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**Etude des paramètres physiologiques et des antioxydants
du latex en réponse aux stress de récolte et à l'encoche
sèche dans le latex chez *Hevea brasiliensis* (Will. ex A.
Juss.) Muell Arg.**

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**THÈSE POUR OBTENIR LE GRADE DE DOCTEUR
DE L'INSTITUT AGRO MONTPELLIER
ET DE L'UNIVERSITE DE MONTPELLIER**

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Portée par**

**Unité de recherche Amélioration Génétique & Adaptation
des Plantes méditerranéennes et tropicales**

**Etude des paramètres physiologiques et des antioxydants
en réponse aux stress de récolte et à l'encoche sèche
dans le latex chez *Hevea brasiliensis* (Will. ex A. Juss.) Muell Arg.**

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Le 28 novembre 2022**

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DECLARATION OF RESEARCH AUTHENTICITY

The author hereby declares that the research in this dissertation is that the author did and is not a duplication of another person's research. To the author's knowledge, the dissertation materials have never been published by other researchers or the result of other research, except for the references contained in the dissertation text. The dissertation materials have never been used to attain any academic degree at other universities.

Yogyakarta, November 10, 2022



Junaidi

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RÉSUMÉ SUBSTANTIEL

Le caoutchouc para (*Hevea brasiliensis* (Will. ex A. Juss.) Muell Arg.) est la principale espèce productrice de caoutchouc naturel. Le caoutchouc est récolté par l'incision de l'écorce pour laisser la solution colloïdale, appelée latex, s'écouler et être collectée. L'éthéphon, un libérateur d'éthylène, est largement utilisé dans les plantations de caoutchouc pour optimiser le rendement. Les études sur l'état physiologique du caoutchouc ont exploré la possibilité de soutenir l'optimisation du rendement, par la modification du tapotement et de la stimulation et d'étudier le mécanisme de réponse au stress, principalement pour atténuer l'apparition de la sécheresse du panneau de tapotement (TPD), un syndrome physiologique induit par la surexploitation et la surstimulation.

Le diagnostic latex (DL) est une méthode d'estimation de l'état physiologique de la plante à un moment de la production de l'arbre. Elle est réalisée en évaluant quatre paramètres principaux, à savoir la teneur totale en solides (TSC) indiquant la régénération du latex entre deux prélèvements, la teneur en saccharose (Suc) indiquant la disponibilité des glucides, le phosphore inorganique (Pi) reflétant l'activité métabolique et la teneur en thiols (RSH) pour la capacité antioxydante et niveau de stress. En ce qui concerne la prévention de la TPD, le comportement RSH n'est toujours pas entièrement compris. Théoriquement, le RSH diminue avec l'augmentation du stress de récolte en raison de la consommation rapide en réponse à l'accumulation de la production d'espèces réactives de l'oxygène (ROS). Cependant, dans certaines études, la teneur en RSH était stable ou augmentée. L'étude de méta-analyse, qui faisait

partie du projet, a révélé que la RSH était mal interprétée, seulement dans 42,8 % des 131 articles scientifiques concernés. L'incohérence des valeurs RSH associées au niveau de stress conduit les auteurs à limiter l'interprétation de leurs données. Il appelle à une étude approfondie de la dynamique RSH dans différents types et niveaux de stress pour une meilleure compréhension de la régulation RSH dans le latex de *H. brasiliensis*.

Le système de piégeage des ROS implique des antioxydants et des enzymes antioxydantes. Dans le latex, les principaux antioxydants sont les thiols (composés de glutathion et de cystéine), l'ascorbate (isomère de la vitamine C) et le tocotriénol (isomère de la vitamine E). De plus, plusieurs enzymes sont impliquées dans la neutralisation des composés radicaux, notamment la superoxyde dismutase (SOD), la catalase (CAT), la glutathion peroxydase (GPX), la glutathion S-transférase (GST) et l'ascorbate peroxydase (APX). Dans des conditions de faibles concentrations de ROS, APX et GPX sont très actifs, tandis que l'activité CAT est augmentée dans des conditions de ROS élevées. La détoxification des ROS se produit par une réaction de réduction-oxydation (redox). La superoxyde dismutase cuivre-zinc (CuZnSOD) est l'enzyme responsable de la conversion du radical superoxyde ($O_2^{\bullet-}$) en peroxyde d'hydrogène (H_2O_2) dans le cytosol. La détoxification de H_2O_2 a généralement impliqué le cycle de l'ascorbate et du glutathion. H_2O_2 est neutralisé en H_2O par transfert d'électrons depuis l'ascorbate réduit (AsA) en présence d'APX. L'ascorbate oxydé (DHA) est produit au cours du processus et régénéré en AsA en utilisant du glutathion réduit (GSH) comme donneur d'électrons. La forme oxydée du glutathion

(GSSG) est produite et recyclée en GSH par le nicotinamide adénine dinucléotide phosphate réduit (NADPH), catalysé par la glutathion réductase (GR).

L'augmentation de la forme oxydée des antioxydants indique une oxydation active due à la présence de stress, tandis que l'augmentation des formes réduites indique une biosynthèse de novo ou une activation du cycle ascorbate-glutathion. Certains travaux sur d'autres espèces végétales ont montré une altération des formes réduites et oxydées d'antioxydants en réponse à un stress abiotique. Cette approche peut donner une meilleure explication des niveaux de stress chez *H. brasiliensis* que l'évaluation du RSH uniquement.

Des études sur d'autres plantes ont révélé que la variabilité de la teneur en antioxydants pouvait être détectée à des niveaux interspécifiques et intraspécifiques. Chez *H. brasiliensis*, plusieurs études ont rapporté une variation génotypique de la teneur en antioxydants et des activités des enzymes antioxydantes telles qu'une variation de la teneur en tocotriénols et thiols ainsi que des activités enzymatiques. Ces variations pourraient être exploitées pour la sélection de clones tolérants au stress oxydatif. L'analyse génétique de RSH, y compris les gènes sous-jacents au Quantitative Trait Locus (QTL), peut être utile pour identifier des marqueurs génétiques, mettre en œuvre une sélection assistée par marqueurs et développer des clones tolérants à la TPD (élevage de clones tolérants à la TPD), en particulier dans un contexte de changement climatique.

Cette recherche de thèse vise à compléter la méthode LD, en particulier dans l'estimation du niveau de stress oxydatif et de la capacité antioxydante. Il s'agit du

premier travail combinant rendement, flux de latex, diagnostic du latex et quantification des antioxydants dans le latex de *H. brasiliensis* afin de mieux comprendre la régulation des antioxydants du latex en réponse au stress de la récolte et de tenter d'identifier d'autres paramètres redox potentiels. Ce travail est également la première tentative d'analyse génétique pour identifier les QTL liés aux principaux traits antioxydants du latex. Les résultats attendus de cette recherche incluent: 1. une meilleure connaissance de la variation de la teneur en antioxydants dans le latex de *H. brasiliensis*, 2. une meilleure compréhension de l'évolution de la teneur en RSH en réponse à différents stress de saignée, de stimulation de l'éthéphon et d'occurrence de TPD, 3 modèle de travail de la dynamique physiologique et antioxydante, y compris RSH, dans différents types de stress et durée, 4. recommandation pour une meilleure interprétation de la valeur RSH en relation avec le stress de récolte et l'occurrence de TPD, 5. nouveau paramètre de niveau de stress oxydatif basé sur le statut en antioxydants, 6. paramètres antioxydants significatifs pour une analyse génétique plus approfondie, et 7. héritabilité et QTL des principaux traits antioxydants du latex. Sur la base de la littérature liée aux objectifs de recherche, les hypothèses proposées sont: 1. la teneur en antioxydants dans le latex varie selon les clones de caoutchouc, 2. la teneur en antioxydants, y compris RSH, se modifie en réponse à différents stress de saignée, de stimulation de l'éthéphon et d'occurrence de TPD, 3. la dynamique des paramètres physiologiques et des antioxydants sous différents types de stress et durée permettra de mieux comprendre la régulation des antioxydants dans différents types de stress et durée, 4. la régulation RSH peut fournir une meilleure interprétation de la

valeur RSH en relation avec le stress de récolte et l'occurrence de TPD, 5. le rapport entre la forme réduite et la forme oxydée des antioxydants peut être un meilleur estimateur du niveau de stress dans le latex de *H. brasiliensis* que le RSH seul, 6. une variation significative de certains paramètres antioxydants peut être appropriée pour une analyse génétique plus approfondie, et 7. la population en ségrégation provenant d'un croisement sensible et tolérant au TPD fournira les données préliminaires sur l'héritabilité et le QTL pour l'antioxydant du latex traits liés aux fourmis.

Pour atteindre les objectifs de la recherche, l'étude sur la variation clonale de la teneur en antioxydants et l'effet du tapotement, de la stimulation et de la TPD sur le rendement, le flux de latex et les paramètres antioxydants ont été réalisées, suivies d'une analyse génétique des principaux paramètres antioxydants dans une population en ségrégation. Le projet a débuté en février 2019. Après la préparation et une série de formations en laboratoire en 2019 - 2020, des expériences sur le terrain ont été menées de janvier à juillet 2021 au Sembawa Research Centre, Indonesian Rubber Research Institute, Palembang, Indonésie. Quatre essais sur le terrain ont été établis pour étudier: 1. la variation clonale des antioxydants dans cinq clones de caoutchouc recommandés, 2. l'effet du tapotement et de la stimulation de l'éthéphon sur le rendement, le flux de latex et les paramètres d'antioxydants, 3. l'effet de l'apparition de TPD, et 4 analyse génétique des principaux paramètres antioxydants.

La variation clonale de cinq clones de caoutchouc recommandés, à savoir IRR 118, BPM 24, IRR 112, RRIC 100 et PB 260 (sélectionnés en fonction du niveau métabolique) a été réalisée sur des arbres sains âgés de dix ans. Les arbres ont été

saignés sur le panneau B0-1 tous les trois jours avec une application d'éthéphon à 2,5 % tous les mois (système de récolte noté S/2 d3 ET2,5 % 12/an). L'effet des systèmes de récolte a été réalisé sur les clones IRR 118 et BPM 24. Quatre systèmes de récolte (S/2 d6, S/2 d3, S/2 d1 et S/2 d3 ET2,5 % 12/a) ont été comparés à l'aide de cinq arbres en répétition pour chaque traitement. La collecte des échantillons de latex a été réalisée en avril 2021, quatre mois après l'application des traitements. L'effet de la TPD a été mené sur cinq clones recommandés, à savoir IRR 118, BPM 24, IRR 112, RRIC 100 et PB 260 exploités avec le système de récolte S/2 d3 ET 2,5 % 12/an sur le panneau B0-1.

La variabilité phénotypique des principaux paramètres antioxydants a été réalisée sur des hévéas âgés de cinq ans afin d'identifier les paramètres antioxydants significatifs pour une analyse génétique plus approfondie. Huit clones de caoutchouc recommandés évalués comprenaient AVROS 2037, GT 1, IRR 112, IRR 39, PB 260, PR 261, RRIC 100 et SP 217. Cinq répétitions ont été utilisées, chaque répétition était la moyenne de deux arbres individuels. Pour l'analyse de l'héritabilité et des QTL, 103 descendances de PB 260 (clone sensible au TPD) x SP 217 (clone tolérant au TPD) ont été utilisées.

Les paramètres observés associés à la production de latex comprenaient le rendement en latex, le débit initial (IF), l'indice de colmatage (PI) et l'indice d'éclatement (BI). Pour le diagnostic du latex, TSC, Suc, Pi et RSH ont été déterminés. Pour les antioxydants, la forme réduite (AsA et GSH) et les formes oxydées (DHA et GSSG) ont été analysées. Les cofacteurs associés à la régénération des antioxydants

ont été évalués, notamment le nicotinamide adénine dinucléotide oxydé et réduit (NAD, NADH) et le nicotinamide adénine dinucléotide phosphate (NADP, NADPH). L'analyse de l'héritabilité et QTL a été réalisée sur les valeurs de RSH, AsA, DHA, AsA total et le rapport AsA/DHA.

L'interaction entre les clones et le système de récolte a été analysée par une analyse de variance à 3 ou 2 voies (ANOVA). Pour étudier l'effet de la variation clonale sur les antioxydants et l'effet de la fréquence de tapotement, de la stimulation de l'éthéphon et de la sévérité du DCL, une ANOVA unidirectionnelle suivie du test Tukey HSD avec $\alpha = 0,05$ a été mise en œuvre. Une analyse discriminante linéaire supervisée avec la sévérité du DCL comme variable catégorielle dépendante a été réalisée impliquant le rendement, le débit de latex, le diagnostic du latex, les antioxydants et les cofacteurs pour trouver des combinaisons de ces paramètres qui pourraient expliquer l'apparition de TPD. Enfin, pour prédire linéairement le pourcentage de DCL, une analyse de régression par étapes multiples utilisant le critère d'information d'Akaike (AIC) a été effectuée.

Le résultat a montré que l'AsA avait une concentration beaucoup plus élevée que le GSH dans le latex de *H. brasiliensis*, ce qui suggère que l'AsA pourrait jouer un rôle important dans le processus de piégeage du stress oxydatif et appelle une enquête plus approfondie. Le rapport GSH/RSH varie de 0,19 à 0,64 selon le clone et le système de récolte inférieur à la littérature de McMullen (1960) qui rapporte qu'au moins 93 % du -SH total dans le latex est sous forme de glutathion et de cystéine.

L'observation de cinq clones de caoutchouc recommandés (IRR 118, BPM 24, IRR 112, RRIC 100 et PB 260) a montré une variation clonale de RSH avec une valeur allant de 0,21 mM à 0,73 mM, inférieure à la littérature rapportant une teneur en RSH comprise entre de 0,5 à 0,9 mM (Jacob et al., 1984). Bien que RSH augmente en réponse à une fréquence de tapotement élevée quels que soient les clones testés, une variation clonale a été observée en réponse à la stimulation à l'éthéphon. Le clone IRR 118 est plus tolérant au TPD que le clone BPM 24. Ces clones de caoutchouc ont montré une réponse clonale spécifique à la stimulation de l'éthéphon avec une augmentation significative du RSH pour le clone IRR 118 alors qu'il est stable pour le clone BPM 24. La caractéristique clonale en réponse à la récolte est un paramètre essentiel pour optimiser le rendement et prévenir la TPD.

Ces données ont été recueillies quatre mois après l'application des traitements du système de récolte, qui peuvent être considérés comme un stress à court terme ou temporaire. L'augmentation du RSH était associée à une plus grande activité métabolique du latex associée à une synthèse élevée de Pi et de glutathion actif en tant que compensation des processus oxydatifs entraînés par le tapotement et la stimulation. De plus, un autre composant non protéique de faible poids moléculaire du RSH non détecté par le réactif d'Ellman pourrait jouer un rôle crucial en réponse aux systèmes de récolte. En cas de stress temporaire, la biosynthèse active de RSH est autorisée en raison d'une disponibilité énergétique suffisante, qui est fournie par un substrat glucidique. Celles-ci ont montré la relation entre RSH, Suc et Pi. Selon la littérature, dans le stress à long terme, le RSH était généralement plus faible pour le traitement à

stress élevé que pour le traitement à faible stress et accompagné de faibles Suc et Pi. Cette condition a montré une faible capacité de biosynthèse RSH en raison d'un manque de substrat énergétique. L'écart de la valeur RSH dans le stress temporaire et le stress long suggère que le RSH est influencé par la durée du stress.

Chez les arbres fortement touchés par la TPD, le RSH a chuté avec d'autres paramètres physiologiques suggérant que l'activité métabolique des laticifères s'est déjà effondrée. La faible énergie réfléchi par un faible Pi pourrait conduire à une biosynthèse limitée d'antioxydants, y compris le RSH. Cette étude a montré que le RSH est influencé par le clone, le stress de récolte et la durée. Cela pourrait expliquer pourquoi RSH est difficile à interpréter en ne connaissant que la valeur dans une certaine condition. La connaissance de la dynamique RSH dans des conditions non stressantes et stressantes pour chaque clone peut aider à déterminer le niveau de stress actuel d'une plante donnée. La littérature a montré que le RSH était également déterminé par l'âge de la plante, l'état de la canopée et les conditions environnementales. Il suggère que pour l'interprétation RSH, les informations relatives à l'historique du système de récolte, à l'âge de la plante, au pourcentage de TPD et à d'autres données de paramètres LD (TSC, Suc et Pi) sont nécessaires. En combinant ces facteurs, il est possible de déterminer si la plante est soumise à un stress temporaire, à une accumulation de stress prolongé ou même déjà dans un état de stress excessif.

Le RSH était le seul facteur antioxydant qui changeait significativement en fonction du niveau de stress chez le laticifère. Il n'y avait pas d'altération significative des autres formes antioxydantes (AsA, DHA, GSH et GSSG) ainsi que des cofacteurs

(NADH, NAD, NADPH et NADP). Il a suggéré que RSH reste le paramètre antioxydant approprié pour caractériser le latex. Le rapport AsA/DHA et GSH/GSSG en réponse au stress de la récolte variait de 1,09 à 2,64 et de 1,11 à 2,65 respectivement. Le résultat n'a pas fourni de données significatives pour décrire le niveau d'oxydation, de régénération et de biosynthèse des antioxydants sous le stress de la récolte. Cela pourrait être dû au niveau et à la durée du stress, qui n'était pas assez fort pour induire une altération significative du métabolisme de l'ascorbate et du glutathion. Cela conduit à supposer que les laticifères sont équipés d'un puissant système de piégeage qui peut neutraliser le stress à un certain niveau et pendant une certaine durée. Cela nécessite des investigations supplémentaires avec un plus grand nombre de répétitions pour déterminer l'effet de la phénologie de l'arbre et le moment de l'application du stress de récolte.

Notre étude a permis de mieux comprendre la régulation des antioxydants, en particulier le RSH, et sa relation avec d'autres paramètres physiologiques. Chez les arbres sains, Suc a significativement diminué en fréquence de frappe élevée en raison d'une consommation rapide en conséquence de la biosynthèse active du caoutchouc pour régénérer le latex expulsé. Un Pi élevé indique un taux élevé d'activités métaboliques chez les laticifères, permettant une augmentation de l'énergie disponible pour la biosynthèse du caoutchouc et des antioxydants, en particulier le RSH. Cependant, l'effet du RSH sur la stabilité du lutoïde était insignifiant car l'IF et l'IP restaient stables. La diminution du TSC suggère un apport actif d'eau dans les laticifères conduisant à un effet de dilution de la teneur en caoutchouc. Elle suggérait également

une régénération limitée du caoutchouc entre deux saignées conduisant à une variation insignifiante du rendement. La stimulation à l'éthéphon a significativement abaissé l'IP, exprimant un long flux de latex. Cela pourrait également résulter d'une teneur élevée en RSH assurant une protection adéquate du lutoïde contre les radicaux libres nocifs. L'énergie disponible élevée réfléchiée par un Pi élevé permet de maintenir un niveau élevé de biosynthèse de RSH. L'augmentation de Suc indique une importation active de saccharose dans les laticifères en compensation du taux élevé de biosynthèse du caoutchouc. L'effet de dilution de la teneur en caoutchouc a également été observé dans la stimulation à l'éthéphon. Dans les arbres fortement touchés par la TPD, le rendement a chuté de façon spectaculaire. L'explication logique est la réduction de l'activité métabolique des laticifères. Dans ces conditions, les disponibilités en glucides et en énergie étaient déjà faibles, ce qui a entraîné des importations limitées de Suc et d'eau, reflétées par un faible Suc et un TSC élevé. Dans cette condition, la biosynthèse du RSH a également été perturbée en raison d'un manque d'énergie entraînant l'éclatement du lutoïde et la coagulation du latex, ce qui est confirmé par un IP élevé.

En conclusion, l'étude a montré que le RSH est un paramètre multifactoriel difficile à interpréter. Il a tendance à augmenter en réponse à un stress temporaire, puis à diminuer lors d'un stress à long terme, généralement accompagné d'une survenue de TPD. Utiliser uniquement la valeur RSH à un moment de la production de l'arbre peut induire en erreur l'interprétation. Pour une meilleure interprétation, la référence de la valeur RSH maximale sans stress et la valeur minimale dans des conditions de stress élevé sont requises, ainsi que des informations relatives à l'historique du système de

récolte, à l'âge de la plante, au pourcentage de TPD et à d'autres données de paramètres LD. L'analyse des formes réduites et oxydées des antioxydants n'a pas pu donner une direction claire du niveau d'oxydation, de régénération et de biosynthèse des antioxydants. Cependant, une autre étude mérite d'être approfondie.

Dans cette étude, le QTL n'a pas pu être détecté pour le RSH, l'AsA, le DHA, l'AsA total et le rapport AsA/DHA. Les données ont montré une faible héritabilité génotypique du trait antioxydant ; le plus élevé était RSH 0,69, les autres traits encore inférieurs à 0,2 indiquant une faible variabilité dans les 103 géotypes observés. Cela peut être dû au faible stress, à la saignée en d3 depuis l'ouverture de la saignée sans stimulation, ou au génotype insuffisant impliqué dans l'analyse. L'analyse dans un stress plus élevé, par exemple, fréquence de tapotement d1, et une population plus importante peut donner un meilleur résultat.

