesting system conducted on the studied trial. Both RSH and GSH tend to increase in response to temporary harvesting stress and then drop for long-term stress and upon TPD occurrence. These results suggest that RSH synthesis is made possible by the capacity of laticifer metabolism to cope with harvesting stress, while a drop in RSH is the sign of long-term stress related to lower metabolic activity and TPD occurrence.

This study also revealed new insights into the regulation of antioxidants in laticifers in response to tapping and ethephon stimulation as well as TPD occurrenceThis first attempt to characterize the oxidation level of antioxidants in latex showed a high variability in antioxidant contents among five rubber clones. The high proportion of reduced antioxidant forms suggests that laticifers have a strong capacity to maintain reduction capacity. Interestingly, ascorbate is the most concentrated antioxidant followed by GSH. This latter accounts for about half of RSH.

For a better understanding of the regulation of antioxidants in laticifers, a larger panel of contrasting clones for latex metabolic activity, a combination of stress treatments in level and duration (tapping and ethephon stimulation), and TPD severity should be analysed to confirm the current status of laticifers. Characterization of enzymes activity involved in the regeneration and biosynthesis of antioxidants is also required. The tolerance to intensive harvesting stress and environmental stress is likely to associate with a complex of genetic factors including RSH. The genetic analysis of RSH including genes underlying QTL may be useful for identifying genetic markers, implementing marker-assisted selection and developing TPD-tolerant clones (breeding for TPD-tolerant clones), especially in a context of climate change.

Declarations

Author contribution statement

Junaidi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tri Rini Nuringtyas: Conceived and designed the experiments; Analyzed and interpreted the data.

Anne Clément-Vidal; Albert Flori: Analyzed and interpreted the data.

Afdholiatus Syafaah; Fetrina Oktavia; Sigit Ismawanto; Martini Aji: Contributed reagents, materials, analysis tools or data.

Siti Subandiyah: Conceived and designed the experiments.

Pascal Montoro: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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