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SYMBOLS AND ABBREVIATION

%	= Percent
μl	= Microliter
¹ O ₂	= Singlet Oxygen
ABA	= Abscisic acid
ADH	= Alcohol dehydrogenase
AIC	= Akaike Information Criterion
ANOVA	= Analysis of Variance
APX	= Ascorbate Peroxidase
ARF	= Auxin Response Factor
AsA	= Ascorbate
ATP	= Adenosine Triphosphate
ATPase	= Adenosine Triphosphatase
B0-1	= First virgin basal panel
BB	= Brown Bast
BI	= Bursting Index
BLUP	= Best Linear Unbiased Prediction
Ca ²⁺	= Calcium Cation
CAT	= Catalase
CIRAD	= Centre de coopération Internationale en Recherche Agronomique pour le Développement
CN	= Cyanide
CPO	= Crude Palm Oil
Cu ²⁺	= Copper Cation
d2	= Alternate tapping frequency, tapping every two days
d3	= Tapping every three days
d4	= Tapping every four days

d5	= Tapping every five days
d6	= Tapping every six days
DCL	= Dry Cut Length
DCPIP	= 2,6-dichlorophenolindophenol
DEGs	= Differentially Expressed Genes
DHA	= Dehydroascorbate
DHAR	= Dehydroascorbate Reductase
DMAPP	= Dimethyl Allyl Pyrophosphate
DNA	= Deoxyribonucleic Acid
DRC	= Dry Rubber Content
DTNB	= 5.5'-dithiobis 2-nitrobenzoic Acid
DTT	= Dithiothreitol
DXP	= 1-deoxy-D-xylulose-5-phosphate
DXPS	= 1-deoxy-D-xylulose 5-phosphate synthase
E	= Transpiration
EDTA	= Ethylenediaminetetraacetic Acid
ET	= Ethylene
F ₀	= Minimal Fluorescence
FAP	= Free Acid Phosphatase
FeCl ₃	= Ferric Chloride
FeSO ₄	= Ferro-sulphate
F _m	= Maximal Fluorescence
FPP	= Farnesyl Pyrophosphate
FPS	= Farnesyl Diphosphate Synthase
FTSW	= Fraction of Transpirable Soil Water
F _v	= Gap between maximal fluorescence to minimal fluoresce
G6PDH	= Glucose 6-phosphate
GA3P	= Glyceraldehyde-3-phosphate
GCL	= Glutamate-cysteine Ligase

GDH	= L-galactose Dehydrogenase
GGP	= GDP-L-galactose Phosphorylase
GGPP	= Geranylgeranyl Pyrophosphate
GGPS	= Geranylgeranyl Diphosphate Synthase
GLDH	= L-galactono-1,4-lactone Dehydrogenase
GME	= GDP-mannose-3',5'-epimerase
GMP	= GDP-mannose Pyrophosphorylase
GPP	= Geranyl Pyrophosphate
GPS	= Geranyl Diphosphate Synthase
GPX	= Glutathione Peroxidase
GR	= Glutathione Reductase
GS	= Glutamine Synthetase
<i>gS</i>	= Stomatal Conductance
GSH	= Reduced Glutathione
GSSG	= Oxidized Glutathione
GST	= Glutathione S-transferase
H ₂ O ₂	= Hydrogen Peroxide
H ₂ SO ₄	= Sulphuric Acid
H ₃ PO ₄	= Phosphoric Acid
<i>HbSUT3</i>	= Sucrose Transporter Gene
HCL	= Hydrochloric Acid
HCN	= Hydrogen Cyanide
HCN _p	= Cyanogenic Potential
HEPES	= 4-1-piperazineethanesulfonic Acid
HK	= Hexokinase
HMBPP	= 4-hydroxy-3-methylbut-2-enyl diphosphate
HMG CoA	= 3-hydroxy-3-methylglutaryl coenzyme A
HMGR	= 3-hydroxy-3-methylglutaryl coenzyme A reductase
<i>hmgs</i>	= Hydroxymethyl Glutaryl-CoA Synthase Gene

HMGS	= 3-hydroxy-3-methylglutaryl coenzyme A synthase
HSD	= Honestly Significant Difference
IDP	= Isopentenyl Diphosphate
IDPI	= IDP Isomerase
IF	= Initial Flow
IPP	= Isopentenyl Pyrophosphate
IRRI	= Indonesian Rubber Research Institute
IRSG	= International Rubber Study Group
K ⁺	= Potassium Cation
KH ₂ PO ₄	= Potassium Phosphate
LD	= Latex Diagnosis
LG	= Linkage Group
LMW	= Low Molecular Weight
LOD	= Log of the Odds
M	= Molar
MAS	= Marker-assisted Selection
MDA	= Malondialdehyde
MDHA	= Monodehydroascorbate
MDHAR	= Monodehydroascorbate Reductase
MEP	= 2-C-methyl-D-erythritol 4-phosphate
mg	= Milligram
Mg ²⁺	= Magnesium Cation
ml	= Milliliter
mM	= Millimolar
MnSOD	= Manganese Superoxide Dismutase
MQM	= Multiple QTL Model
MRB	= Malaysian Rubber Board
MVA	= Mevalonate
MVD	= Mevalonate Diphosphate Decarboxylase Gene

N	= Normality
NAD	= Nicotinamide Adenine Dinucleotide
NADH	= Reduced Nicotinamide Adenine Dinucleotide
NADP	= Nicotinamide Adenine Dinucleotide Phosphate
NADPH	= Reduced Nicotinamide Adenine Dinucleotide Phosphate
NaH ₂ PO ₄	= Sodium dihydrogen phosphate
NaOH	= Sodium Hydroxide
NEM	= n-ethylmaleimide
nm	= Nanometer
NO ³⁻	= Nitrate
NPQ	= Non-photo-chemical quenching
O ₂ ⁻	= Superoxide Anions
O ₃	= Ozone
°C	= Degree Celsius
OH ⁻	= Hydroxyl Radical
PCD	= Programmed Cell Death
PGI	= Glucose-6-phosphate Isomerase
pH	= Potential of hydrogen, acidity/alkalinity scale
Pi	= Inorganic Phosphorus
PI	= Plugging Index
PIPs	= Plasma membrane Intrinsic Proteins
PMI	= Mannose-6-phosphate Isomerase
PMM	= Phosphomannomutase
PMS	= Phenazine methosulfate
P _n	= Net Photosynthetic Rate
PNPP	= Sodium p-nitrophenyl Phosphate
POD	= Phenol oxidase
PX	= Peroxydase
QTL	= Quantitative Trait Locus

RBOHs	= Respiratory Burst Oxidase Homologs
Redox	= Reduction-oxidation
REF	= Rubber Elongation Factor
RNA	= Ribonucleic Acid
ROS	= Reactive Oxygen Species
RP	= Redox Potential
rpm	= Revolutions per minute
RSH	= Thiols Content
RTV1	= Rubber Tree Virus 1
SIPs	= Small basic Intrinsic Proteins
SOD	= Superoxide Dismutase
SRPP	= Small Rubber Particle Protein
SSR	= Simple Sequence Repeats
Suc	= Sucrose Content
SWCE	= Shrimp Waste-enriched Compost
TAP	= Total Acid Phosphatase
TCA	= Trichloroacetic Acid
TIPs	= Tonoplast Intrinsic Proteins
TPD	= Tapping Panel Dryness
TPN	= Tapping Panel Necrosis
TSC	= Total Solid Content
UGM	= Universitas Gadjah Mada
USD	= United States Dollar
USDA	= United States Department of Agriculture
VINV	= Vacuolar Invertase
VPD	= Vinylpyridine
VTE1	= Tocopherol Cyclase
VTE2	= Homogentisate Phytyltransferase
VTE3	= 2-methyl-6-phytyl-1,4-benzoquinol Methyltransferase

VTE4 = γ -tocopherol Methyltransferase
 β -CAS = β -cyanoalanine Synthase
 γ -ECS = γ -glutamylcysteine Synthase

ABSTRACT

The reduced thiols content (RSH) is one of the biochemical parameters of latex Diagnosis (LD) in rubber trees (*Hevea brasiliensis* (Will. ex A. Juss.) Muell. Arg.) associated with the risk of Tapping Panel Dryness (TPD), a physiological syndrome induced by over-accumulation of Reactive Oxygen Species (ROS). However, RSH is difficult to interpret due to the influence of the environment. The dissertation research aims to observe the variation of antioxidant content in latex of rubber clones, to study the evolution of RSH content in response to different stress of tapping, ethephon stimulation, and TPD occurrence, to understand the RSH regulation in different stress types and duration, to identify the better way to interpret RSH value in relation with harvesting stress and TPD occurrence, and to examine the ratio of reduced to oxidised form of antioxidants as the estimator of stress level in *H. brasiliensis* latex. This work is also the first attempt at genetic analysis to identify Quantitative Trait Locus (QTL) related to antioxidant traits. The project was started in February 2019. Following preparation and a series of laboratory training in 2019 – 2020, field experiments were carried out from January to July 2021 at Sembawa Research Centre, Indonesian Rubber Research Institute, Palembang, Indonesia. Four field trials were established to study: 1. the clonal variation of antioxidants in five recommended rubber clones, 2. the effect of tapping and ethephon stimulation on the yield, latex flow, and antioxidants parameters, 3. the effect of TPD occurrence, and 4. genetic analysis of main antioxidant parameters. Clonal variation was performed on ten-year-old trees of five recommended rubber clones i.e. IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260 which were selected based on latex metabolism characteristics. The trees were tapped on the B0-1 with S/2 d3 ET2.5% 12/y tapping system. The effect of harvesting systems was studied on clones IRR 118 and BPM 24 using four harvesting systems (S/2 d6, S/2 d3, S/2 d1, and S/2 d3 ET2.5% 12/y). Data were collected four months after the application of treatments. The effect of TPD was examined on five recommended clones i.e. IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260 tapped with S/2 d3 ET 2.5% 12/y on the B0-1 panel. Heritability and QTL analysis was performed on 103 PB 260 (TPD susceptible clone) x SP 217 (TPD tolerant clone) progenies. Observed parameters associated with latex production included latex yield, initial flow (IF), plugging index (PI), and bursting index (BI). For LD, total solid content (TSC), sucrose content (Suc), inorganic phosphorus content (Pi), and RSH were determined. The observed antioxidants included reduced and oxidised forms of ascorbate (AsA and DHA) and glutathione (GSH and GSSG). Cofactors associated with antioxidants regeneration were assessed, including oxidised and reduced nicotinamide adenine dinucleotide (NAD, NADH), and nicotinamide adenine dinucleotide phosphate (NADP, NADPH). Heritability and QTL analysis was performed on RSH, AsA, DHA, total AsA, and the ratio of AsA/DHA. The results showed that the concentration in AsA is about ten times higher than GSH in laticifer, while GSH accounts for about 50 % of RSH. The RSH and its related parameters (GSH and GSSG) varied among rubber clones, while AsA, DHA, and antioxidant cofactors (NADH, NAD, NADPH, and NADP) were not

significantly different among observed clones. For short-term harvesting stress, RSH increases with tapping frequency and ethephon stimulation and then drops in long-term stress, usually accompanied by TPD occurrence which is associated with high latex viscosity and luteoid bursting. 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 a sign of prolonged stress related to lower metabolic activity and TPD occurrence. For better interpretation, reference of maximum RSH value under short-term high-stress and minimum value in long-term high-stress conditions are required as well as information related to harvesting system history, plant age, TPD percentage, and other LD parameters data (TSC, Suc, and Pi). The analysis of the reduced and oxidised forms of antioxidants could not give a clear direction of the oxidation, regeneration, and biosynthesis level of antioxidants. The high proportion of reduced antioxidant forms suggests that laticifers have a strong capacity to maintain reduction capacity. The highest heritability was RSH 0.69, while other traits were lower than 0.2. The QTL could not be detected for RSH, AsA, DHA, total AsA, and the ratio of AsA/DHA. Further investigation under higher and longer stresses, as well as larger samples, is recommended for a clearer effect of tapping, ethephon stimulation, and TPD on the antioxidants and cofactors evolution and QTL mapping.

Keywords: ascorbate, ethephon, glutathione, latex flow, rubber, tapping, thiols, yield

RÉSUMÉ

La teneur réduite en thiols (RSH) est l'un des paramètres biochimiques du latex Diagnostic (LD) chez l'hévéa (*Hevea brasiliensis* (Will. ex A. Juss.) Muell. Arg.) associé au risque de Tapping Panel Dryness (TPD), un syndrome physiologique induit par une suraccumulation de Espèces réactives de l'oxygène (ROS). Cependant, RSH est difficile à interpréter en raison de l'influence de l'environnement. La recherche de thèse vise à observer la variation de la teneur en antioxydants dans le latex de clones de caoutchouc, à étudier l'évolution de la teneur en RSH en réponse à différents stress de tapotement, de stimulation de l'éthéphon et d'occurrence de TPD, à comprendre la régulation du RSH dans différents types de stress et durée, identifier la meilleure façon d'interpréter la valeur RSH en relation avec le stress de récolte et l'occurrence de TPD, et examiner le rapport entre la forme réduite et la forme oxydée des antioxydants comme estimateur du niveau de stress dans le latex de *H. brasiliensis*. Ce travail est également la première tentative d'analyse génétique pour identifier le locus quantitatif des traits (QTL) lié aux traits antioxydants. Le projet a débuté en février 2019. Après la préparation et une série de formations en laboratoire en 2019 - 2020, des expériences sur le terrain ont été menées de janvier à juillet 2021 au Sembawa Research Centre, Indonesian Rubber Research Institute, Palembang, Indonésie. Quatre essais sur le terrain ont été établis pour étudier : 1. la variation clonale des antioxydants dans cinq clones de caoutchouc recommandés, 2. l'effet du tapotement et de la stimulation de l'éthéphon sur le rendement, le flux de latex et les paramètres d'antioxydants, 3. l'effet de l'apparition de TPD, et 4 analyse génétique des principaux paramètres antioxydants. La variation clonale a été réalisée sur des arbres de dix ans de cinq clones de caoutchouc recommandés, à savoir IRR 118, BPM 24, IRR 112, RRIC 100 et PB 260, sélectionnés en fonction des caractéristiques du métabolisme du latex. Les arbres ont été saignés sur le B0-1 avec le système de saignée S/2 d3 ET2.5% 12/y. L'effet des systèmes de récolte a été étudié sur les clones IRR 118 et BPM 24 en utilisant quatre systèmes de récolte (S/2 d6, S/2 d3, S/2 d1 et S/2 d3 ET2,5 % 12/an). Les données ont été recueillies quatre mois après l'application des traitements. L'effet de la TPD a été examiné sur cinq clones recommandés, à savoir IRR 118, BPM 24, IRR 112, RRIC 100 et PB 260 tapotés avec S/2 d3 ET 2,5 % 12/an sur le panel B0-1. L'héritabilité et l'analyse QTL ont été réalisées sur 103 des descendances PB 260 (clone sensible au TPD) x SP 217 (clone tolérant au TPD). Les paramètres observés associés à la production de latex comprenaient le rendement en latex, le débit initial (IF), l'indice de colmatage (PI) et l'indice d'éclatement (BI). Pour LD, la teneur totale en solides (TSC), la teneur en saccharose (Suc), la teneur en phosphore inorganique (Pi) et la RSH ont été déterminées. Les antioxydants observés comprenaient des formes réduites et oxydées d'ascorbate (AsA et DHA) et de glutathion (GSH et GSSG). Les cofacteurs associés à la régénération des antioxydants ont été évalués, notamment le nicotinamide adénine dinucléotide oxydé et réduit (NAD, NADH) et le nicotinamide adénine dinucléotide phosphate (NADP, NADPH). L'analyse de l'héritabilité et du QTL a été effectuée sur RSH, AsA, DHA, AsA total et le rapport AsA/DHA. Les résultats ont montré que la concentration en

AsA est environ dix fois supérieure à celle du GSH dans le laticifère, alors que le GSH représente environ 50 % du RSH. Le RSH et ses paramètres associés (GSH et GSSG) variaient selon les clones de caoutchouc, tandis que l'AsA, le DHA et les cofacteurs antioxydants (NADH, NAD, NADPH et NADP) n'étaient pas significativement différents parmi les clones observés. Pour le stress de récolte à court terme, le RSH augmente avec la fréquence de tapotement et la stimulation de l'éthéphon, puis diminue le stress à long terme, généralement accompagné de l'apparition de TPD qui est associée à une viscosité élevée du latex et à l'éclatement du lutoïde. Ces résultats suggèrent qu'un niveau élevé de RSH montre la capacité du métabolisme des laticifères à faire face au stress de la récolte, tandis qu'une baisse de RSH est un signe de stress prolongé lié à une activité métabolique plus faible et à l'apparition de TPD. Pour une meilleure interprétation, la référence de la valeur RSH maximale dans des conditions de stress élevé à court terme et de la valeur minimale dans des conditions de stress élevé à long terme est requise, ainsi que des informations relatives à l'historique du système de récolte, à l'âge de la plante, au pourcentage de TPD et à d'autres données de paramètres LD. (TSC, Suc et Pi). L'analyse des formes réduites et oxydées des antioxydants n'a pas pu donner une direction claire du niveau d'oxydation, de régénération et de biosynthèse des antioxydants. La forte proportion de formes antioxydantes réduites suggère que les laticifères ont une forte capacité à maintenir la capacité de réduction. L'héritabilité la plus élevée était RSH 0,69, tandis que les autres caractères étaient inférieurs à 0,2. Le QTL n'a pas pu être détecté pour le RSH, l'AsA, le DHA, l'AsA total et le rapport AsA/DHA. Une enquête plus approfondie sous des contraintes plus élevées et plus longues, ainsi que des échantillons plus grands, est recommandée pour un effet plus clair du tapotement, de la stimulation de l'éthéphon et de la TPD sur l'évolution des antioxydants et des cofacteurs et la cartographie QTL.

Mots-clés : ascorbate, éthéphon, glutathion, flux de latex, saignée, thiols, rendement

ABSTRAK

Kadar tiol tereduksi (RSH) adalah salah satu parameter biokimia yang diukur dalam *Latex Diagnose* (LD) pada tanaman karet (*Hevea brasiliensis* (Will. ex A. Juss.) Muell. Arg.) yang terkait dengan risiko Kering Alur Sadap (KAS), yaitu suatu sindrom fisiologis yang disebabkan oleh akumulasi *Reactive Oxygen Species* (ROS) yang berlebihan. Namun demikian, RSH sulit diinterpretasikan karena pengaruh lingkungan. Disertasi ini bertujuan untuk mengamati variasi kandungan antioksidan pada lateks klon karet, mempelajari evolusi kandungan RSH tanggap cekaman penyadapan, stimulasi etefon dan kejadian KAS, memahami regulasi RSH pada berbagai jenis dan durasi cekaman, mengidentifikasi metode yang lebih baik untuk menginterpretasikan nilai RSH dalam kaitannya dengan cekaman penyadapan kejadian KAS, dan untuk menguji rasio antioksidan tereduksi terhadap antioksidan teroksidasi sebagai penduga tingkat cekaman pada lateks *H. brasiliensis*. Disertasi ini juga merupakan upaya pertama dalam analisis genetik untuk mengidentifikasi *Quantitative Trait Locus* (QTL) yang terkait dengan sifat antioksidan. Proyek penelitian ini dimulai pada Februari 2019. Setelah persiapan dan serangkaian pelatihan laboratorium pada 2019 – 2020, percobaan lapangan dilakukan pada Januari hingga Juli 2021 di Pusat Penelitian Sembawa, Pusat Penelitian Karet, Palembang, Indonesia. Empat pengujian lapangan dilakukan untuk mempelajari: 1. variasi klon terhadap kadar antioksidan pada lima klon karet yang direkomendasikan, 2. pengaruh penyadapan, stimulasi etefon, 3. pengaruh kejadian KAS, dan 4. analisis genetik parameter terhadap parameter antioksidan utama. Studi variasi klon dilakukan pada tanaman berumur sepuluh tahun dari lima klon karet yang direkomendasikan yaitu IRR 118, BPM 24, IRR 112, RRIC 100, dan PB 260 yang dipilih berdasarkan karakteristik metabolisme lateks. Tanaman yang diamati disadap pada panel B0-1 dengan sistem sadap S/2 d3 ET2,5% 12/y. Pengaruh sistem sadap diamati pada klon IRR 118 dan BPM 24 menggunakan empat sistem sadap (S/2 d6, S/2 d3, S/2 d1, dan S/2 d3 ET2,5% 12/y). Data dikumpulkan empat bulan setelah penerapan perlakuan. Pengaruh KAS diamati pada lima klon yang direkomendasikan yaitu IRR 118, BPM 24, IRR 112, RRIC 100, dan PB 260 yang disadap dengan S/2 d3 ET 2,5% 12/y pada panel B0-1. Analisis heritabilitas dan QTL dilakukan pada 103 progeni dari PB 260 (klon rentan KAS) x SP 217 (klon toleran KAS). Parameter yang diamati terkait dengan produksi lateks yaitu produksi lateks, aliran awal (IF), indeks penyumbatan (PI), dan *bursting index* (BI). Untuk LD, kadar padatan total (TSC), kadar sukrosa (Suc), kadar fosfat anorganik (Pi), dan RSH ditentukan. Antioksidan yang diamati meliputi askorbat tereduksi dan teroksidasi (AsA dan DHA) dan glutathione (GSH dan GSSG). Kofaktor yang terkait dengan regenerasi antioksidan yang diamati yaitu nikotinamida adenin dinukleotida teroksidasi dan tereduksi (NAD, NADH), dan nikotinamida adenin dinukleotida fosfat teroksidasi dan tereduksi (NADP, NADPH). Analisis heritabilitas dan QTL dilakukan terhadap parameter RSH, AsA, DHA, total AsA, dan rasio AsA/DHA. Hasil penelitian menunjukkan bahwa konsentrasi AsA sekitar sepuluh kali lebih tinggi dibanding GSH dalam latisifer, sedangkan GSH sekitar 50 % dari RSH. Parameter RSH, GSH dan GSSG bervariasi antar klon karet, sedangkan

AsA, DHA dan kofaktor antioksidan (NADH, NAD, NADPH, dan NADP) tidak berbeda nyata antar klon yang diamati. Dalam cekaman jangka pendek, RSH meningkat seiring peningkatan frekuensi penyadapan dan stimulasi etefon dan kemudian turun dalam cekaman jangka panjang disertai kejadian KAS yang dikaitkan dengan peningkatan viskositas lateks dan ledakan lutoid. Hasil ini mengindikasikan bahwa tingkat RSH yang tinggi menunjukkan kapasitas metabolisme latisifer untuk mengatasi cekaman, sementara penurunan RSH merupakan tanda cekaman berkepanjangan terkait aktivitas metabolisme yang lebih rendah dan terjadinya KAS. Untuk interpretasi yang lebih baik, diperlukan referensi nilai RSH maksimum pada cekaman tinggi jangka pendek dan nilai minimum pada kondisi cekaman tinggi jangka panjang serta informasi terkait riwayat sistem sadap, umur tanaman, persentase KAS, dan data parameter LD lainnya (TSC, Suc, dan Pi). Analisis rasio antioksidan tereduksi dan teroksidasi tidak dapat memberikan gambaran yang jelas terhadap tingkat oksidasi, regenerasi, dan biosintesis antioksidan. Tingginya proporsi bentuk antioksidan tereduksi menunjukkan bahwa latisifer memiliki kapasitas yang kuat untuk mempertahankan kapasitas reduksi. Heritabilitas tertinggi dijumpai pada parameter RSH (0,69), sedangkan sifat lainnya lebih rendah dari 0,2. QTL tidak dapat dideteksi untuk RSH, AsA, DHA, total AsA, dan rasio AsA/DHA. Penelitian lebih lanjut direkomendasikan dengan tingkat cekaman yang lebih tinggi dan durasi lebih lama serta sampel yang lebih besar untuk dapat mengetahui efek penyadapan, stimulasi etefon, dan KAS yang lebih jelas serta untuk mengetahui evolusi antioksidan dan kofaktor dan juga untuk pemetaan QTL.

Kata kunci: askorbat, etefon, glutathione, aliran lateks, penyadapan, tiol, produksi

I. INTRODUCTION

A. Background

Para rubber (*Hevea brasiliensis* (Will. ex A. Juss.) Muell. Arg.) is the main natural rubber-producing species. It is mainly cultivated in Southeast Asia and some in South Asia, Africa, and Tropical American countries. In 2019, Indonesia had 3.68 million hectares of rubber plantations. Of this area, 88.93 % were smallholding plantations, while private and state-owned companies were only 6.57 % and 4.50 %, respectively. Provisional data showed that in 2020 the rubber plantations reached 3.68 million hectares and in 2021 were estimated to reach 3.69 million hectares. Indonesia's rubber production in 2019 reached 3.30 million tons, while in 2020 and 2021 was estimated to reach 2.88 million tons and 3.12 million tons, respectively. Most of the production was exported. A total of 2.50 million tons of rubber was exported in 2019 with a value of 3.53 billion United States Dollars (USD). The total labour involved in rubber plantations in 2019 reached 2.26 million people, reflecting the importance of this commodity to the Indonesian economy (Ditjenbun, 2020).

The tree is harvested through the incision of the bark to let the colloidal solution, called latex, flow out and be collected. Latex contains 25-50 % dry matter, 95 % of which are natural rubber particles (d'Auzac and Jacob 1989; Jacob et al., 1993; Bottier, 2020). In rubber plantations, the latex production cycle is 25 to 30 years. The sustainability of latex production is imperative for economic viability. To optimize the yield and maintain the lifespan, the harvesting system implicates some factors, including tapping frequency, panel management, and ethephon stimulation.

The harvesting practice of *H. brasiliensis* that applies mechanical wounding and exogenous ethylene (ethephon) stimulation invited scientific studies to understand the effect of harvesting systems on plant physiological status. The studies have explored the opportunity to support yield optimisation through tapping and stimulation modification and investigate the stress-response mechanism, mainly to mitigate the occurrence of tapping panel dryness (TPD), a physiological syndrome induced by over-exploitation and over-stimulation. The symptom of this syndrome is that the latex does not flow out upon tapping leading to a significant yield drop. It is induced by the over-accumulation of reactive oxygen species (ROS), leading to an oxidative burst and cellular damage. The partial dryness of the tapping cut indicates the early stage of TPD, whilst the severe occurrence leads to a total dryness and oftentimes exhibits a brownish bark tissue. Other observers encountered that a TPD-affected tree may experience a vertical cracking on the outer layer of the bark or infestation of insect borers (Mathew and Gnanaharan, 2000; Mathew et al., 2006). In the field extent, the percentage of TPD in the mature plant was estimated to reach 12 – 50 % of the tree population (Chen et al., 2002; Abraham et al., 2005) leading to 10 – 40 % of yield losses (Chen et al., 2002; Okoma et al., 2011). Therefore, TPD is considered an important limiting factor for rubber yield (Abraham et al., 2005).

Besides abiotic stress, tapping activity induces biotic stress in the form of fungal infections on tapped bark; for example, *Phytophthora palmivora* (Butl.) Butl. (black stripe disease) (Sdoodee, 2004) and *Ceratocystis fimbriata* Ell. & Halst. (moldy rot), and insect borers (Mathew and Gnanaharan, 2000; Hiremath and Prathapan, 2019). In

response to biotic stress, *H. brasiliensis* accumulates pathogenesis-related proteins such as chitinase and heveamine as well as increases antioxidants and antioxidative enzymes (Das and Roychoudhury, 2014; Krishnan et al., 2019). Regarding TPD, the previous study failed to confirm any biotic causal agent (Pellegrin et al., 2007). A consensus view is that TPD is mainly caused by the abiotic stress of tapping and ethephon stimulation. However, other studies revealed the possibility of the association of viroid-like ribonucleic acid (RNA) with TPD-affected rubber trees (Ramachandran et al., 2000). Many of the trees where low molecular weight RNA (LMW RNA) bands were detected before the trees were tapped showed TPD after tapping (Ramachandran et al., 2006). An observation by Abraham and collaborators suggested that there was a chance of the spread of TPD from one tree to the neighbouring tree (Abraham et al., 2005). The LMW RNA isolated from TPD-affected rubber trees was found infectious on seedlings of tomato cv Pusa Ruby (Kumar et al., 2013). Li and collaborators determined the complete genome sequences of a novel virus identified in a TPD-affected tree, tentatively called “rubber tree virus 1” (RTV1). The genome architecture is similar to that of members of the genus Capillovirus (family Betaflexiviridae) (Li et al., 2020).

Numerous studies had been conducted on latex physiology and were reviewed in the book “Physiology of Rubber Tree Latex” (d’Auzac et al., 1989). Latex diagnosis (LD) was introduced to estimate the physiological status at one moment of tree production. It is performed by assessing 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). LD is mainly used for clonal metabolic typology determination, yield optimisation and TPD prevention. Clonal metabolic typology distinguishes rubber clones based on their latex metabolic level into low metabolic clones such as AVROS 2037, PB 217, and PR 107 (Adou et al., 2018b; Gohet et al., 2019), moderate metabolic clones such as RRIC 100, BPM 24, IRR 112, and IRR 118 (Herlinawati et al., 2016; Diarrassouba et al., 2021; Aji et al., 2021), and high metabolic clones such as PB 260, PB 235, and RRIM 911 (Adou et al., 2018a; Atminingsih et al., 2019). According to the physiological status, monitored by LD, the harvesting system is customised to obtain high yield and prevent TPD.

Concerning TPD mitigation, 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 decrease (Dian et al., 2016a,b; Gohet et al., 2019). This inconsistency leads to difficulty interpreting RSH and calls for a comprehensive study of RSH dynamics in different stress types and levels to better understand RSH regulation in *H. brasiliensis* latex and better interpretation. Clonal variability of LD parameters in response to the harvesting system has been widely studied; however, knowledge of the variability of other physiology and antioxidant parameters in latex *H. brasiliensis* is still limited.

The ROS scavenging system occurs through a reduction-oxidation (redox) mechanism involving antioxidants and enzymes, and mainly relies on the ascorbate-

glutathione cycle (Zhang et al., 2017). The redox reaction evolves antioxidants and enzymes from reduced form to become oxidised in the process of radical neutralization (Sharma et al., 2012). Two molecules of ascorbate (AsA) are used to convert hydrogen peroxide (H_2O_2) to water, in the present of ascorbate peroxidase (APX), generating two molecules of monodehydroascorbate (MDHA) which are rapidly disproportionated to AsA and dehydroascorbate (DHA), catalysed by monodehydroascorbate reductase (MDHAR). DHA is recycled to AsA using reduced glutathione (GSH) as the reducing substrate, in the present of dehydroascorbate reductase (DHAR). The oxidized glutathione (GSSG) is generated in this process and recycled to GSH by reduced nicotinamide adenine dinucleotide phosphate (NADPH), catalysed by glutathione reductase (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). Indeed, a study by Marok et al. (2013) detected the alteration of reduced and oxidised antioxidants proportion in response to environmental stress. 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). It suggested that the ratio of

reduced to oxidised form of antioxidants may be suitable as a diagnostic parameter for plant physiological stress estimation. It can be an alternative for stress estimation besides RSH. To our knowledge, the study of the alteration of reduced and oxidized forms of antioxidants in the latex of *H. brasiliensis* particularly in response to the harvesting stress has not been performed.

The main strategy to minimize the latex yield decline due to oxidative stress, including TPD, is through the TPD-resistant breeding program. The endogenous antioxidant content and anti-oxidative enzymatic activities could be biomarkers for oxidative stress (Sanoubar et al., 2016; Fatima et al., 2019). The variability was encountered in inter and intra-specific levels (Hale et al., 2008; Hanen et al., 2009; Sarker et al., 2014; Mageney et al., 2016). Several studies reported genotypic variation in antioxidants content and antioxidative enzymes activities in *H. brasiliensis*, such as a variation in tocotrienols content (Nadarajah et al., 1971), thiols content (Syafaah et al., 2015; Phearun et al., 2016), and enzymatic activities including superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Santos et al., 2021). However, variability in AsA and glutathione contents has not been reported. These variabilities could be exploited for the selection of oxidative stress-tolerant clones. Therefore, the genetic analysis of RSH, including genes underlying Quantitative Trait Locus (QTL), may be useful for identifying genetic markers of antioxidant traits in *H. brasiliensis*. (Chandra and Pandey, 2017). To date, QTL detection in *H. brasiliensis* is mainly for growth and latex production-related traits (Rattanawong et al., 2008; Rosa et al., 2018; Xia et al., 2018; An et al., 2019; Hou et

al., 2021), while QTL identification on antioxidants-related traits has not been performed.

B. Research Questions

The LD is widely used in rubber research and plantations. However, the RSH parameter is still difficult to interpret due to its inconsistency. The research was established to answer the following questions:

1. How is the variation of antioxidants contained in the latex of rubber clones under standard tapping practices?
2. How does RSH behave under different stress of tapping, ethephon stimulation, and TPD occurrence?
3. How is RSH regulated in different stress types and duration?
4. What is the better way to interpret RSH value?
5. Can the ratio of reduced to oxidised form of antioxidants be the estimator of stress level in *H. brasiliensis* latex?
6. What are the significant antioxidant parameters in *H. brasiliensis* latex?
7. What is the heritability of the main antioxidant traits in latex? and can the QTL of the traits be detected?

C. Research Objectives

The objectives of the dissertation research include:

1. To observe the variation of antioxidants contained in the latex of rubber clones under a standard tapping system.
2. To study the evolution of RSH content in response to different stress of tapping, ethephon stimulation, and TPD occurrence.
3. To understand the RSH regulation in different stress types and duration.
4. To identify the better way to interpret RSH value in relation to harvesting stress and TPD occurrence.
5. To examine the ratio of reduced to oxidised form of antioxidants as the estimator of stress level in *H. brasiliensis* latex.
6. To observe the phenotypic variation of antioxidant content in latex and to select the most significant antioxidant parameters for genetic analysis.
7. To observe the heritability and identify the QTLs of latex's main antioxidants traits.

This dissertation aims to complement the LD method, especially in oxidative stress level and antioxidant capability estimation. This is the first work combining yield, latex flow, latex diagnosis, and antioxidant quantification in *H. brasiliensis* latex in order to have a better understanding of the regulation of latex antioxidants in response to harvesting stress and attempt to identify other potential redox parameters. This work also carried out a genetic analysis to identify QTL related to main latex antioxidant traits, which are useful for TPD-tolerant breeding programs through the marker-assisted selection method.

The strategy to achieve the dissertation objectives is presented in Figure 1.1. The project started with meta-analysis studies on LD in rubber research to understand the use of LD and the interpretation of its parameters. As this is the first work to quantify the reduced and oxidised forms of main latex antioxidants, some experiments were performed to analyse the influence of sample collection, handling, and quantification protocols on antioxidant parameters. Field experiments were established to figure out the effect of the tapping system, ethephon stimulation, and TPD occurrence on the LD and antioxidant parameters. The QTL mapping was carried out to identify genes underlying the QTL of main latex antioxidants.

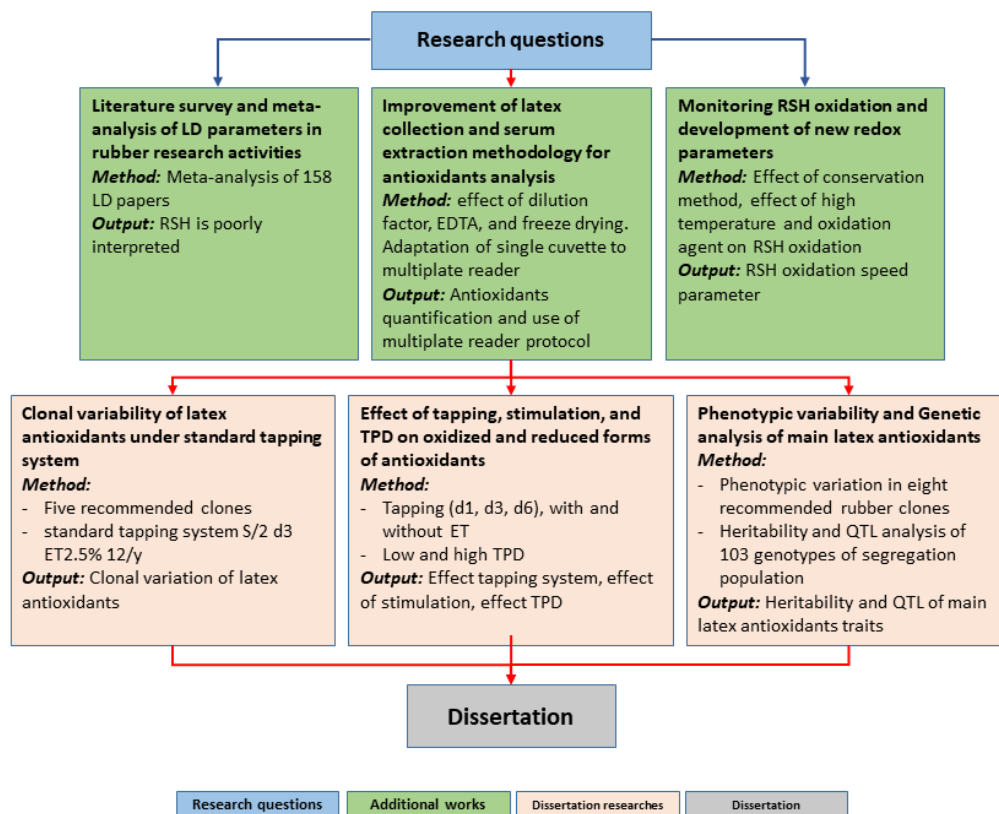


Figure 1.1. Scheme of research steps for achieving the dissertation objectives

D. Research Authenticity

The LD technology is adopted for yield optimisation and TPD prevention. Previous studies usually applied LD on the combination of tapping and stimulation under a particular condition. To date, physiological stress estimation is performed by assessing the RSH content; however, several studies indicated that RSH was not consistent with the stress extent experienced by the plants leading to difficulty in interpreting RSH values. On the other hand, the ratio of reduced/oxidised forms of antioxidants had been studied for estimating the physiological stress level in other plants (Hodges and Forney, 2000; Xie et al., 2018). To our knowledge, these typical works have not been performed in *H. brasiliensis*.

This study is the first work 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 oxidised 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). Previous works on LD, TPD, and antioxidants related to oxidative stress are presented in Table 1.1.

Antioxidants and scavenging enzymes are key actors in the ROS detoxification process. Breeding for rubber clones tolerant to oxidative stress and TPD is vital in relation to the harvesting system and climate change. Molecular marker-assisted selection (MAS) allows the breeders to select superior genotypes with desired traits in the earlier stage of the plants. This method offers a shorter time for breeding activities. A QTL is defined as a region of the genome or locus of a gene associated with an effect on a quantitative trait (Chandra and Pandey, 2017). QTL detection for *H. brasiliensis* has been performed mainly for growth and latex production-related traits (Rattanawong et al., 2008; Rosa et al., 2018; Xia et al., 2018; An et al., 2019; Hou et al., 2021). To our knowledge, QTL identification on antioxidants-related genes has not been performed. This work is the first attempt at QTL analysis on latex antioxidant traits.

Table 1.1. Previous studies related to the research topics and the findings

No.	Reference	Title	Finding
I Studies on the physiological status of <i>H. brasiliensis</i> in relation to tapping and ethephon stimulation			
1	de Faÿ (2011)	Histo- and cytopathology of trunk phloem necrosis, a form of rubber tree (<i>Hevea brasiliensis</i> Müll. Arg.) tapping panel dryness	<ul style="list-style-type: none"> • Numerous structural features of Tapping Panel Necrosis (TPN) are common to TPD, notably tylosoids associated with in situ coagulated latex, which are also known to be early structural markers of TPD and cyanide-induced. • It is therefore concluded that TPN is identical to or a variant of TPD and is a degenerative disease of rubber tree trunk phloem resembling plant stress response, programmed cell death (PCD), and plant tumourigenesis in some aspects.
2	Okoma et al. (2011)	Seasonal variation of tapping panel dryness expression in rubber tree <i>Hevea brasiliensis</i> Muell. Arg. in Cote d'Ivoire	<ul style="list-style-type: none"> • The results showed that <i>Hevea brasiliensis</i> clones do not have the same sensitivity to TPD. • They also showed that the expression of TPD varies seasonally and is positively correlated to the water deficit and the temperature.

No.	Reference	Title	Finding
3	Obouayeba and Soumahin (2011)	Relationship between tapping intensity and tapping panel dryness susceptibility of some clones of <i>Hevea brasiliensis</i> in Southwestern Côte d'Ivoire	<ul style="list-style-type: none"> • The rubber yield strongly depends on the intensity of tapping. • The intensity of tapping governed the TPD rate among the clones having active metabolisms, such as IRCA 18, IRCA 111, PB 235, and PB 255.
4	Krishnakumar et al. (2011)	A modified stimulation method in <i>Hevea brasiliensis</i> for reducing oxidative stress	<ul style="list-style-type: none"> • Stress response in the tapping panel was studied by analyzing hydrogen peroxide (H₂O₂), peroxidase, cyanide (CN), β-Cyanoalanine synthase (βCAS), and malondialdehyde (MDA) which have shown variations with treatments. • Minimum in trees stimulated with the modified method; 150 cm above the bud union and on the bud union together.
5	Moraes et al. (2014)	Relationship between cyanogenesis and latex stability on tapping panel dryness in rubber trunk girth	<ul style="list-style-type: none"> • The positive correlations between cyanogenic potential (HCNp) values and luteoid BI indicate that cyanogenic glycosides present in the bark reduce latex stability, resulting in low yield due to the short duration of flow during tapping. • The largest amount of hydrogen cyanide (HCN) is released by trunk bark tissues in TPD trees.
6	Putranto et al. (2015)	Involvement of ethylene in the latex metabolism and tapping panel dryness of <i>Hevea brasiliensis</i>	<ul style="list-style-type: none"> • The TPD susceptibility of <i>Hevea</i> clones was found to be related to some biochemical parameters, such as low Suc and high Pi. • High tapping frequency and ethephon stimulation induced the early TPD occurrence in a high latex metabolism clone and late occurrence in a low latex metabolism clone.
7	Liu et al. (2016)	Molecular mechanism of ethylene stimulation of latex yield in rubber tree (<i>Hevea brasiliensis</i>) revealed by de novo sequencing and transcriptome analysis	<ul style="list-style-type: none"> • The increase in the yield in ethephon stimulation might be due to the rapid acceleration of the glycolytic pathway supplying precursors for the biosynthesis of isopentenyl pyrophosphate (IPP) and natural rubber, instead of rubber biosynthesis per se. • The elevated rate of flux throughout the Calvin cycle may account for some durability of ethylene-induced stimulation.
8	Adou et al. (2017)	Latex micro diagnosis, a modern management tool for rubber plantations of clones	<ul style="list-style-type: none"> • Tapping system S/2 d4 6d/7 Pa1 (1) ET2.5 4/y is most suitable for moderate clones • The micro latex diagnosis, through the physiological index, played a preponderant

No.	Reference	Title	Finding
		with moderate metabolism GT 1, RRIC 100 and BPM 24	role in the choice of latex harvesting technologies adapted to moderate metabolism clones
9	Adou et al. (2018)	Contribution of latex micro diagnosis to modern management of rubber plantations: a case of clones with low or slow metabolism PB 217 and PR 107	<ul style="list-style-type: none"> • Tapping system S/2 d3 6d/7 Pa1(1) ET2.5% 4/y, S/2 d4 6d/7 Pa1(1) ET2.5% 4/y and S/2 d4 6d/7 Pa1(1) ET2.5% 8/y is the most suitable for low metabolic clones • The latex micro-diagnosis, through the physiological index, played a predominant role in the choice of latex harvesting technologies suitable for slow metabolism clones: PB 217 and PR 107.
10	Suwandi et al. (2018)	Curative activity of watery fermented compost extract as a bark treatment against tapping panel dryness	<ul style="list-style-type: none"> • Shrimp waste-enriched compost (SWCE) reduced tapping cut dryness and increased latex yield, suggesting a recovery. • Slight recovery from TPD in trees treated without bark scraping and in trees with a history of ethephon stimulation. • No curative effect of SWCE was demonstrated in treated trees without a tapping rest period.
11	Gohet et al. (2019)	Meta-analysis of a large industrial latex diagnosis database provides insight into <i>Hevea brasiliensis</i> clonal adaptation and site-specific yield potential in Western Africa	<ul style="list-style-type: none"> • Positive correlation between RSH and Suc. Clones with high latex Suc maintain higher RSH latex concentrations than clones with lower Suc • A hypothesis is that the latex RSH and the interaction Pi x RSH value might be accurate indicators to describe the local clonal suitability and global stress conditions.
12	Tistama et al. (2019)	Physiological status of high and low metabolism <i>Hevea</i> in the different stages of tapping panel dryness	<ul style="list-style-type: none"> • The physiological pattern of IRR 42 (low metabolism clone) was different from that of IRR 118 (high metabolism clone) for the increase in the TPD stages. • Pi in the latex and bark was a decline in the TPD-affected trees. • Peroxidase activity in the latex and bark negatively correlated with the TPD stage. • The decline of Pi and peroxidase activity can be used to identify the TPD incident in rubber trees.
13	Liu et al. (2019)	Involvement of <i>HbMCI</i> -mediated cell death in tapping panel dryness of rubber tree (<i>Hevea brasiliensis</i>)	<ul style="list-style-type: none"> • The expression level of <i>HbMCI</i> was increased along with TPD severity in the rubber tree. • Overexpression of <i>HbMCI</i> in yeast and tobacco reduced cell survival after high-concentration H₂O₂ treatment and resulted in enhanced H₂O₂-induced leaf cell death.

No.	Reference	Title	Finding
			<ul style="list-style-type: none"> • A total of 11 proteins, rbcL, TM9SF2-like, COX3, ATP9, DRP, HbREF/Hevb1, MSSP2-like, SRC2, GATL8, CIPK14-like, and STK, were identified and confirmed to interact with <i>HbMCI</i> by yeast two-hybrid screening and co-transformation in yeast.
14	Rukkhun et al. (2020)	Effect of high-frequency tapping system on latex yield, tapping panel dryness, and biochemistry of young hillside tapping rubber	<ul style="list-style-type: none"> • The high-frequency tapping system positively affected the rubber latex yield formation. • Using a high-frequency tapping system increased bark consumption and stimulated TPD. • Latex diagnosis (Suc, Pi, and RSH levels) showed a relatively unhealthy rubber tree as impacted by a high-frequency tapping system.
15	Chen et al. (2020)	Effects of ethephon stimulation and cut length by vertically tapping on rubber yield and latex quality	<ul style="list-style-type: none"> • The increase of cut length and ethephon stimulation concentration increased dry rubber yield, Copper cation (Cu^{2+}), and RSH, yet decreased Magnesium cation (Mg^{2+}) content. • Ethephon stimulation decreased consistently tear strength values, and increased tensile strength and tensile permanent set values.
16	Rukkhun et al. (2021)	Latex diagnosis at the whole trunk level under different tapping systems in young-tapping rubber trees	<ul style="list-style-type: none"> • S/6 d3 with RRIMFLOW (ethylene gas releaser) tapping system in young-tapping rubber tree provided significantly highest averaged latex yield per tapping yet the average cumulative latex yield was no significant difference compared with the traditional tapping system S/3 2d/3. • Girth increment had no significant difference among treatments. • An averaged sucrose distribution in the trunk level of none stimulation treatments was high to very high Suc; however, it was medium Suc in the stimulation treatments. • Pi distribution in the trunk level showed medium to high values.
II Antioxidants and anti-oxidative enzymes activity in response to oxidative stress in <i>Hevea brasiliensis</i>			
1	Sreelatha et al. (2003)	Antioxidant defence systems and drought tolerance in <i>Hevea brasiliensis</i>	<ul style="list-style-type: none"> • The glutathione content in the bark of high-yielding trees was higher than in low-yielding trees. • High-yielding trees showed higher peroxidase (PX), ascorbate peroxidase

No.	Reference	Title	Finding
			(APX), and lower phenol oxidase activities (POD) than low-yielding trees.
			<ul style="list-style-type: none"> • The antioxidant activity can be a marker for drought tolerance.
2	Mai et al. (2009)	Effect of chilling on photosynthesis and antioxidant enzymes in <i>Hevea brasiliensis</i> Muell. Arg.	<ul style="list-style-type: none"> • The net photosynthetic rate (P_n) decreased dramatically at 1 h, and Fv/Fm was slightly affected. NPQ reached its maximal level between 4 and 7 h. Lipid peroxidation and membrane lysis were observed between 48 and 96 h. • Activities of antioxidant enzymes increased, along with the induction of antioxidant gene expression. • The plants were capable to recover (net photosynthetic rate, photochemical efficiency, antioxidant enzymes activities) when placed back to 28°C showing that PB260 can withstand long-term chilling.
3	Chen et al. (2009)	Inhibition of monoterpene biosynthesis accelerates oxidative stress and leads to enhancement of antioxidant defense in leaves of rubber trees (<i>Hevea brasiliensis</i>)	<ul style="list-style-type: none"> • The results showed that monoterpene biosynthesis in leaves was considerably inhibited by fosmidomycin. • The activities of antioxidant enzymes (SOD, CAT, POD, APX, and GR) were enhanced in the fosmidomycin. • The contents of antioxidant metabolites (ascorbate and glutathione) were also elevated in the fosmidomycin.
4	Sanier et al. (2013)	Influence of water deficit on the physiological and biochemical parameters of <i>in vitro</i> plants from <i>Hevea brasiliensis</i> clone PB 260	<ul style="list-style-type: none"> • Proline and ascorbate contents increased from the same level of water deficit. Antioxidant enzyme activities were more stable than GR activity, which dramatically increased in line with the water deficit to peak at 0.12 UI/mg for a Fraction of Transpirable Soil Water (FTSW) of 0.2. • When plants were rehydrated, enzyme activities decreased. The accumulation of proline for the lowest FTSW suggested water stress. Antioxidant systems did not play a predominant role in this genotype.
5	Krishnakumar et al. (2014)	Plant hormones and oxidative stress in <i>Hevea brasiliensis</i>	<ul style="list-style-type: none"> • Both ethylene (ET) and abscisic acid (ABA) concentrations were high in trees that are exposed to oxidative stress. • The amount of PX in the bark tissue was inadequate under a certain physiological state of the tree (TPD). • Accumulation of MDA was evidenced as the peroxidative damage that occurred in the bark tissues of stressed trees. The tissue

No.	Reference	Title	Finding
			CN level was very high in stressed trees due to the low levels of CN scavenging enzyme, β -CAS.
6	Martin et al. (2015)	Engineering rubber plants with high antioxidant capacity	<ul style="list-style-type: none"> • <i>Hevea</i> transgenic lines overexpressing the <i>HbCuZnSOD</i> gene showed dramatically affected the somatic embryogenesis process and plant development. • The TS5T8An and TS 12T3A82 lines overexpress <i>HbCuZnSOD</i> and <i>EcGSHJ</i>, respectively. • TS5T8An plants showed a higher SOD activity and proline content than control plants. • TS 12T3A82 plants had higher thiols and proline contents as well as a higher glutathione reductase activity in leaves.
7	Zou et al. (2015)	Genome-wide identification of rubber tree (<i>Hevea brasiliensis</i> Muell. Arg.) aquaporin genes and their response to ethephon stimulation in the laticifer, a rubber-producing tissue	<ul style="list-style-type: none"> • 51 full-length aquaporin (AQP) genes were identified from the rubber tree genome. • The phylogenetic analysis assigned these AQPs to five subfamilies, including 15 plasma membrane intrinsic proteins (PIPs), 17 tonoplast intrinsic proteins (TIPs), 9 NOD26-like intrinsic proteins (NIPs), 4 small basic intrinsic proteins (SIPs), and 6 X intrinsic proteins (XIPs).
8	Carvalho et al. (2018)	Effect of nitrogen source and oxygen deficiency on carbon metabolism and antioxidant system of rubber tree plants (<i>Hevea</i> spp.)	<ul style="list-style-type: none"> • P_n, stomatal conductance (g_s), and transpiration rate (E) in stressed plants decreased significantly when compared to the control plants, regardless of nitrogen source. • Under hypoxia, plants treated with NO_3 showed significantly higher CO_2 assimilation, overcoming the treatment with NH_4. • Under hypoxia, NO_3-treated plants also showed higher antioxidant system efficiency.
9	Fipriani et al. (2019)	Physiological response of TPD-affected rubber plant to growth regulator and antioxidant treatments	<ul style="list-style-type: none"> • The difference in antioxidant activities in the bark and latex rubber plants change according to environmental factors, clones, and physiological disorders (TPD). • Increasing the value of RSH can reduce the value of protein and POD activity. • Some parameters that can be used as early indicators for detecting TPD are bark POD activity and thiols latex value

No.	Reference	Title	Finding
10	Santos et al. (2021)	Performance of <i>Hevea brasiliensis</i> under drought conditions on osmoregulation and antioxidant activity through evaluation of vacuolar invertase and reducing sugars	<ul style="list-style-type: none"> • The greater tolerances of clones RRIM600 and GT1 to drought were related to greater vacuolar invertase (VINV) activity in their leaves • Glycine betaine accumulations were related to osmoprotection and to reducing oxidative damage (lipidic peroxidation). The antioxidant enzyme (SOD and APX) activities increased in the leaves in response to water deficit conditions.
11	Yuan et al. (2021)	The variation of reactive oxygen species scavenging enzymes and related gene expressions during the occurrence and recovery of rubber tree tapping panel dryness	<ul style="list-style-type: none"> • The activities of these ROS scavenging enzymes and expressions of the related genes changed during the TPD occurrence and recovery. • The genes were also regulated by H₂O₂ and various hormone treatments, such as methyl jasmonate, salicylic acid, abscisic acid, and so on. • The ROS scavenging genes and their corresponding enzymes are involved in responses to TPD, H₂O₂, and different hormones in rubber trees.
12	Lubis et al. (2021)	Activities of ascorbic peroxidase (Apx) and tannin clones PB 260 on tapping panel dryness (TPD)	<ul style="list-style-type: none"> • The result showed the application of AsA 150 ppm can increase the production of latex and tannins in rubber plants that are exposed to TPD, but reduced the activity of APX.
III Antioxidants variability and QTL analysis in <i>Hevea brasiliensis</i>			
1	Chaendaekattu and Mydin (2014)	Inheritance of tapping panel dryness in a full-sib population of <i>Hevea brasiliensis</i>	<ul style="list-style-type: none"> • The study showed high narrow-sense heritability ($h^2 = 0.50$) for TPD. • Progenies from hybridization between clones with very low TPD incidences (e.g. RR11 33) exhibited very low TPD incidences; progenies from hybridization between clones with very high TPD (e.g. PB 235) possessed more TPD incidences. • The study indicated that TPD may be governed by heritable gene action.
2	Syafaah et al. (2015)	Keragaman sifat pertumbuhan, fisiologi, dan daya hasil progeni karet (<i>Hevea brasiliensis</i> Muell. Arg.) hasil persilangan antara klon PB 260 dan RRIC 100	<ul style="list-style-type: none"> • The progenies of the cross between PB 260 and RRIC 100 had a high degree of variability in yield, Suc, Pi, and number of latex vessels • Stem girth, bark thickness, and RSH had low variability.
3	Conson et al. (2018)	High-resolution genetic map and QTL analysis	<ul style="list-style-type: none"> • Linkage group 4 showed the greatest number of QTLs (7), with phenotypically

No.	Reference	Title	Finding
		of growth-related traits of <i>Hevea brasiliensis</i> cultivated under suboptimal temperature and humidity conditions	explained values varying from 7.67 to 14.07 %. <ul style="list-style-type: none"> • A total of 53 significant effects for stem diameter were observed, and these effects were mostly related to additivity in the GT1 clone.
4	Oktavia et al. (2017)	Genetic diversity and population structure of IRRDB 1981 and Wickham rubber germplasm based on EST-SSR	<ul style="list-style-type: none"> • The evaluated SSR primers yielded polymorphic markers. • The USSR 268 primer pairs yielded the most informative markers while HBE 280 primers generated the lowest ones. • Results of the genetic diversity analysis supported that the PN'81 population belonged to a single large natural population of rubber trees while the Wickham clones belonged to a different group than that of PN'81.
5	Rosa et al. (2018)	QTL detection for growth and latex production in a full-sib rubber tree population cultivated under suboptimal climate conditions	<ul style="list-style-type: none"> • A total of 86 distinct QTLs were identified. • Latex production (eleven QTLs). Most important QTL in linkage group 16. • Girth increment (nine QTLs).
6	Xia et al. (2018)	An ultra-high-density map allowed for mapping QTL and candidate genes controlling dry latex yield in rubber tree	<ul style="list-style-type: none"> • The whole-genome QTL scanning for dry latex yield trait was performed and seventeen repQTLs were obtained, among which qDFY-10 and qDFY-18-4 could explain up to 38.3 % and 33.3 % phenotypic variability, respectively. • Numerous highly-promising QTL candidate genes were identified and then verified significantly associated with the DLY trait, including some first found in rubber trees, such as <i>thioredoxin h</i>, <i>plastin-like protein</i>, <i>calmodulin-binding protein</i>, <i>cytochrome-c oxidase</i>, and <i>methylglutaconyl-CoA hydratase</i>.
7	An et al. (2019)	A high-density genetic map and QTL mapping on growth and latex yield-related traits in <i>Hevea brasiliensis</i> Müll.Arg	<ul style="list-style-type: none"> • Eleven QTLs for stem growth and 12 QTLs for latex yield, distributed in 15 linkage groups (LGs), were detected, explaining 3.1 % – 18.4 % of the phenotypic variance. • Two QTLs, qSG-8-2 for stem growth and qLD- 8-3 for latex yield, were mapped within the same marker interval of LG 8 with a genetic distance of 3.0 cM.
8	Oktavia (2020)	Studi pendahuluan konstruksi peta tautan	<ul style="list-style-type: none"> • Three of the QTLs linked to the early growth of the girth rubber plant 22

No.	Reference	Title	Finding
		genetik dan identifikasi Quantutative Trait Loci yang terpaut dengan pertumbuhan awal tanaman karet	months after planting were successfully identified on LG 14 with LOD 4.64, LG 13 with LOD 3.5, and LG 1 with LOD 3. <ul style="list-style-type: none"> • QTL linked to the girth from 16 to 22 months old was in LG 14 with LOD 4.1. • The position of the loci of genes or a DNA sequence that affected the growth of girth was indicated by the TAs2185 marker where the marker gives an 11.7 % effect on the growth of the girth rubber plant.
9	Zain et al. (2021)	Antioxidant activity, total phenolic and flavonoid content from leaves and seed extracts of <i>Hevea brasiliensis</i> clone	<ul style="list-style-type: none"> • Each clone gives a different result on extraction yield, antioxidant activity, total phenolic, and flavonoid content. • The highest amount for total phenolic and flavonoid contents were clone RRIM 2020 (0.020) and RRIM 3001 (0.200).
10	Hou et al. (2021)	Construction and integration of genetic map and quantitative trait locus (QTL) mapping of yield-related traits	<ul style="list-style-type: none"> • 3 QTLs related to dry rubber content. 3 QTLs related to dry rubber yield/tree. 4 QTLs related to the unit secant length dry rubber yield • 5 QTLs related to the number of conducting phloem laticifer. 4 QTLs related to the number of laticifers

E. Research Outcomes

The expected outcomes of this research include:

1. Better knowledge of the variation of antioxidants content in *H. brasiliensis* latex.
2. A better understanding of the evolution of RSH content in response to different stress of tapping, ethephon stimulation, and TPD occurrence.
3. Working model of physiological and antioxidant dynamics, including RSH, in different stress types and duration.
4. Recommendation for better interpretation of RSH value in relation to harvesting stress and TPD occurrence.
5. New parameter of oxidative stress level based on antioxidants status.

6. Significant antioxidant parameters for further genetic analysis.

7. Heritability and QTLs of latex main antioxidants traits.

This research leads to a better understanding of antioxidant regulation in response to harvesting stress and provides other potential redox parameters in latex. It improves the LD method and better estimation of the level of oxidative stress and antioxidant capacity, which is pivotal for maintaining high rubber yield. It also provides valuable information related to QTLs associated with key latex antioxidant properties, which are useful for TPD-tolerant breeding programs.

II. LITERATURE STUDY

A. Biology of *Hevea brasiliensis*

1. History of rubber cultivation

Hevea brasiliensis is believed to have originated in the upper stream of the Amazon River in South America, currently covering parts of Brazil, Columbia, Guyana, Ecuador, and Venezuela. Columbus described the first rubber plant in 1496. Natural rubber began to be considered a beneficial material when Charles Macintosh discovered the waterproof ability of this material in 1818, followed by the discovery of the mastication method by Thomas Hancock in the 1820s. The milestone in the widespread usage of rubber was the discovery of vulcanization by Charles Goodyear in 1839 (Encyclopaedia Britannica, 2019). The early industrial utilization of rubber was limited to the manufacture of waterproof fabrics and shoes. The development of the vulcanization method and the discovery of pneumatic tires by John Boyd Dunlop in 1888 led to rubber demand booming in the late 19th century (Jones and Allen, 1992).

The high demand and limited supply of natural rubber provoked some countries to cultivate and spread the *H. brasiliensis* species. The milestone of the rubber plant spread was when Charles Marie de La Condamine sent some rubber samples for the first time to Europe in 1736 (Baker, 1996). In 1876, Sir Henry Wickham collected 70,000 rubber seeds from the Amazon basin in South America and sent them to the Kew Botanical Gardens in London. Out of 2,899 germinated seeds, 1,911 seedlings were sent to the Sri Lanka Botanical Gardens in 1877, and 90 % of them survived. The seedlings were further distributed to the British colonies, including Malaysia,

Singapore, and India (Simmons, 2008; Priyadarshan, 2011). The first rubber species introduced to Indonesia was believed to originate from Singapore and was planted in the Bogor Botanical Gardens. At the beginning of the 20th century, the establishment of rubber plantations on a large scale was carried out in tropical regions of southeast Asia as recorded by the Swiss Botanist Jacques Huber (Castro et al., 2009).

At present, most rubber is not cultivated in its origin. Pinizzotto (2019) reported that approximately 12 million hectares of rubber are cultivated all over the world, 91 % are in Southeast Asia, South Asia, and Southern China, the remaining 6 % are in tropical Africa, and the other 3 % in tropical America. Malaysian Rubber Board (MRB, 2019) reported that in 2018 natural rubber production reached 13.9 million tons while consumption reached 13.9 million tons. International Rubber Study Group (IRSG) reported that Thailand and Indonesia accounted for 61 % of total world natural rubber production, the remaining 29 % was produced by Vietnam, Malaysia, China, India and Ivory Coast, the rest 10 % were from minor producing countries.

Most of the rubber productions are absorbed into automotive tire industries (\pm 71.4 %), while the rest are processed for broad-spectrum products, including medical equipment, construction, and daily household tools. China solely consumes 40 % of the world's natural rubber; other main consumer countries are the European Union (9 %), India (8 %), and the United States of America (7 %) (Pinizzotto, 2019).

2. Biology of *Hevea brasiliensis*

Hevea brasiliensis is a tree-shaped perennial species (Figure 2.1.). According to the United States Department of Agriculture (USDA, 2019), a rubber plant is included in the Euphorbiaceae family with the following classification:

Kingdom: Plantae

Sub-kingdom: Tracheobionta

Super-division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Ordo: Euphorbiales

Family: Euphorbiaceae

Genus: *Hevea* Aubl.

Species: *Hevea brasiliensis* Müll. Arg.

Under optimum conditions, a rubber tree can grow up to 40 m in height (Wychherley, 1992). The rubber tree is a monoecious plant with lateral inflorescences bearing both staminate and pistillate. *Hevea* normally attains the reproductive stage in 4 – 5 years. Staminate flowers have ten anthers arranged over a staminal column in rows of five each. The pistillate flower consists of a three-celled ovary with three short sessile stigmas. For each pistillate flower, about 70 staminate flowers are found. The pollen grains are triangular, measuring about 35–40 µm on each side with a sticky surface (Priyadarshan, 2011). The flowering of the rubber tree is induced by high solar

radiation intensity; thus, the flowering season varied based on geographical location (Yeang, 2007).

The mature fruit is a large three-lobed capsule, 3–5 cm in diameter, having a woody endocarp and a thin, leathery mesocarp, and contains three seeds. The fruit reaches its maximum size in about 80–90 days, the endocarp becomes woody in about 110 days and the endosperm matures in about 130 days (Priyadarshan, 2011). *Hevea* seed behaves as a recalcitrant with no dormancy undergoes drying when physiologically ripe, and loses its viability in a short time (Shuib et al., 2018).



Figure 2.1. Rubber plantation in Indonesia

The rubber tree leaves grow in a rhythmic-flush growth pattern. Whorls of leaves in successive cycles are formed annually. An elongation of the internodes separates the whorls from each other. Each whorl consists of 9–15 leaves configuring a $3/8$ spiral phyllotaxy pattern. The entire canopy is shed at the beginning of the dry season and the refoliation starts at the beginning of the rainy season (Lieberei, 2007). This seasonal

phenomenon influences the annual yield pattern. The development of rubber leaves is distinguished into four phases i.e. A, B, C, and D. In phase A, the leaves are very small with red-brown colour. In phase B, the leaves are bright green-brown with soft-textured. These early stages of rubber leaf development are susceptible to foliar pathogens attack. The leaves grow to near full size and are sturdy in phase C. The mature leaves (phase D) have fully developed with a position close to horizontal to the petiole. Research by Miguel et al. (2007) reported that net photosynthesis in phase B was still negative, then started to be positive in phase C.

The most economically valuable part of *H. brasiliensis* is latex, a milky colloidal compound that can be processed into various products. Naturally, the roles of latex in plants are suggested to be part of the defense system from herbivores and pathogens (Konno, 2011; Ramos et al., 2019). Latex is synthesised in a special cell named laticifer. This cell is possessed by over 20,000 species of 40 families; in monocots and dicots, 22 families ($\pm 12,500$ species) are reported to have laticifer cells (Lewinsohn, 1991). Among all species, *H. brasiliensis* is the most economical latex producer.

3. The laticifer cell

Latex is an exudate that is synthesized and kept in the elongated cells called laticifer, which is distributed in roots, stems, petioles, and leaves (Konno, 2011). A laticifer can be found as a single cell or a row of anastomosed cells. When it is composed of a single cell, it is classified as a non-articulated laticifer (Figure 2.2A.); while when it is formed by a row of cells, it is classified as an articulated laticifer

(Figure 2.2B.) (Rudall, 1987; Prado and Demarco, 2018). Non-articulated laticifers arise from a single cell in the embryo, elongate, and grow along with plant development via tip growth; while articulated laticifers are derived from rows of neighbouring cells in which the walls between individual cells become perforated or completely removed, resulting in the laticifer tubing structures (Castelblanque et al., 2017).

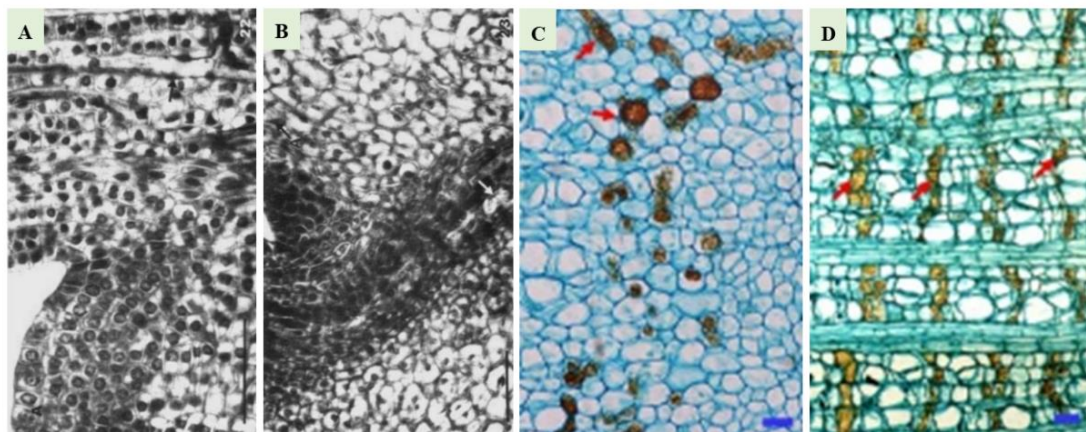


Figure 2.2. Non-articulated laticifers of *Baloghia lucida* (A) and articulated laticifer of *Cnidoscolus chayamansa* (B), scale bar = 50 μm . Primary (C) and secondary (D) laticifers of *Hevea brasiliensis* (Rudall, 1987; Tan et al., 2017)

Laticifer also can be distinguished based on the stages of laticifer growth into primary and secondary laticifers. The primary laticifers (Figure 2.2C.) are differentiated from the meristematic zones, while the secondary laticifers (Figure 2.2D.) are differentiated from the vascular cambium (Tan et al., 2017). Castelblanque et al. (2017) suggested that the capacity to produce latex has evolved more than once since the latex is restricted to a small number of plant families with various taxonomic relationships. In the tapping activity of *H. brasiliensis*, the latex is extracted from articulated secondary laticifer that is present in rings parallel to the vascular cambium

in the secondary phloem of the trunk. Its number, controlled by genetic and environmental factors, determines the yield potency of rubber clones (Hao and Wu, 2000). A study by Tian and collaborators revealed that tapping activity induced the dehydration of the wounded bark and triggered a burst of H₂O₂, ABA, and jasmonates. Furthermore, the team suggested that jasmonate, ABA, and cytokinin played an essential role in the differentiation and division of vascular cambial in *H. brasiliensis* (Tian et al., 2015).

Laticifers contain organelles including the nucleus, ribosome, mitochondrion, cytoplasm, plastid, vacuole, lutoid, and Frey-Wyssling complex (Dickenson, 1969). In some species, latex is stored in the vacuole, the most occupying organelle in laticifer (Konno, 2011); in *H. brasiliensis*, latex is essentially the cytoplasmic content. The laticifers are the recipient sink cells for metabolites.

4. The lutoid

Lutoid is a latex lysosomal vacuome, 1 – 5 µm in diameter and enveloped by a unit of bio-membrane. It occupied up to 20 % of latex volume. Lutoid contains various cations (K⁺, Mg²⁺, Ca²⁺, and Cu²⁺), soluble phosphates, sucrose, citrate, malate, and amino acids. The lutoid membrane possesses an Mg²⁺-dependent adenosine triphosphatase (ATPase) that leads to an influx of protons and vacuolar acidification (d'Auzac et al., 1982).

An enzymatic activity of NAD(P)H oxidase occurred at the level of the lutoidic tonoplast. Sreelatha et al. (2016) found a clonal variation of lutoid membrane activity. It induced superoxide anions (O_2^-) production leading to membrane alteration (Chrestin et al., 1984). The lutoid membrane lysis allows for the lutoid bursting, a liberation of coagulating factors resulting in latex coagulation, and ceases the latex flow. Lutoid stability is an important parameter for latex flow and consequently yield. Bursting index (BI), a portion of acid phosphatase before and after an induced bursting, is one of the physiological parameters for assessing lutoid stability.

B. Rubber Biosynthesis and Latex Harvesting System

1. Rubber biosynthesis pathways

Fresh latex consists of 30 % - 45 % rubber particles where take place *cis*-polyisoprene polymerisation (Figure 2.3.). Rubber biosynthesis occurs through the isoprenoid pathway via isopentenyl diphosphate (IDP) as a common intermediate (Figure 2.4.). The IDP is synthesized using the mevalonate (MVA) pathway, the well-known pathway for isoprenoid biosynthesis in the cytosol, and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastid (Chow et al., 2012).

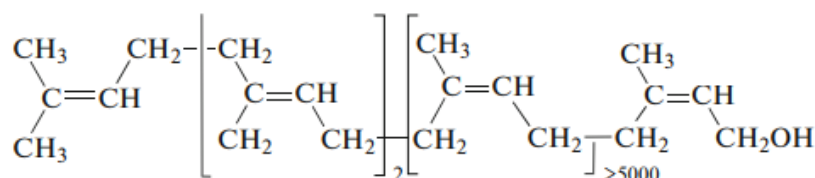


Figure 2.3. Chemical structure of natural rubber (*cis*-polyisoprene) (Amerik et al., 2018)

The MVA pathway occurs in the cytosol, which is supported by the evidence of radiolabelled intermediates incorporation such as MVA and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) into rubber. The MEP pathway occurs in the plastid with the glyceraldehyde-3-phosphate (GA3P), 1-deoxy-D-xylulose-5-phosphate (DXP), MEP, and 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) as the intermediates. The expression of 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) in *Hevea* latex suggests that the MEP pathway exists in the laticifer. MVA and MEP pathways produce IDP, the primary precursor for rubber biosynthesis in the cytosol. Rubber particle synthesis involves dimethyl allyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) as intermediates.

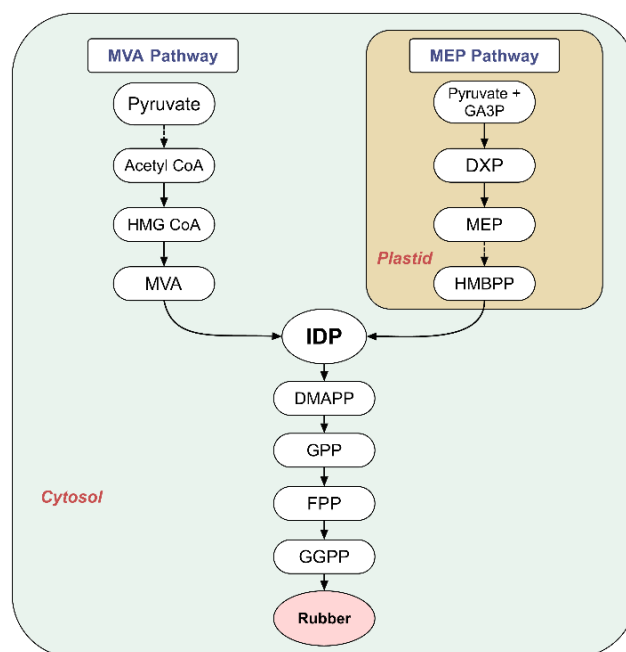


Figure 2.4. Biosynthesis of *Hevea* rubber through the mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways (Chow et al., 2012; Yamashita and Takahashi, 2020)

2. Rubber biosynthesis-related genes

According to Tang et al. (2016), there are 84 genes from 20 gene families were involved in rubber biosynthesis, including 18 genes in the MVA pathway, 22 in the MEP pathway, 15 for initiator synthesis in the cytosol, and 39 for ‘rubber elongation’ on rubber particles. Six enzymes in the MEP pathway are involved in the conversion of pyruvate into IDP, they are encoded by 12 genes. In the MVA pathway, six enzymes are involved in acetyl-CoA into IDP conversion, encoded by 18 genes. The cis-polyisoprene particle synthesis from the IDP requires four enzymes, IDP isomerase (IDPI), geranyl diphosphate synthase (GPS), geranylgeranyl diphosphate synthase (GGPS), and farnesyl diphosphate synthase (FPS). Ambily et al. (2018) suggested that the variation of enzyme activities related to rubber biosynthesis might result in the clonal variation of latex yield. The expression of sucrose transporter gene (*HbSUT3*), hydroxymethyl glutaryl-CoA synthase (*hmgs*), HMG-CoA (*hmgr*), and mevalonate diphosphate decarboxylase (*MVD*) were higher in high-yielding clones than low-yielding clones.

Sucrose is the main precursor for rubber biosynthesis. The sucrose transporter gene *HbSUT3* is involved in sugar loading and responsible for raw material availability (Ambily et al., 2018; Zhang et al., 2021). The enzymes 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is involved in the early steps of rubber biosynthesis by forming HMG-CoA. Three genes encoding HMGR in *Hevea* were identified namely, *hmg1*, *hmg2*, and *hmg3*. The *hmg1* gene is a key gene in the rubber biosynthesis process

(Venkatachalam et al., 2009). The rubber elongation factor (REF) and small rubber particle protein (SRPP) are involved in the final polymerization of natural rubber biosynthesis. Both REF and SRPP were positively correlated with the latex yield (Klaewklad et al., 2016) and encoded by 18 genes in *Hevea* latex named *SRPP1* to *10* and *REF1* to *8* (Tang et al., 2016). Latex is harvested through tapping activities, slicing the stem bark of the plant, to allow the latex to flow out and be collected.

3. Latex harvesting practice

Among the latex-producing species, *H. brasiliensis* is the most economical for its large quantities of latex production, long lifespan, and easiness of latex extraction and processing. Tapping activity is generally carried out on the trunk due to the easiness and high-yield extraction (Figure 2.5.). Open tapping generally starts when the girth has reached 45 - 50 cm, measured at 100 cm from the collar. In good environmental conditions and agronomic practices, it can be achieved in 4.5 – 5 years, yet it may be longer in a sub-optimal region. The tapping activity is usually carried out in the early morning to run into high cell turgor pressure in order to acquire a high latex flow rate (Yeang, 2005; An et al., 2014).



Figure 2.5. The tapping activity of the young rubber tree

In daily practice, the term tapping intensity indicates how intensive the tapping is applied. The parameter was previously popular for comparing tapping systems. However, it has very little application nowadays due to the increase in tapping system complexity adopted by rubber plantations (Vijayakumar et al., 2009). The intensity of tapping includes the length of tapping cut, frequency of tapping, and stimulant application. The higher intensity tends to give a higher yield, yet it is more susceptible to physiological fatigue. Several tapping techniques have been tested, including downward tapping (Figure 2.6A.), upward tapping (Obouayeba et al., 2008), double cut (Figure 2.6B.), alternate tapping, and alternate double-tapping system (Nhean et al., 2017). The tapping techniques aim to utilize the bark optimally and to obtain a high yield throughout the plant's lifespan. Although the longer the tapping cut tends to produce the higher latex per tree, it has consequences of a shortened lifespan and an increase in plant stress due to a larger wounding area (Duan et al., 2010; Junaidi et al., 2019).



Figure 2.6. The downward (A) and double-cut (B) tapping system of *Hevea brasiliensis*

The alternate tapping frequency (d2) was originally a common practice as *H. brasiliensis* needs about 48 hours to regenerate latex. However, harvesting rubber requires a lot of labour, on average, the tapper is able to complete 400-600 trees per task, depending on the tapping method (de Jonge and Westgarth, 1962). High labour costs forced to lower the frequency of tapping every three days (d3) while the decrease in annual cumulative yield due to reduced tapping days is compensated by the latex stimulant application usually using ethephon (2-chloroethyl phosphoric acid).

On a practical basis, many factors are considered, including commodity price, labour availability, crop conditions, phenology, disease, agro-climate, and plant physiological status. The current rubber agribusiness condition requires a high yield with an efficient cost of production (labour productivity) due to low rubber prices in recent years. Thus, the low-frequency tapping systems with d4, d5, or d6 tapping interval accompanied by high concentration and frequency of stimulation is likely preferable (Sainoi et al., 2017; Zaw et al., 2017; Atminingsih et al., 2019).

4. Ethephon Stimulation

Ethephon, an ethylene releaser compound, is usually applied in a certain amount and frequency in order to gain a high yield. It can use water or crude palm oil (CPO) as the carrier and applied on the renewing bark close to the tapping cut (panel application), on scraped bark to be tapped (bark application, on the tapping cut over tree lace (lace application), or on the tapping cut after removal of tree lace (groove application).

Several previous studies reported the effect of using ethephon (latex stimulant) on the increase in latex yield. The increase in the yield by ethephon stimulation is mainly due to the prolonged latex flow and the activation of latex regeneration between two tappings (Coupe and Chrestin, 1989; Zhu and Zhang, 2009). Plugging index (PI), a ratio of initial latex flow rate to total latex upon tapping day, is a parameter to estimate latex flow duration; high PI indicates short latex flow. The increase in the yield in stimulated trees is usually accompanied by a low PI, for example, in Yang et al. (2017). Concerning rubber biosynthesis, Liu et al. (2016) reported the rapid acceleration of IPP synthesis in stimulated trees through the glycolytic pathway.

Other physico-chemical and biochemical effects of ethephon stimulation included the extension of the drainage area, increasing sugar loading, latex regeneration intensification, increasing protein synthesis, and increasing total adenine nucleotide pools (Coupe and Chrestin, 1989). The increase in stimulant concentration significantly reduced the dry rubber content (DRC) and volatile fatty acid content, as well as other latex properties such as latex viscosity and serum magnesium content. The lower serum

magnesium content was suggested to be involved in the increase in latex flow time (Attanayake et al, 2018). However, the effect of ethephon stimulation on the yield depends on the clones, tapping system, and dosage (Dian et al., 2016; Dian et al., 2017). Rubber production and TPD incidence are positively affected by increasing ethephon dosage (da Cruz et al., 2017).

Ethylene significantly improves rubber latex rubber yield by inhibiting some enzymes related to rubber particle aggregation leading to latex flow prolongation (Wang et al., 2015). It also increases the generation of small rubber particles (SRPs), regulates protein accumulation, and activates enzyme activities (Wang et al., 2019). Tang et al. (2016) identified 509 differentially expressed genes (DEGs) in latex that respond to ethephon treatment. The primary ethylene signalling genes include *ETR*, *CTR1*, *EIN2*, and *EIN3/EIL1*. The *ETR*, *EIN2*, and *EIN3/EIL1* genes enhanced expression after ethephon treatment, indicating active ethylene signalling in laticifers. Seven downregulated *ERF* genes, four belong to repressor subgroups (*ERF-IIa* and *ERF-VIIa*) of ethylene response. The depression of their expression is expected to be beneficial for activating downstream ethylene response. Montoro et al. (2018) identify the DEGs involved in ethylene response from stimulated compared to unstimulated trees (Table 2.1.). The most responsive gene families include Ethylene Response Factors (*ERF*), Auxin Response Factor (*ARF*), *WRKY*, Homeobox superfamily, and *bHLH*.

Table 2.1. Number of differentially expressed genes (DEGs) in response to ethephon stimulation from the comparison of stimulated and control (non-stimulated) trees (Montoro et al., 2018)

Gene ontology		Number of DEGs	
Level 1	Level 2	Over expressed	Under expressed
Biological process	biological regulation	187	135
	cellular process	740	472
	localization	111	99
	metabolic process	768	571
	regulation of the biological process	182	129
	response to stimulus	0	86
	single-organism process	305	317
Cellular component	cell part	355	158
	cell	355	158
	membrane part	72	95
	membrane	121	149
	macromolecular complex	209	48
	organelle	257	99
	organelle part	96	35
Molecular function	binding	912	768
	catalytic activity	589	583
	nucleic acid binding transcription factor activity	0	0
	transporter activity	0	0

A study by Nie et al. (2016) exhibited that transcript levels of 163 latex genes were significantly altered in ethylene-treated compared to non-treated trees (92 genes were up-regulated and 71 genes down-regulated). It is suggested that ethylene increases latex production by regulating the expression of many genes that are involved in laticifer water circulation, ROS production and scavenging, sugar metabolism, and actin cytoskeleton assembly, rather than by regulating genes that express directly in latex regeneration or rubber biosynthesis. Zhu and Zhang (2009) noted that ethylene is involved in Glutamine synthetase (GS), Manganese superoxide dismutase (MnSOD), and HMG-CoA synthase activities. Putranto et al. (2015) reported that transcripts of *HbERF-IXc4*, *HbERF-IXc5*, and *HbERF-IXc6* were dramatically accumulated by combining wounding, methyl jasmonate, and ethephon treatments. *HbERF-IXc5* is an

ortholog to *ERF1*, which is at the crosstalk of the ethylene and jasmonate pathway. Jasmonate production is induced by mechanical wounding. This suggests a strong interaction between the effect of ethephon stimulation and tapping. The ethephon stimulation significantly diluted the corresponding latex fractions due to the up-regulation of *HbPIP1*, *HbPIP2;1*, and *HbPIP2;3* genes (An et al., 2015). Ethephon, an ethylene releaser, is used widely in rubber plantations to obtain a high yield. However, over-stimulation was reported to induce TPD, a physiological disorder in which latex does not flow out upon tapping, leading to a detrimental yield drop (Putranto, et al., 2015).

C. Tapping Panel Dryness (TPD)

1. TPD symptom

Latex will flow upon tapping in a healthy tree (Figure 2.7A.). A TPD-affected tree may exhibit a partial dry cut where part of the tapping cut releases latex while another part is dry, followed by colour alteration to brownish (Figure 2.7B.). In this condition, latex may not flow unless tapping was conducted deeper near the cambium. To a severe extent, the tapping cut would completely dry. Some observers reported that the symptom oftentimes was followed by vertical cracks (Mathew et al., 2006). Unfortunately, the TPD occurrence is usually reported when it has been affecting the yield. Early observations revealed that no pathogens were involved in this disorder (Ramachandran et al., 2006).



Figure 2.7. A. Tapping cut of a healthy tree is flowing latex upon tapping. B. A TPD-affected tree is exhibiting partial dry cut and brownish bark

The TPD occurrence was reported to affect 12 % – 50 % of the population of rubber-mature plants; although the percentage might depend on the type of clone, age, and place of observation. Yield losses incurred in the form of a decrease of 10 % – 40 % depended on the level of occurrence in the field (Chen et al., 2002; Abraham et al., 2005).

This physiological syndrome is predicted to increase in the future as farmers tend to adopt high-production clones (Deka et al., 2006), which are more susceptible to TPD, and the high tapping intensity, including ethephon stimulation (Putranto et al., 2015; Dian et al., 2017) in order to gain adequate yield in current low price condition. The increase is also fuelled by climate change, which drives a broad spectrum of environmental stress (Cassia et al., 2018; Golbon, et al., 2018).

2. Two types of TPD

The *in situ* coagulation of rubber particles leads to the cessation of latex flow. It is induced by luteoid bursting, which releases rubber coagulant factors, such as *Hevein*. The increasing activity of enzyme NAD(P)H oxidase, also called respiratory burst oxidase homologs (RBOHs), in luteoid membrane is associated with the production of ROS, leading to membrane damage, luteoid bursting and consequent *in situ* latex coagulation (Wititsuwannakul et al., 2008). This symptom, named the ROS-induced TPD, shows reversible and the latex yield can recover after resting the tress for a while (d'Auzac et al., 1982; Lacrotte et al., 1997; Putranto et al., 2015).

In case the oxidative stress and the ROS accumulation continue, the plant responds through the extreme defence mechanism of programmed cell death (PCD). This mechanism includes DNA degradation, cell degeneration, and the release of glucosyl cyanide. The loss of turgor pressure allows the over-growth of surrounding cells to invade the affected laticifers cell through outgrowths of the associated parenchyma cells (tylosoids). Full latex coagulation was observed in severe TPD-affected trees, followed by the browning of phloem tissues or even a vertical cracking on the outer layer of the bark (Mathew et al., 2006; Lacrotte et al., 1997; de Faÿ et al., 2010; de Faÿ, 2011). This type of TPD was confirmed to have identical symptoms to bark necrosis (de Faÿ, 2011). The syndrome tends to spread and seems to be barely irreversible. Since its symptoms are easily recognized by the browning colour of the bark, the healthy barks are usually green to light yellow, it is called Brown Bast or brown bast-TPD (BB-TPD) (Lacrotte et al., 1997; Putranto et al., 2015).

3. Reactive oxygen species (ROS) related to TPD

Reactive oxygen species (ROS) are oxygen compounds that have one or more unpaired electrons, including singlet oxygen ($^1\text{O}_2$), radical superoxide ($\text{O}_2^{\bullet -}$), hydroxyl radical (OH^\bullet), hydrogen peroxide (H_2O_2), and ozone (O_3), which are triggered by environmental stress. Activation of O_2 into ROS may occur by absorption of sufficient energy to reverse the spin on one of the unpaired electrons and stepwise monovalent reduction mechanism. An energy transfer to O_2 leads to the formation of $^1\text{O}_2$; while a stepwise monovalent reduction of O_2 results in the formation of $\text{O}_2^{\bullet -}$. The $\text{O}_2^{\bullet -}$ is the primary ROS formed in the cell which is dismutated to H_2O_2 by mitochondrial enzyme superoxide dismutase (MnSOD) (Sharma et al., 2012). Hydrogen peroxide generates hydroxyl radicals through the Fenton reaction. A superoxide molecule and a hydrogen peroxide molecule could interact to produce reactive hydroxyl radicals (Patel et al., 2018).

Zhang and collaborators predicted the subcellular localization of ROS production based on a genome-wide analysis (Zhang et al., 2019). The result suggested that ROS production occurs in the plastid (in form of H_2O_2 , $\text{O}_2^{\bullet -}$, and *o-quinone*), lutoid (in form of H_2O_2), peroxisome (in form of H_2O_2), nucleus (in form of H_2O_2), mitochondria (in form of H_2O_2 and $\text{O}_2^{\bullet -}$), and cytosol (in form of H_2O_2 and $\text{O}_2^{\bullet -}$). The NADPH oxidase activity, at the outer surface of the lutoid membrane in contact with cytosol, is considered the main source of ROS and is mostly encoded by the *Rboh2* gene which is more expressed under ethephon stimulation (Zhang et al., 2019). The ROS compounds

are very reactive and generate oxidative damage to lipids, proteins, mitochondria, and nucleic acids (Slimen et al., 2014). They are also involved in plant development and signal transduction system (Zhu, 2016; Mhamdi and Van Breusegem, 2018).

4. Genomic and transcriptomic analyses of TPD

Previous studies indicated that TPD is triggered by the over-accumulation of ROS at the cellular level. ROS accumulation alters the redox homeostasis that can be detected through the transcription of redox-related genes. The complex TPD syndrome may be induced by plant response to mechanical wounding and exogenous ethylene.

Li et al. (2010) suggested that the genes related to TPD, investigated in latex, involved ROS production and scavenging, ubiquitin-proteasome, PCD, and rubber biosynthesis-related genes. In addition, Li et al. (2016) reported that 5652 genes were up-regulated and 2485 genes were down-regulated in the bark of TPD-affected trees compared to healthy trees. Liu et al. (2015) suggested that in the bark of severely TPD-affected trees, the expression of genes associated with latex biosynthesis and jasmonate synthesis was significantly inhibited. Chen et al. (2002) reported that *HbMyb1* transcription factor expression is significantly decreased in barks of TPD trees. Liu et al. (2019) noted that the *HbMCI* expression in the bark correlated with TPD severity through the PCD mechanism. A deep RNA sequencing of TPD-affected trees by Montoro et al. (2018) showed a significant decrease in regulated transcription factors in severe TPD trees compared to slight TPD trees (Table 2.2.). The under/over-

expressed transcription factor ratio increased, highlighting a general decrease in plant signalling.

A study by Tian et al. (2015) suggested that mechanical wounding did not significantly induce the burst of H₂O₂ and jasmonates. However, Broekaert et al., (1990) reported that wounding resulted in the accumulation of hevein transcripts in leaves, stems, and latex. The hevein protein is involved in rubber particle agglutination, which is the onset of TPD. Duan et al. (2010) reported that the mechanical wounding increased the *HbSAUR*, *HbGP*, and *HbACR* genes expression, while noting the decrease in expression of *HbMAPK*, *HbBTF3b*, *HbCAS1*, *HbLTPP*, *HbPLD*, *HbCO11*, and *HbDef*.

Table 2.2. Number of main transcription factor families regulated in response to the severity of ethephon-induced TPD (Montoro et al., 2018)

Transcription factor family	Ethephon vs control		Total
	Up-regulated	Down-regulated	
Ethylene Response Factors (ERF)	1	2	3
Auxin Response Factor (ARF)	0	3	3
WRKY	4	0	4
Homeobox	0	5	5
bHLH	0	2	2
MYB	1	5	6
MADSbox	0	1	1
TCP	0	2	2
GATA	0	1	1
MYC	0	1	1
HSF	0	2	2
NAC	0	1	1
GRAS	2	0	2
Other	3	14	17
Total	11	39	50

This physiological syndrome is predicted to increase in the future as the farmer tends to adopt high-production clones (Deka et al., 2006), which are more susceptible to TPD, and the high tapping intensity including ethephon stimulation (Putranto et al., 2015; Dian et al., 2017) in order to gain adequate yield in current low price condition. The increase is also fueled by climate change, which drives a broad spectrum of environmental stress (Cassia et al., 2018; Golbon, et al., 2018). However, the recommended clones in Indonesia IRR 112 and IRR 118 showed a moderately high metabolism and were highly tolerant to TPD.

D. Latex Diagnosis (LD)

1. LD parameters

The physiological characteristics of *H. brasiliensis* latex have been widely studied and reviewed in the book 'Physiology of Rubber Tree Latex' (d'Auzac et al., 1989). The latex flow and *in-situ* regeneration between two tappings are the main limiting factors in latex production. The latex flow is associated with TSC, BI, RSH, and Mg²⁺ contents; while latex regeneration is linked with TSC, Suc, latex pH, Pi, Mg²⁺, RSH, and redox potential (RP) (Jacob et al., 1989). These parameters were the basis of LD, a method for assessing rubber plant physiological conditions according to some physiological parameter status (Jacob et al., 1995). At the beginning of the LD method was introduced, the parameters used included TSC which reflects biological activity, Suc which is a major element in cell functioning and raw material for rubber synthesis, Pi which shows the cellular energy level, RSH which indicates protective ability agents

against cellular senescence, latex pH which is directly related to metabolic activity, and BI which reflects luteoids stability. Nowadays, only four parameters i.e. Suc, Pi, RSH, and TSC are used in LD assessment.

2. LD parameters evolution in relation to the harvesting system

Gohet et al. (2019) summarized the effect of stimulation frequency on the LD parameter using 12 years of observation data from some rubber plantations in Africa (Figure 2.8.). The increase in ethephon stimulation would decrease the TSC in a linear or hyperbolic pattern (Figure 2.8A.). The decrease in TSC is due to enhanced water importation (An et al., 2015); although in some cases a transient increase might occur due to the effect of increasing rubber biosynthesis. The increase in ethephon stimulation generally results in a hyperbolic decrease in Suc due to its consumption in response to latex regeneration (Figure 2.8B.). Sucrose provides the energy for cellular activities and is the source of carbon for rubber particle biosynthesis.

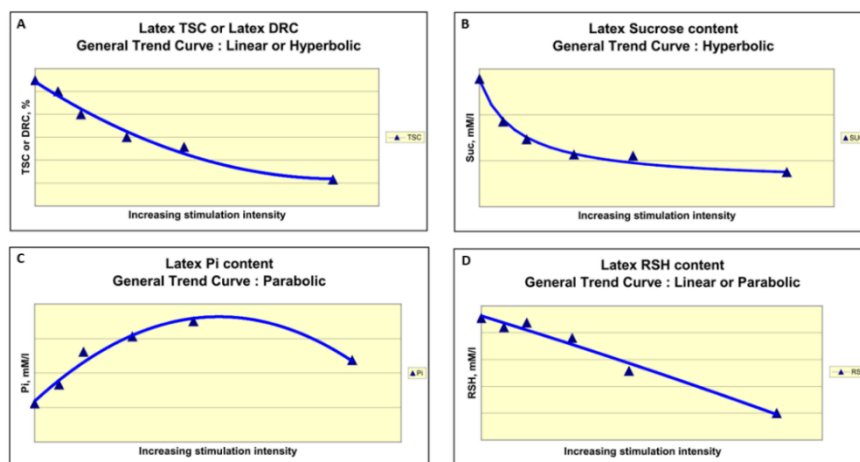


Figure 2.8. LD parameters evolution in response to ethephon stimulation (Gohet et al., (2019)

The evolution of Pi generally exhibited an inverse parabolic pattern (Figure 2.8C.). The increase in Pi content was found in the lower stimulation intensities as a result of the metabolic activation. However, for high stimulation frequency, the Pi would drop significantly, indicating overexploitation. It is usually associated with the yield drop. The increase in ethephon stimulation intensity would decrease the RSH content due to rapid consumption in response to the increase in ROS production during latex regeneration (Figure 2.8D.). A transient increase may occur as a result of the RSH biosynthesis activation.

3. LD application in rubber research and plantations

LD technology was introduced in the late 1980s and has been widely adopted in natural rubber-producing countries. To understand the application of LD in rubber research and plantations, a meta-analysis study was performed. The literature was collected from online and printed journals, and conference proceedings, and some papers were also obtained directly from authors. From 413 papers that were collected, 158 papers that assessed at least one of four main LD parameters (TSC or DRC, Suc, Pi, and RSH) were used for meta-analysis. The papers were grouped by the discipline of agronomy, physiology, and breeding (Figure 2.9.). The information extracted from each paper included authors, publication year, country, discipline, genotype, tapping system, assessed physiological parameters, and LD values.

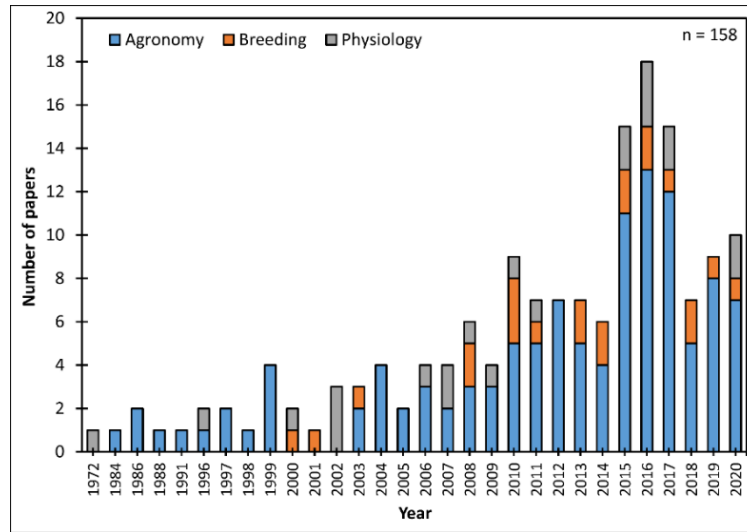


Figure 2.9. Number of papers by year published and discipline used for statistical analysis

In agronomy works, latex diagnosis application was mainly for yield optimisation, for example, the use of double cutting (Chantuma et al., 2011) and low-frequency tapping accompanied by stimulation modification (Sainoi et al., 2017). In physiology studies, LD parameters were used for physiological mechanism understanding, environmental stress adaptation, and tapping stress mitigation including the seasonal variation of physiological parameters (Nguyen et al., 2016), environmental stress impacts (Kudaligama et al., 2017), and the production of both free radicals and scavengers due to the TPD extent (Tistama et al., 2019). In breeding works, LD was involved in growth and yield-related traits investigation (Vinod et al., 2000), progenies evaluation (Phearun et al., 2016), and performance assessment of rubber clones (Cardoso et al., 2014). Interestingly, the meta-analysis reveals that the adoption of the LD method in physiology and breeding works (reported in 22 papers for each discipline) is lower than in the agronomy discipline (assessed in 114 papers).

The papers in this work came from various countries, disciplines, plant age, clone, and tapping systems so the LD values showed a high variation (coefficient variation of 19.6 % - 72.9 %) (Figure 2.10.). Among the four main parameters used in latex diagnosis, Suc and Pi were the most used and interpreted in scientific papers.

Of the 158 scientific papers included in our database, Suc was the most frequently analysed parameter (96.2 %) followed by Pi (89.9 %), RSH (82.9 %), and TSC (73.4 %) (Figure 2.11.). Following a thoughtful reading, we identified the authors' interpretation of each LD parameter such as the effect of genotypes, harvesting systems, or environmental conditions. The Suc was interpreted in 63.8 % of the papers, Pi 60.6 %, TSC 56.0 %, and RSH 42.8 %. According to our assessment, Suc was the most frequently interpreted parameter, while the RSH was poorly interpreted.

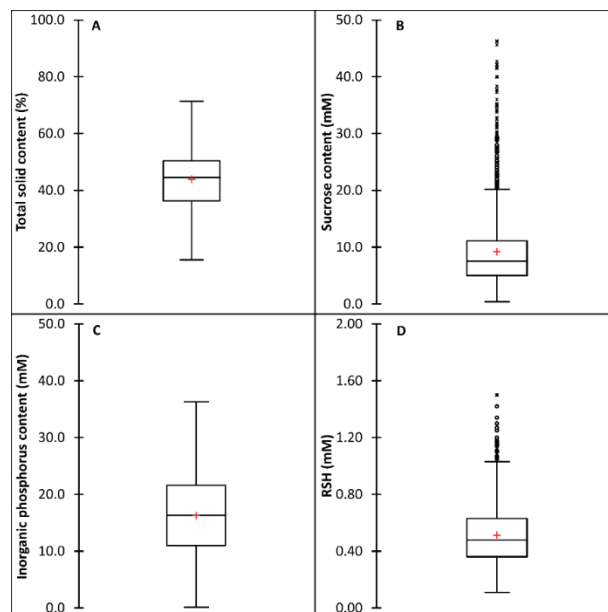


Figure 2.10. Box plot representative of the variability of four main latex physiological parameters, A. total solid content, B. Sucrose content, C. Inorganic phosphate content, and D. Thiols (RSH) content

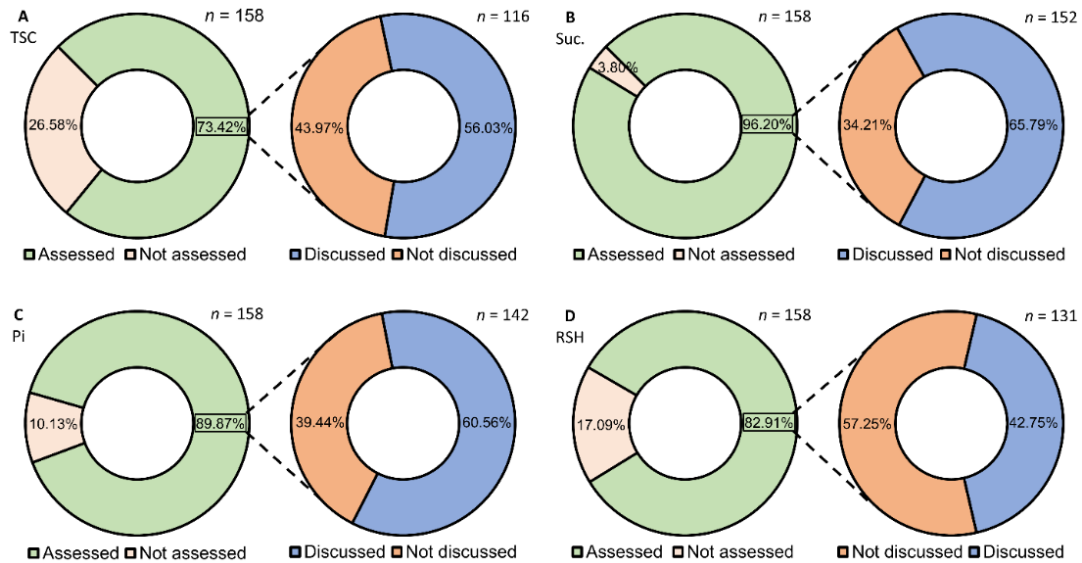


Figure 2.11. The proportion of the paper listed and interpreted by the authors for TSC (A), Suc (B), Pi (C), and RSH (D)

According to our dataset, RSH is still low-interpreted both in plantation monitoring and research disciplines. RSH was interpreted only in 42.8 % of the 131 papers. Some RSH values were not associated with the level of stress leading the authors to limit the interpretation of their data. For example, in (Herlinawati and Kuswanhadi, 2012) and (She et al., 2013), the content of the RSH remained constant whilst the stress increased. Some papers mentioned that Suc and Pi might be better stress indicators (Gohet et al., 2019; Tistama et al., 2019). The low interpreting of RSH questions whether this parameter is relevant in LD and calls for the development of a new parameter.

E. Latex Antioxidants and Scavenging Enzymes

The concentration of ROS is maintained by the scavenging system involving enzymes, antioxidants, and phytohormones (Dar et al., 2017; Wani et al., 2016). In latex, the three main antioxidants are RSH (consisting of glutathione and cysteine), AsA (vitamin C isomer), and tocotrienol (vitamin E isomer). In addition, several enzymes are involved in neutralizing radical compounds including SOD, CAT, APX, glutathione peroxidase (GPX), and glutathione S-transferase (GST).

1. Ascorbate biosynthesis

Ascorbate is synthesized through the L-galactose pathway with the D-glucose being the main precursor (Figure 2.12.). The process produces nine intermediate products i.e. D-glucose-6-P, D-fructose-6-P, D-mannose-6-p, D-mannose-1-p, GDP-D-mannose, GDP-l-galactose, L-galactose-1-p, L-galactose, and L-galactono lactone before finally converted to AsA. The reaction requires ten enzymes namely hexokinase (HK), Glucose-6-phosphate isomerase (PGI), mannose-6-phosphate isomerase (PMI), phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GMP), GDP-mannose-3',5'-epimerase (GME), GDP-L-galactose phosphorylase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH), and L-galactono-1,4-lactone dehydrogenase (GLDH). Other biosynthetic pathways, through galacturonate and glucuronate, were proposed but the regulation of these pathways is still not deeply known (Smirnoff, 2000; Bulley and Laing, 2016).

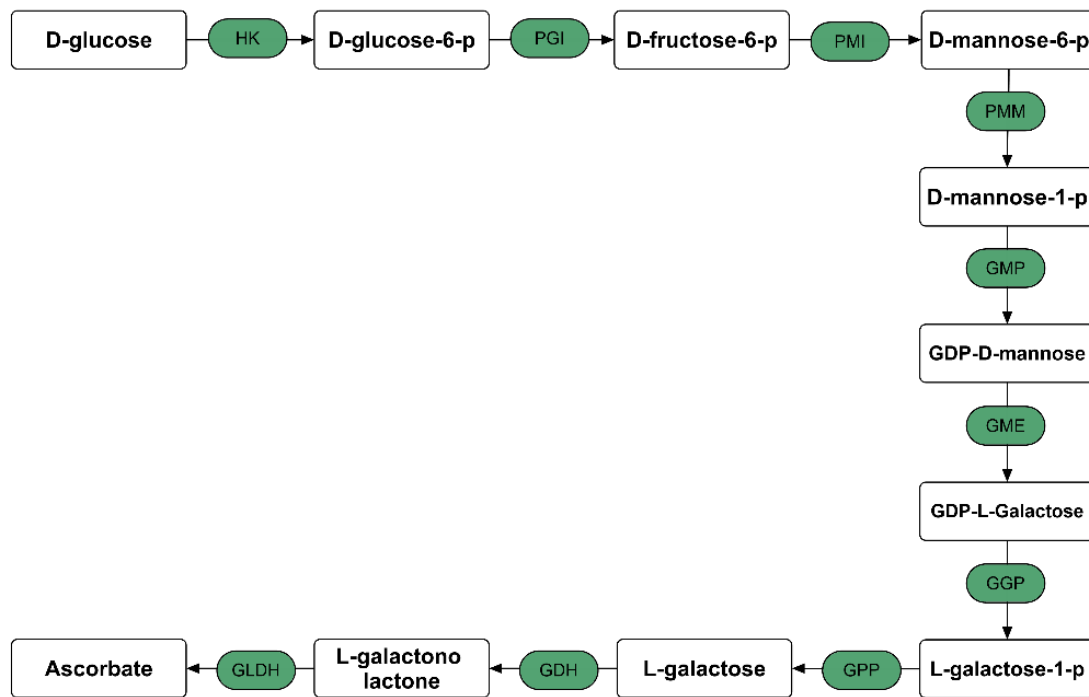


Figure 2.12. Ascorbate biosynthesis in plants through the L-galactose pathway (Bulley and Laing, 2016)

2. Glutathione biosynthesis

Glutamate, cysteine, and glycine are required for GSH biosynthesis (Figure 2.13.). The synthesis commences with the reaction of γ -carboxyl glutamine and α -amino group cysteine to generate γ -glutamylcysteine 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 (Noctor and Foyer, 1998; Hasanuzzaman et al., 2017).

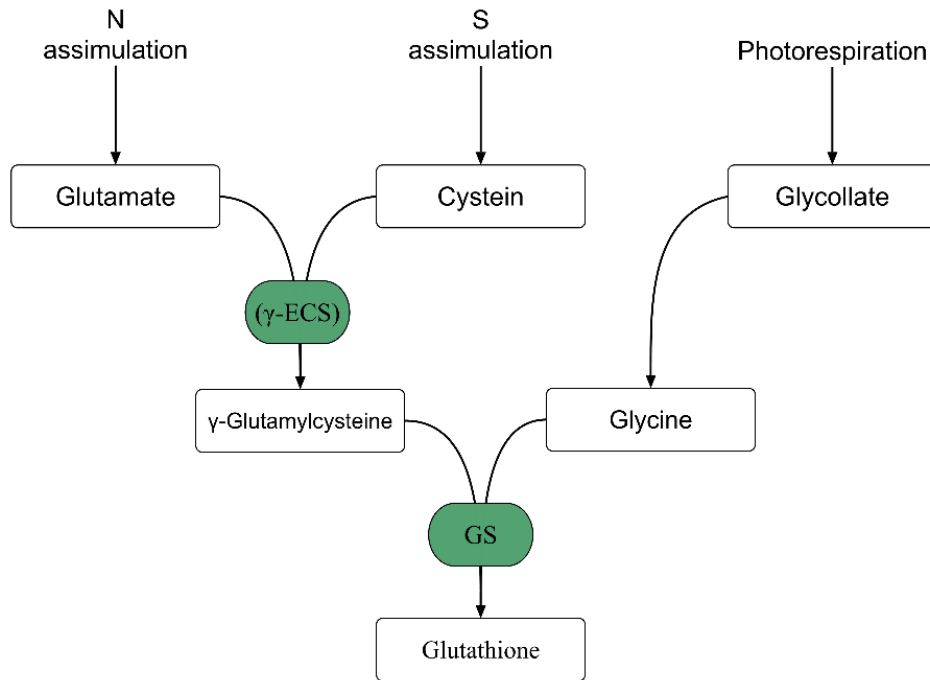


Figure 2.13. Glutathione biosynthesis from its constituent amino acids. γ -ECS = γ -glutamylcysteine synthase, GS = Glutamine Synthetase (Noctor and Foyer (1998))

3. Tocotrienol biosynthesis

Tocotrienol is a lipid-soluble antioxidant mostly located in the plastid membrane (Munné-Bosch and Alegre, 2002). The tocotrienol isomers in *H. brasiliensis* latex include α -tocotrienol, γ -tocotrienol and δ -tocotrienol; the γ -tocotrienol is the most abundant variant up to 8 % of the total lipids (Dunphy et al., 1965). The 2-methyl-6-phytyl-1,4-benzoquinol, synthesized through isoprenoid and shikimate pathways, is the main precursor for tocotrienol biosynthesis (Figure 2.14.).

Homogentisate phytyltransferase (VTE2) catalysed the conversion of phytyldiphosphate and homogentisate into 2-methyl-6-phytyl-1,4-benzoquinol. The 2-

methyl-6-phytyl-1,4-benzoquinol methyltransferase (VTE3) is involved in the conversion of 2-methyl-6-phytyl-1,4-benzoquinol to 2,3-dimethyl-5-phytyl-1,4-benzoquinol followed by tocopherol cyclase (VTE1) and γ -tocopherol methyltransferase (VTE4) to converts γ -tocotrienol and α -tocotrienol. On the other side, VTE1 and VTE4 are involved in the conversion of 2-methyl-6-phytyl-1,4-benzoquinol to δ -tocotrienol and β -tocotrienol.

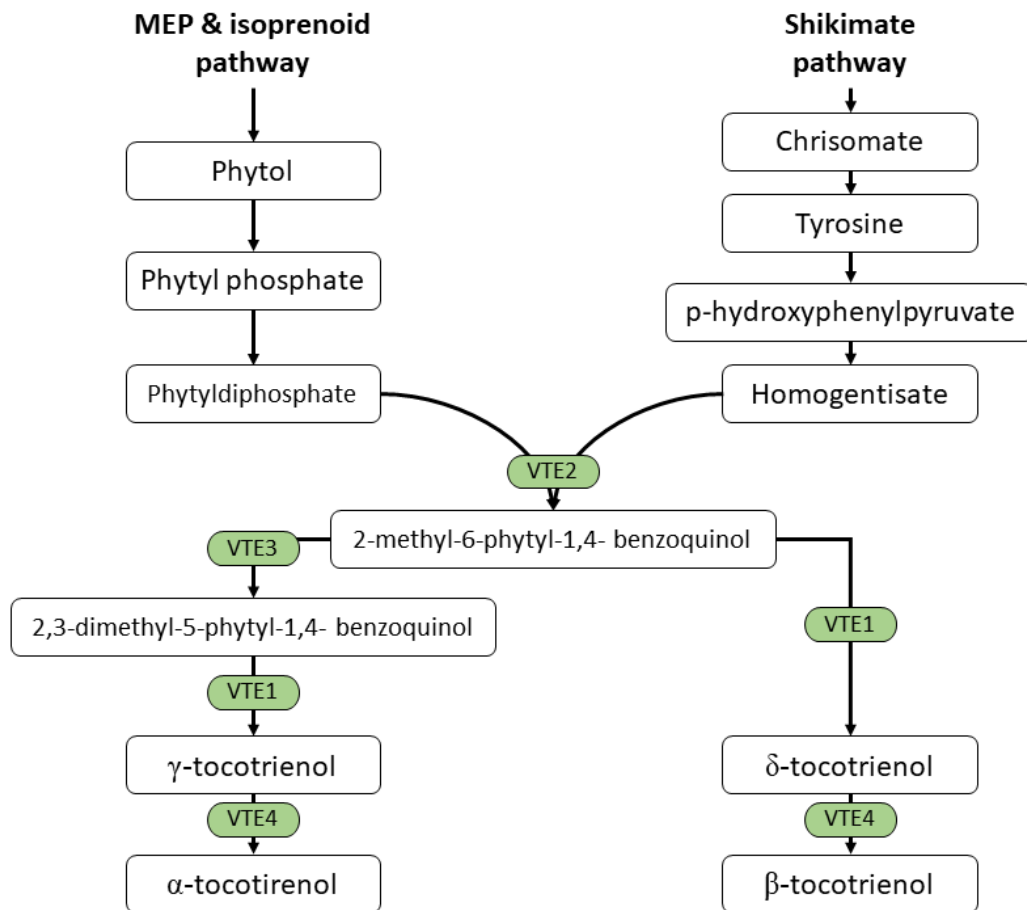


Figure 2.14. Tocotrienol biosynthesis pathway in the plant. VTE1 = tocopherol cyclase, VTE2 = Homogentisate phytoltransferase, VTE3 = 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase, VTE4 = γ -tocopherol methyltransferase (Hussain et al., 2013)

4. Redox mechanism in ROS scavenging process

Under the condition of low ROS concentration, APX and GPX are very active, whereas CAT, due to its low affinity, is active in high ROS conditions (Zhang et al., 2016). ROS production and scavenging, as well as antioxidant biosynthesis, occur through a redox reaction. CuZnSOD is the enzyme of defence against ROS by converting $O_2^{\bullet-}$ into H_2O_2 in the cytosol of laticifers. The detoxification process of H_2O_2 has generally involved the ascorbate and glutathione cycle (Figure 2.15.). The AsA is the reducing agent for detoxifying H_2O_2 to water, catalysed by APX. The process generates DHA which is recycled back to AsA using GSH as the reducing substrate in the presence of DHAR. The GSSG is produced in the process and recycled back to GSH by NADPH, catalysed by GR (Noctor and Foyer, 1998; Foyer and Noctor, 2011).

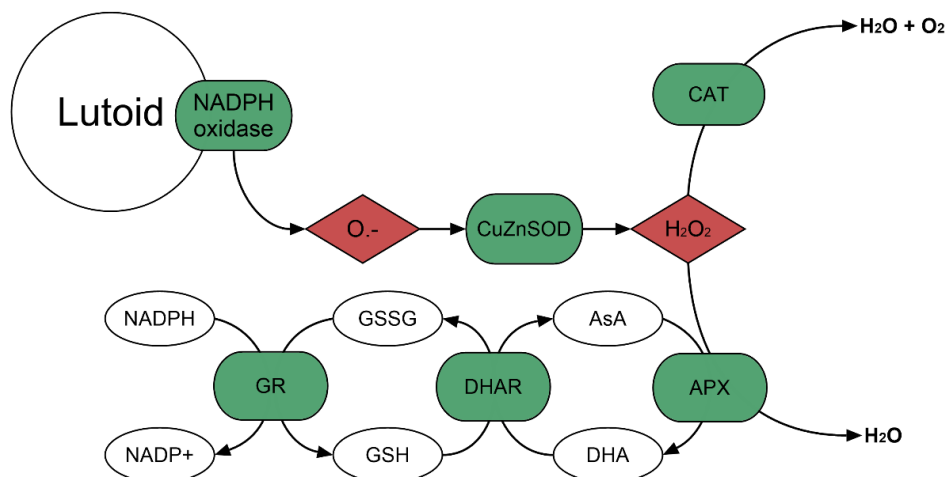


Figure 2.15. Scheme of ROS production and scavenging in the plant (Wititsuwannakul et al., 2008; Foyer and Noctor, 2011)

Genome analysis in *Hevea latex* by Zhang et al. (2019) suggested that the main redox-related genes involved in ROS production and scavenging system are ascorbate biosynthesis, glutathione biosynthesis, and tocotrienol biosynthesis. Some important expressed genes in latex include:

1. *Respiratory burst oxidase homolog 2 (Rboh2)* which encodes the NADPH oxidase
2. *Cu/Zn superoxide dismutase 1 (CuZnSOD1)* gene for maintaining the SOD activity
3. *Catalase 1 (Cat1)* gene which is related to the CAT activity
4. *Thioredoxin H5 (TrxH5)* which encodes thioredoxins
5. *Monodehydroascorbate reductase 2 (MDHAR2)*, *dehydroascorbate reductase 2 (DHAR2)*, and *glutathione reductase 1 and 2 (GR1 and GR2)* for regenerating the reduced ASA and GSH.
6. *Ascorbate peroxidase 1 and 5 (HbAPx1 and HbAPx5)* encode ascorbate peroxidase.
A study by Chao et al. (2015) showed that ethephon application disturbed the redox homeostasis in laticifer cells by down-regulating the expression of *HbAPX* genes.
7. *Glutathione S-transferase U1 and F1 (GSTU3 and GSTF1)* encode glutathione transferase
8. *Tocopherol cyclase (VTE1)* and *homogentisate phytyltransferase (VTE2)* which are involved in δ -tocotrienol biosynthesis
9. *Tocopherol cyclase (VTE1)*, *MPBQ/MSBQ methyltransferase 1 (VTE31)*, and *MPBQ/MSBQ methyltransferase 2 (VTE32)* which are involved in γ -tocotrienol biosynthesis

5. Variability of antioxidants in plant and *Hevea brasiliensis*

Studies on other plants revealed that the variability of antioxidant content could be detected in interspecific levels (Hale et al., 2008; Hanen et al., 2009) and intraspecific levels (Sarker et al., 2014; Mageney et al., 2016). Roy et al. (2017) suggested that the inherent genetic variation in endogenous antioxidants content in wheat (*Triticum aestivum* L.) is due to the variation in biosynthesis and utilization pathways; 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 (Sanoubar et al., 2016; Fatima et al., 2019).

In *H. brasiliensis*, several studies reported genotypic variation in antioxidants content and antioxidative enzymes activities; such as a variation in tocotrienols content (Nadarajah et al., 1971), RSH (Syafaah et al., 2015; Phearun et al., 2016), and enzymatic activities (SOD, APX, and CAT) (Santos et al., 2021). A variation was also reported between different tissue; for example, the RSH in TPD-affected trees was higher in bark than in latex (Fipriani et al., 2019; Tistama et al., 2019).

F. Theoretical Background

ROS-scavenging systems involve enzymes and antioxidants in laticifers (for review: Zhang et al., 2017). The main scavenging enzymes identified in *H. brasiliensis* latex are CAT, APX, GPX, GR, DHAR, MDHAR, GS, and glutamate-cysteine ligase (GCL). The main antioxidants are AsA, GSH, carotenoid, and vitamin E. ROS-

scavenging mechanism mainly relies on the ascorbate-glutathione cycle (Zhang et al., 2017). Two molecules of AsA are used to H_2O_2 to water in the presence of APX, generating two molecules MDHA, which are rapidly disproportionated to AsA and DHA, catalysed by MDHAR. DHA is recycled to AsA using GSH as the reducing substrate in the presence of DHAR. The GSSG is generated in this process and recycled to GSH by 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). AsA 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 oxidised to ascorbate by L-galactono-1,4-lactone dehydrogenase. Other biosynthetic pathways, through galacturonate and glucuronate, have been proposed but the regulation of these pathways is still not deeply known (Smirnoff, 2000; Bulley and Laing, 2016). GSH synthesis commences with a reaction of γ -carboxyl glutamine and α -amino group cysteine to generate 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 oxidised form of antioxidants indicates active oxidation due to the presence of stress, while the increase in 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 oxidised forms of antioxidants in response

to abiotic stress (Marok et al., 2013; Borisova et al., 2016). To our knowledge, these typical works have not been performed in *H. brasiliensis*, particularly in response to the harvesting stress. This approach may give a better explanation of stress levels in *H. brasiliensis* than assessing RSH solely.

The tolerance to harvesting and environmental stresses is likely to associate with a complex of genetic factors, including RSH. Several studies reported genotypic variation in antioxidants content and antioxidative enzyme activities in *H. brasiliensis* as well as variation in different tissues. These variations may be exploited for genetic analysis of antioxidant-related genes underlying QTL. Typical works of QTL mappings on the yield and growth-related traits have been performed in *H. brasiliensis* (An et al., 2019; Oktavia, 2020; Hou et al., 2021). A QTL mapping on latex antioxidant traits will be useful for identifying oxidative stress genetic markers.

G. Hypotheses

Based on the literature of the previous research related to the research questions, the hypotheses proposed are:

1. The antioxidant content in latex varies among rubber clones.
2. Antioxidant content, including RSH, alters in response to different stress of tapping, ethephon stimulation, and TPD occurrence.
3. The dynamic of physiological parameters and antioxidants, including RSH, under different stress types and duration, will give a better understanding of antioxidants regulation in different stress types and duration.

4. The RSH regulation may provide a better interpretation of RSH value in relation to harvesting stress and TPD occurrence.
5. The ratio of reduced to oxidised form of antioxidants may be a better estimator of stress level in *H. brasiliensis* latex than RSH solely.
6. A significant variation in some antioxidant parameters may be appropriate for further genetic analysis.
7. The segregating population from TPD susceptible and tolerant cross will provide the preliminary heritability and QTL data for latex antioxidants-related traits.

III. RESEARCH METHODOLOGY

A. Research Scope

This study observed the effect of harvesting, tapping, and ethephon stimulation, stress, and TPD on antioxidant levels and several physiological parameters in the latex of rubber trees. Although harvesting practices can also induce biotic stresses, such as fungi and insects, this study was limited to abiotic stress. Observations were focused on biochemical compounds contained in latex. Other organs, such as bark or leaf, were not the objects of the research. Parameters observed in this study included latex production (latex yield, IF, PI, and BI), LD parameters (TSC, Suc, Pi, and RSH), antioxidants (AsA, DHA, GSH, GSSG), and cofactors (NAD, NADH, NADP, and NADPH). Antioxidant enzyme activities were not assessed in this research.

B. Research Sites

The project was started in February 2019. Following preparation and a series of laboratory training in 2019 – 2020 at Universitas Gadjah Mada (UGM) and Centre de coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) Montpellier, field experiments were carried out from January to May 2021 at Sembawa Research Centre, Indonesian Rubber Research Institute (IRRI), Palembang, Indonesia (2°55'38''S, 104°32'19''E). The location is \pm 10 m above sea level with rainfall ranging from 154.48 – 435.80 mm/month with rainy days 18 – 25 days/month during the study. Research fields are receiving standard agronomical practices including fertilizing and weeding. Four field trials were established to study:

1). clonal variation of antioxidants in five recommended rubber clones, 2). the effect of tapping and ethephon stimulation on the yield, latex flow, and antioxidant parameters, 3). the effect of TPD occurrence on the yield, latex flow, and antioxidant parameters, and 4). phenotypic variability and genetic analysis of main antioxidant parameters.

C. Experimental Design and Planting Materials

If not otherwise indicated, healthy trees without any pathogenic infection and TPD throughout experiments were used. All experimental trees were tapped on basal virgin bark (panel B0). Detailed treatments are explained in each following trial.

1. Clonal variation of latex antioxidants in five recommended rubber clones

Clonal variation of latex antioxidants was performed in five recommended rubber clones selected based on latex metabolic level i.e. IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260. Clones IRR 118, BPM 24, and IRR 112 are moderate metabolic clones from Indonesia, derived from cross LCB 1320 \times FX 2784, GT 1 \times AVROS 1734, and IAN 873 \times RRIC 110 respectively (Adou et al., 2017; Darojat and Sayurandi, 2018). Clone RRIC 100 originated from Sri Lanka. The moderate metabolic clone came from crossing RRIC 52 \times PB 86 (Adou et al., 2017). PB 260 is a high metabolic clone, that originated from Malaysia as a result of crossing PB 5/51 \times PB 49 (Pulchérie et al., 2021). Sample trees were ten-year-old (5th year of tapping), planted in 6 \times 3 m of space, and tapped on the first virgin basal panel (B0-1) every three days with one application

of 2.5 % ethephon every month (harvesting system noted S/2 d3 ET2.5% 12/y). Three healthy trees, with no dry cut and homogeneous girth as possible, were selected as replications for each clone, except for PB 260 which consisted of four replicates. Latex sample collection for physiological and antioxidant analysis was performed three weeks after ethephon stimulation in May 2021.

2. Study of the effect of tapping and stimulation on the yield, latex flow, and antioxidants parameters

The effect of harvesting systems was carried out at different locations using ten-year-old trees of clones IRR 118 (relatively TPD-tolerant clone) and BPM 24 (TPD-sensitive clone). Prior to the experiment, all trees were under the S/2 d3 (without stimulation) tapping system in the B0-1 panel. Due to the workability to process the samples, 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 replicates for each treatment. The experiment was started in January 2021 and latex sample collection was performed in April 2021, four months after treatments were applied.

3. Study of the effect of TPD occurrence

Evaluation of the effect of TPD was conducted 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. The TPD occurrence was assessed by measuring

the Dry Cut Length (DCL). To analyse the TPD severity for subsequent data analysis, trees were divided into two groups, low DCL and high DCL, using the k-means clustering method. Latex samples were collected three weeks after ethephon stimulation to eliminate the stimulation effect. Latex sample collection was performed in May 2021.

4. Phenotypic variability and genetic analysis of main antioxidant parameters

This observation aimed to find significant antioxidant parameters for further genetic analysis. Phenotypic variability assessment was carried out on five-year-old rubber trees planted in 6 m × 3 m space. Eight recommended rubber clones assessed included AVROS 2037, GT 1, IRR 112, IRR 39, PB 260, PR 261, RRIC 100, and SP 217. These clones were the control clones of a segregating population. Five replications were used with each replication being the average of two individual trees.

The heritability and QTL analysis were applied to RSH, AsA, and DHA parameters. The quantification of AsA and DHA allowed for calculating the total AsA and ratio of AsA/DHA, which were also involved. The assessment was carried out on 103 progenies of PB 260 (TPD susceptible clone) × SP 217 (TPD tolerant clone). The data was the average of three biological replications. Latex samples were collected in April 2021.

D. Materials

1. Latex and serum

Latex was collected immediately after tapping until 10 minutes in a 15 ml plastic tube on ice. After the initial latex volume was recorded, 1 ml of latex was put in 9 ml of 2.5 % trichloroacetic acid (TCA) and kept on ice for Suc, Pi, and RSH quantifications. Other 100 μ l of latex, used for AsA, DHA, GSH, GSSG, NAD, and NADP assessment, was added to 900 μ l of 0.2N hydrochloric acid (HCl) supplemented with 1mM ethylenediaminetetraacetic acid (EDTA) and stored in liquid nitrogen prior to centrifugation. For total NAD and total NADP, 100 μ l of latex was added to 900 μ l of 0.2N sodium hydroxide (NaOH) supplemented with 1 mM EDTA and stored in liquid nitrogen. The remaining latex was kept on ice for TSC and BI measurement. The latex collection handling steps is presented in Figure 3.1.

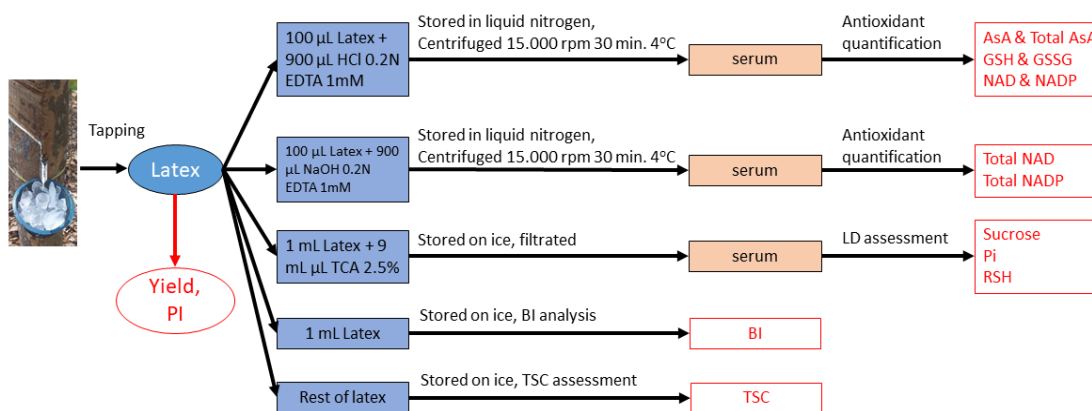


Figure 3.1. Latex collection and handling procedure for LD and antioxidants analysis

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 minutes on ice and 30 - 40 minutes in the refrigerator. The samples were then centrifuged at 15.000 rpm at 4 °C for 20 minutes. The clear serum was transferred into a new 1 ml tube and returned to liquid nitrogen before quantification.

2. Chemicals

Chemicals for ethephon stimulation and quantification of reduced and oxidised forms of antioxidants, cofactors, BI, and LD are presented in Table 3.1.

Table 3.1. Chemicals required for ethephon stimulation, quantification of ascorbate, glutathione, cofactors, bursting index, and latex diagnosis

No.	Name	Formula	Manufacturer	MW (g/mole)
Chemicals for ethephon stimulation				
1	Ethephon (Ethrel 10PA®)	C ₂ H ₆ ClO ₃ P	Bayer	144,50
2	Distilled water	H ₂ O	nd	nd
Chemicals for AsA and total AsA quantification				
1	2,2'-dipyridyl	C ₁₀ H ₈ N ₂	Sigma	156.19
2	Ascorbic acid (AsA)	C ₆ H ₈ O ₆	Sigma	176.12
3	Dithiothreitol (DTT)	C ₄ H ₁₀ O ₂ S ₂	Sigma	154.25
4	Ethanol absolute 99.8 %	C ₂ H ₆ O	Sigma	nd
5	Ethylenediaminetetraacetic acid (EDTA)	C ₁₀ H ₁₆ N ₂ O ₈	Fluka	372.00
6	Ferric chloride	FeCl ₃ ·6H ₂ O	Sigma	162.20
7	Hydrochloric acid	HCl	Panreac	36.46
8	N-ethylmaleimide (NEM)	C ₆ H ₇ NO ₂	Merck	125.13
9	Phosphoric acid	H ₃ PO ₄	Sigma	97.99
10	Potassium dihydrogen phosphate	KH ₂ PO ₄	Sigma	136.09
11	Sodium hydroxide	NaOH	Sigma	40.00
12	Trichloroacetic acid (TCA)	Cl ₃ CCOOH	Sigma	163.38
Chemicals for GSH and total GSH quantification				
1	5, 5'-Dithio-bis (DTNB)	C ₁₄ H ₈ N ₂ O ₈ S ₂	Sigma	396.34
2	Ethylenediaminetetraacetic acid (EDTA)	C ₁₀ H ₁₆ N ₂ O ₈	Fluka	292.24
3	Hydrochloric acid	HCl	Panreac	36.46
4	Oxidised glutathione (GSSG)	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	Boehringer	612.63
5	Reduced glutathione (GSH)	C ₁₀ H ₁₇ N ₃ O ₆ S	Sigma	307.32
6	Nicotinamide adenine phosphate (NADPH)	C ₂₁ H ₂₆ N ₇ O ₁₇ P ₃ Na ₄	Roche	883.4
7	Glutathione reductase (GR)	nd	Sigma	nd
8	Sodium hydrogen carbonate	NaHCO ₃	Labosi	84.01
9	Sodium hydroxide	NaOH	Sigma	40.00
10	Sodium dihydrogen phosphate, monobasic	NaH ₂ PO ₄ , H ₂ O	Sigma	137.99
11	Vinylpyridine	C ₇ H ₇ N	Sigma	105.14

No.	Name	Formula	Manufacturer	MW (g/mole)
Chemical for NAD, NADH, NADP, and NADPH quantification				
1	2,6-Dichlorophenolindophenol (DCPIP)	C ₁₂ H ₆ Cl ₂ NNaO ₂	Sigma	290.10
2	Alcohol dehydrogenase (ADH)	nd	Sigma	nd
3	Distilled water	H ₂ O	nd	nd
4	Ethanol 99 %	CH ₃ CH ₂ OH	Honeywell	46.07
5	Ethylenediaminetetraacetic acid (EDTA)	C ₁₀ H ₁₆ N ₂ O ₈	Fluka	292.25
6	HEPES	C ₈ H ₁₈ N ₂ O ₄ S	Sigma	238.30
7	Nicotinamide adenine dinucleotide (NAD)	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂ · xH ₂ O	Boehringer	717.4
8	Phenazine methosulfate (PMS)	C ₁₃ H ₁₁ N ₂ · CH ₃ SO ₄	Sigma	306.34
9	Glucose 6-phosphate	C ₆ H ₁₁ O ₈ PNa ₂	Roche	304.2
10	Glucose 6-phosphate dehydrogenase (G6PDH)	nd	Sigma	nd
11	Hepes	C ₈ H ₁₈ N ₂ O ₄ S	Sigma	238.30
12	Nicotinamide adenine dinucleotide phosphate (NADP)	C ₂₁ H ₂₆ N ₇ O ₁₇ P ₃ Na ₂	Roche	787.4
Chemicals for BI determination				
1	Acetic acid	CH ₃ COOH	PanReac	60.05
2	Distilled water	H ₂ O	nd	nd
3	Mannitol	C ₆ H ₁₄ O ₆	Sigma	182.17
4	Sodium acetate	C ₂ H ₃ O ₂ Na	Sigma	82.03
5	Sodium hydroxide	NaOH	Sigma	39.99
6	Sodium p-nitrophenyl phosphate (PNPP)	nd	Thermo	371.1
7	Trichloroacetic acid (TCA)	Cl ₃ CCOOH	Sigma	163.38
8	Triton X-100		Sigma	nd
Chemicals for LD				
1	Trichloroacetic acid (TCA)	Cl ₃ CCOOH	Sigma	163.38
2	Tris	H ₂ CN(CH ₂ OH) ₃	Merck	121.14
3	Ethylenediaminetetraacetic acid (EDTA)	C ₁₀ H ₁₆ N ₂ O ₈	Fluka	292.24
4	5, 5'-Dithio-bis (DTNB)	C ₁₄ H ₈ N ₂ O ₈ S ₂	Sigma	396.34
5	Reduced glutathione (GSH)	C ₁₀ H ₁₇ N ₃ O ₆ S	Sigma	307.32
6	Amonium heptamolibdat	(NH ₄) ₆ Mo ₇ O ₂₄	Sigma	1,163.9
7	Sulphuric acid	H ₂ SO ₄	Sigma	98.08
8	Ferrous sulfate	FeSO ₄ 7H ₂ O	Sigma	278.01
9	Potassium phosphate	KH ₂ PO ₄	Sigma	136.09
10	Anthrone	C ₁₄ H ₁₀ O	Sigma	194.23

E. Equipment

The Hermle Z 216 centrifuge (HERMLE Labortechnik GmbH, Wehingen-Germany) was used for serum extraction. A series of micropipettes (Eppendorf SE, Hamburg-Germany) was used for chemical preparation and multi-channel pipettes (Thermo Fisher Scientific, Waltham-USA) were used for LD and antioxidant

quantification. For AsA and total AsA determination, incubation was performed in the Memmert U30 Oven (Memmert GmbH, Büchenbach-Germany). LD and antioxidants were determined using the SPECTROstar Nano spectrometer (BMG Labtech, Ortenberg, Germany).

F. Methods

Observed parameters associated with latex production included latex yield, IF, PI, and BI. For latex diagnosis, TSC, Suc, Pi, and RSH were determined. For antioxidants, both the reduced (AsA and GSH) and oxidised forms (DHA and GSSG) were analysed. Cofactors associated with antioxidants regeneration were assessed including NAD, NADH, NADP, and NADPH.

1. Yield calculation

The yield was defined as dry rubber produced per tree per cm of the cut length (g/t/cm). The values were derived from the total weight of fresh latex multiplied by the DRC and divided by the cut length of the associated tree. The DRC was estimated at 90 % of TSC for these trials.

2. Initial latex flow (IF) and plugging index (PI)

To obtain sufficient volume for all parameters, latex was collected in a 15 ml plastic tube until 10 minutes after tapping. The IF was the average latex volume per minute in the first 10 minutes after tapping. The PI was calculated by dividing the IF

by the total latex volume according to Pakianathan et al. (1989). The total volume was measured at noon from each tree.

3. Bursting index (BI)

The BI is the ratio of free acid phosphatase (FAP) to total acid phosphatase (TAP). The FAP indicated the content of acid phosphatase before luteoid bursting, using Triton X100; while TAP is acid phosphatase after luteoid bursting. The FAP substrate was prepared by mixing 50 ml of 0.8M pH 5.0 sodium p-nitrophenyl phosphate (PNPP) with 200 ml of 0.6 M mannitol and 145 ml of distilled water, while the TAP substrate was prepared by mixing 50 ml of 0.8M pH 5.0 PNPP with 100 ml 0.5 % Triton X-100 and 145 ml distilled water. These substrates were used to establish standards and samples of FAP and TAP.

Standard FAP consisted of 2.9 ml of FAP substrate added by 1 ml of 2N TCA and 0.1 ml of fresh latex of the associated sample. After 10 minutes of incubation, the solution was filtered. A similar procedure for standard TAP was applied using a TAP substrate. For sample FAP and TAP preparations, the latex was put first into the corresponding substrate then TCA was added 10 minutes later to stop the enzymatic process. For the absorbance measurement, 1 ml of the FAP or TAP solutions were added with 1 ml of 1N NaOH and made up to 10 ml with distilled water. Absorbance measurement was performed in 96 wells plates using SPECTROstar Nano spectrometer (BMG Labtech, Ortenberg, Germany) at 400 nm with 200 μ l of standard/sample per channel. The FAP and TAP are absorbances different between the

sample and standard for the associated sample, while BI is the ratio of FAP to TAP in percentage (Thepchalerm et al., 2015).

4. Total solid content (TSC)

TSC quantification was performed by pouring 5.0 ± 0.2 g fresh latex on the aluminum disk and then oven-dried at a stable temperature of 100 °C for 3 hours. The TSC was the result of the dry weight divided by the fresh weight of each sample.

5. Sucrose content (Suc)

The measurement of the Suc protocol was adopted from Dische (1962). The 150 μ L of latex serum was added by 2.5 % TCA to reach 500 μ L of total volume, followed by the addition of 3 ml of 5 mM anthrone (dissolved in sulphuric acid (H_2SO_4)). Following 15 minutes of submergence in the boiling water, the absorbance was measured at 627 nm wavelength with 200 μ l of the solution in each well of the plate.

6. Inorganic phosphorus content (Pi)

The procedure for Pi quantification followed Taussky and Shorr (1953). Following four times dilution using 2.5 % TCA, 100 μ l diluted serum was added to Ferro-sulphate (FeSO_4) solution and incubated for 5 minutes at ambient temperature. FeSO_4 solution consisted of 5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 ml of 10 % sulpho-molybdenum, and diluted by distilled water up to 100 ml. The absorbance was measured at 750 nm wavelength.

7. Thiols content (RSH)

The procedure for RSH determination adopted the Ellman assay. The 100 μ l sample serum was added with 55 μ L of 10 mM 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and 100 μ l of 0.5 M Tris buffer. After 30 minutes of incubation at ambient temperature, the absorbance was measured at 412 nm.

8. Reduced ascorbate (AsA) and oxidised ascorbate (DHA) quantification

The AsA determination was performed by pipetting 50 μ l of HCl serum into 96 wells plate added by 50 μ l of 200 mM pH 7.5 potassium phosphate (KH_2PO_4) and incubated at ambient temperature for 30 minutes. After 20 μ l of distilled water addition, the sample was incubated once again at ambient temperature for 5 minutes and then reacted with 40 μ l of 10 % TCA, 50 μ l of 44 % phosphoric acid (H_3PO_4), 40 μ l of 65 mM 2,2'-dipyridyl, and 20 μ l of 110 mM ferric chloride (FeCl_3). Before absorbance measurement at 492 nm, the sample was incubated in the oven at 40 $^\circ\text{C}$ for one hour.

Total AsA was determined by adding 50 μ l HCl serum into 96 wells plate and adding 50 μ l of 200 mM pH 7.5 KH_2PO_4 and 10 μ l of 10 mM dithiothreitol (DTT). Following 30 minutes of incubation at ambient temperature, 10 μ l of 0.5 % n-ethylmaleimide (NEM) 0.5 % was added, and another 5 minutes of incubation at ambient temperature was applied. The sample was then reacted with 40 μ l of 10 % TCA, 50 μ l of 44 % H_3PO_4 , 40 of 65 mM μ l 2,2'-dipyridyl, and 20 μ l of 110 mM FeCl_3 . Absorbance measurement (at 492 nm) was performed after one hour of incubation in

the oven at 40 °C. The DHA content was calculated by subtracting the total AsA with AsA.

9. Reduced glutathione (GSH) and oxidised glutathione (GSSG) quantification

The total glutathione and GSSG assessment were started with HCl serum neutralization. The 200 µl of HCl serum was reacted with 20 µl of 0.2M pH 5.6 sodium dihydrogen phosphate (NaH₂PO₄) and 200 µl of 0.2N 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 for 30 minutes at ambient temperature. The clear supernatant was obtained by 10 minutes of centrifugation at 13,000 rpm.

For total glutathione determination, 20 µl of the neutralized sample was reacted with 100 µl buffer A (consisting of 9.5 mg DTNB, 19.6 ml NaH₂PO₄ pH 7.0, and 400 µl of 0.5M EDTA), 10 µl of 20U GR, and 60 µl distilled water. Following the absorbance measurement at 405 nm, the sample was reacted with 10 µl NADPH 20 mM, then absorbance measurement at 405 nm was performed five times at 30 seconds intervals. For GSSG determination, 50 µl of the sample was reacted with 100 µl buffer A, 10 µl of 20 U GR, 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 seconds intervals. The total glutathione and GSSG content were determined by calculating the absorbance slope. The GSH was derived by subtracting the total glutathione with GSSG (total glutathione = GSH + 2 GSSG).

10. Nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide (NADH) determination

The protocol was adopted from Queval and Noctor (2007). Sample preparation for NAD determination was carried out by incubating 200 μ l of the HCl serum in the boiling water for two minutes followed by rapid cooling and 20 μ l of 0.2M NaH_2PO_4 (pH 5.6) addition. The pH was then adjusted with 0.5 M NaOH to 5 – 6. For total NAD, 200 μ l of NaOH serum was incubated in the boiling water for two minutes followed by rapid cooling and 20 μ l of 0.2M NaH_2PO_4 (pH 5.6) addition. The pH was adjusted with 0.5 M HCl to 7 – 8.

Each sample (20 μ l in volume) was reacted with 100 μ l of 10 mM 4-1-piperazineethanesulfonic acid (HEPES) 2 mM EDTA, 20 μ l of 1.2 mM 2,6-dichlorophenolindophenol (DCPIP), 10 μ l of 10 mM phenazine methosulfate (PMS), 25 μ l distilled water, and 10 μ l of 2500 U/ml alcohol dehydrogenase (ADH). The reaction was started with 15 μ l of absolute ethanol addition. The absorbance was measured at 600 nm five times at 30-second intervals. The NAD and total NAD contents were determined by calculating the absorbance slope. The NADH was derived by subtracting the total NAD value from the NAD value.

11. Nicotinamide adenine dinucleotide phosphate (NADP), and reduced nicotinamide adenine dinucleotide phosphate (NADPH)

The neutralization procedure for NADP and total NADP quantification was the same as NAD and total NAD quantification protocol. For absorbance measurement, a 30 µl sample was reacted with 100 µl of HEPES 10 mM EDTA 2 mM, 20 µl DCPIP 1.2 mM, 10 µl PMS 10 mM, 30 µl distilled water, and 10 µl glucose 6-phosphate 10 mM. The reaction was started with 10 µl of glucose 6-phosphate (G6PDH) 200 U/ml. The absorbance was measured five times with 30-second 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 from the NADP value.

12. Dry Cut Length (DCL) Measurement

The DCL was observed immediately after tapping. The percentage of DCL was calculated by dividing the dry cut length by the total cut length.

13. Heritability and Best Linear Unbiased Prediction (BLUP) calculation

The heritability and BLUP calculation were performed using R statistic software (R Foundation) in the R Studio interface version (R Studio Inc., Boston – USA). A mixed model was applied and all factors were considered random.

14. Quantitative Trait Locus (QTL) mapping on antioxidants-related traits

The QTL analysis was performed using MapQTL®6 software (Kyazma B.V, Wageningen – The Netherlands). The genetic map was provided by CIRAD which consisted of 18 groups. The analysis started with a permutation test to determine the Log of the Odds (LOD) score significant threshold, followed by an interval mapping analysis. Following the cofactors selection, the Multiple QTL Model (MQM) mapping and Kruskal-Wallis analysis were carried out.

G. Data Analysis

Statistical analysis was carried out using the XLSTAT program (Addinsoft Inc., New York, USA). The interaction between Clones and the Harvesting system (2d experiment) was analysed through a 3-ways or 2-way analysis of variance (ANOVA). For these analyses, a pairwise comparison of harvesting systems for each clone was performed by the Bonferroni method with $\alpha = 0.05/(n((n-1)/2))$, 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, a one-way ANOVA followed by Tukey Honestly Significant Difference (HSD) test with $\alpha = 0.05$ was implemented. A supervised Linear Discriminant Analysis with DCL severity as the 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 the occurrence of TPD. Finally, to predict linearly the

DCL percentage, a multiple stepwise regression analysis using the Akaike Information Criterion (AIC) was performed.

IV. RESULTS

A. Clonal Variability of Antioxidants in Latex *Hevea brasiliensis*

The antioxidant contents in five recommended rubber clones showed a significant clonal variation in RSH, GSH, and GSSG, while AsA, DHA, NADH, NAD, NADPH, and NADP were not significant (Table 4.1). Clones BPM 24 and PB 260 had higher RSH (0.70 mM and 0.73 mM, respectively) compared to the other three clones i.e., IRR 118 (0.39 mM), IRR 112 (0.27 mM), and PB 260 (0.73 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 the 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 mM to 5.11 mM, while DHA from 0.07 mM to 0.43 mM. The cofactors' determination resulted in no significant differences among clones. The NADH content ranged from 0.46 μ M to 0.81 μ M and NAD ranged from 0.26 μ M to 0.58 μ M. The NADPH ranged from 0.51 μ M to 0.60 μ M, while NADP from 2 nM to 4 nM. This result showed that the concentration in ascorbate was higher than in thiols and glutathione. Cofactor contents were much lower than antioxidants.

Table 4.1. Antioxidant and cofactor content in 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 $\alpha = 0.05$

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

B. Effect of Tapping Frequency and Ethephon Stimulation

The two rubber clones IRR 118 and BPM 24 were selected for further studies for their contrasting value in RSH content and level of susceptibility to TPD, with 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 the application of the treatments. Interaction analysis showed that the clone factor had a strong effect on IF, Suc, Pi, RSH, and AsA (Table 4.2.). 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

Tapping frequency significantly affected TSC, Suc, Pi, and RSH but not yield, IF, and PI parameters (Figure 4.1.). High tapping frequency (d1) significantly decreased TSC in clones IRR 118 and BPM 24, while d6 and d3 were comparable. For clone IRR 118, TSC decreased from 46.55 % to 34.63 %, and in PB 260 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). For clone BPM 24, the Suc was relatively stable in d6 (4.24 mM) and d1 (6.24 mM); interestingly, a drop occurred in d3 (3.50 mM).

Table 4.2. 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 significance according to the Bonferroni test at $\alpha = 0.017$

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 × 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 × 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

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 from d1 (0.34 mM and 0.46 mM respectively); while in d3 was significantly lower than d1 (0.33 mM).

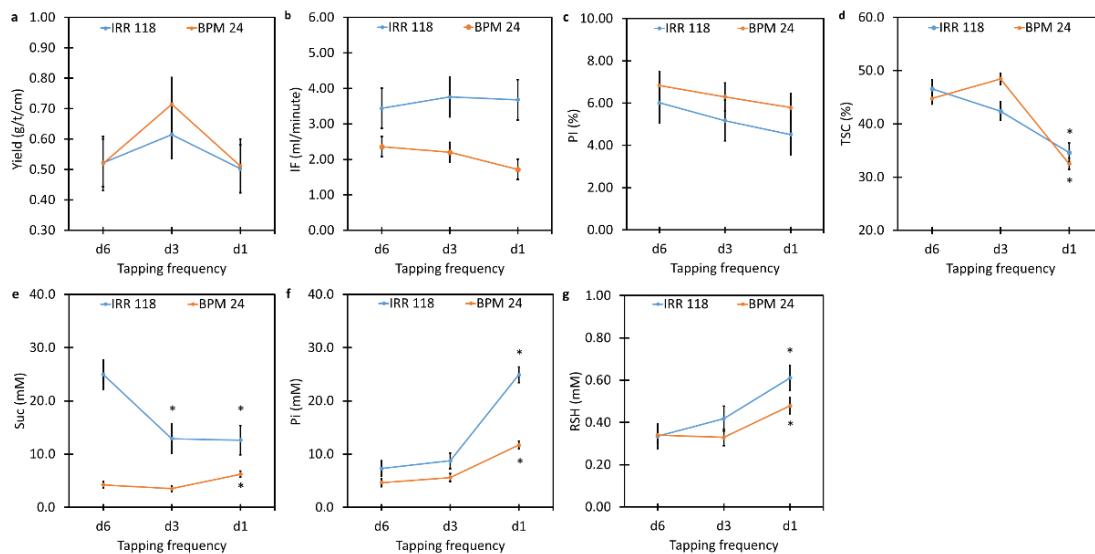


Figure 4.1. Effect of tapping frequency on yield, latex flow, and physiological parameters. The effect of tapping was analysed separately by clone. Data are least-squares means \pm standard error. * indicated significant differences from other tapping frequencies in the same clone according to Tukey Multiple Comparison Method at $\alpha = 0.05$

The effect of tapping frequency on reduced and oxidised forms of antioxidants was not significant for both rubber clones (Figure 4.2.). The 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 to 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 to 0.42 μM and for BPM 24 from 0.24 μM to 0.48 μM .

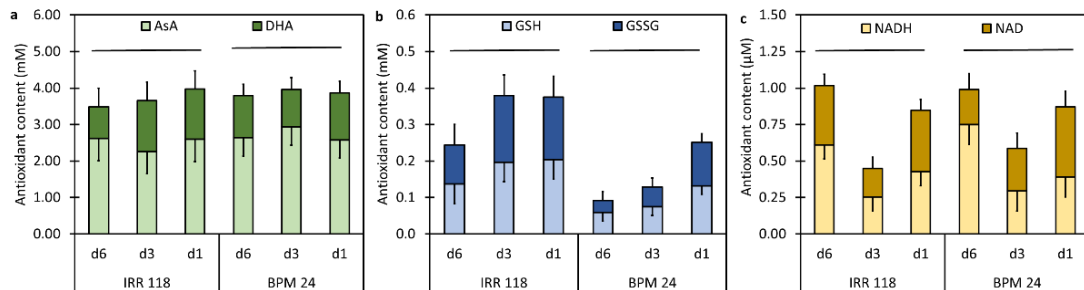


Figure 4.2. Effect of tapping frequency on reduced and oxidised forms of antioxidant and cofactor contents. The effect of tapping was analysed separately by clone. Data are least-squares means \pm standard error

The calculation of ratios between reduced by oxidised forms of antioxidants did not reveal oxidation. 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 it 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 it 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 frequencies i.e. 1.66 in d6, 1.30 in d3, and 1.08 in d1.

The ethephon stimulation had a significant effect on yield, latex flow, and physiological parameters except IF (Figure 4.3.). 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 difference 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 % to 1.72 % for clones IRR 118 and BPM 24, respectively.

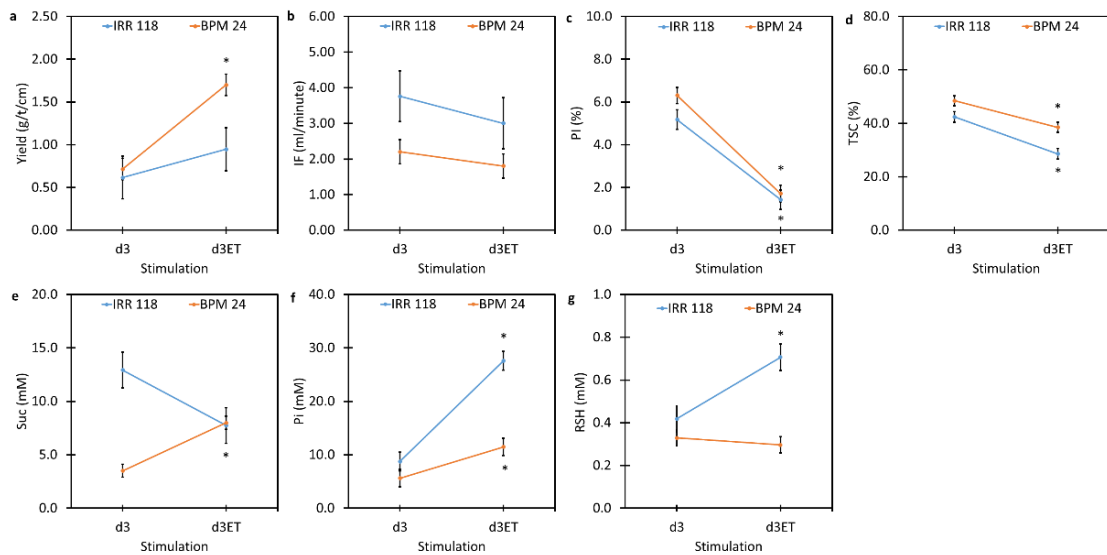


Figure 4.3. Effect of ethephon stimulation on yield, latex flow, and physiological parameters. The effect of stimulation was analysed separately by clone. Data are least-squares means \pm standard error. * indicated a significant difference from non-stimulated treatment in the same clone according to Tukey Multiple Comparison Method at $\alpha = 0.05$

TSC dropped from 42.4 % to 28.6 % for clone IRR 118 and from 48.5 % to 38.5 % for clone BPM 24. Suc increased significantly in stimulated trees (7.99 mM) compared with non-stimulation (3.50 mM) for 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 oxidised forms of antioxidants and cofactors were not significantly affected by ethephon stimulation except for an increase in NAD for clone IRR 118 (Figure 4.4.). 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 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 in clone BPM 24, it did not show a 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 μ M to 0.39 μ M in clone IRR 118 and from 0.30 μ M to 0.40 μ M 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 μ M instead of 0.20 μ M for IRR 118 and 0.39 μ M instead of 0.25 μ M for BPM 24.

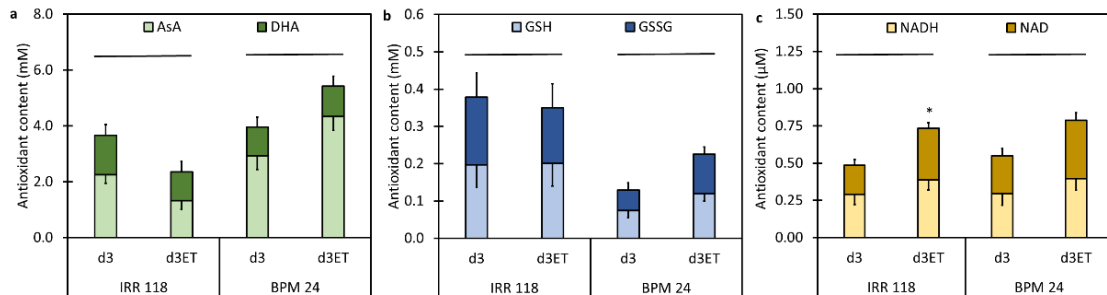


Figure 4.4. Effect of ethephon stimulation on reduced and oxidised forms of antioxidant and cofactor contents. The effect of stimulation was analysed separately by clone. Data are least-squares means \pm standard error. * indicated a significant difference from non-stimulated treatment in the same clone according to Tukey Multiple Comparison Method at $\alpha = 0.05$

There was no significant effect of ethephon stimulation on the ratio between the reduced and oxidised forms of ascorbate and glutathione. The AsA/DHA ratio of non-stimulated and stimulated trees were 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), and 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), and for BPM 24 was 1.10 (non-stimulation) and 1.00 (ethephon stimulation).

C. 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 the 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 4.3.). 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.

Linear discriminant analysis of high and low DCL severity for five recommended rubber clones showed several subgroups more or less clustered (Figure 4.5.). 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.

Table 4.3. Interaction analysis between the effect of clone, DCL severity, and the combination of factors. Numbers in bold indicate significance according to the 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 × 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

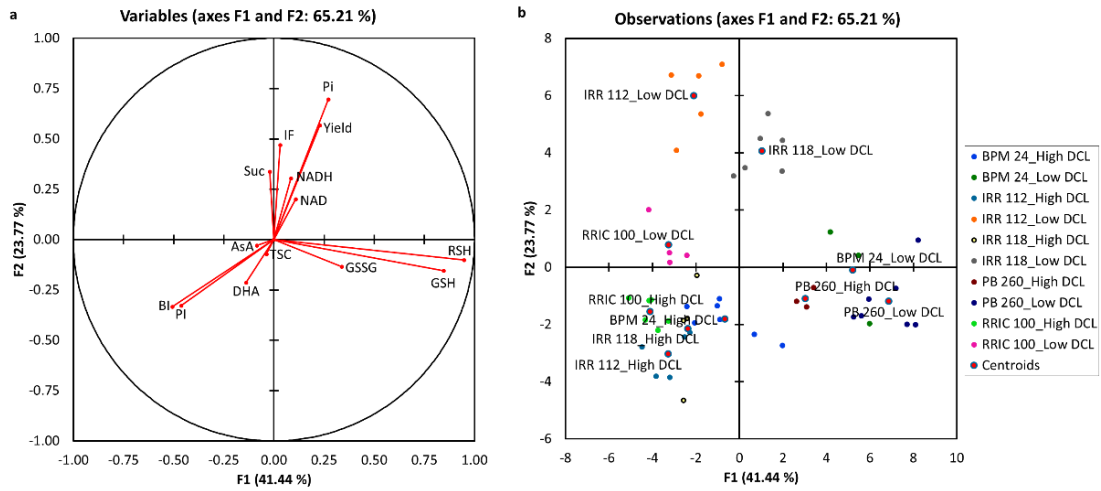


Figure 4.5. Determinant analysis of different DCL severity on five recommended rubber clones

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

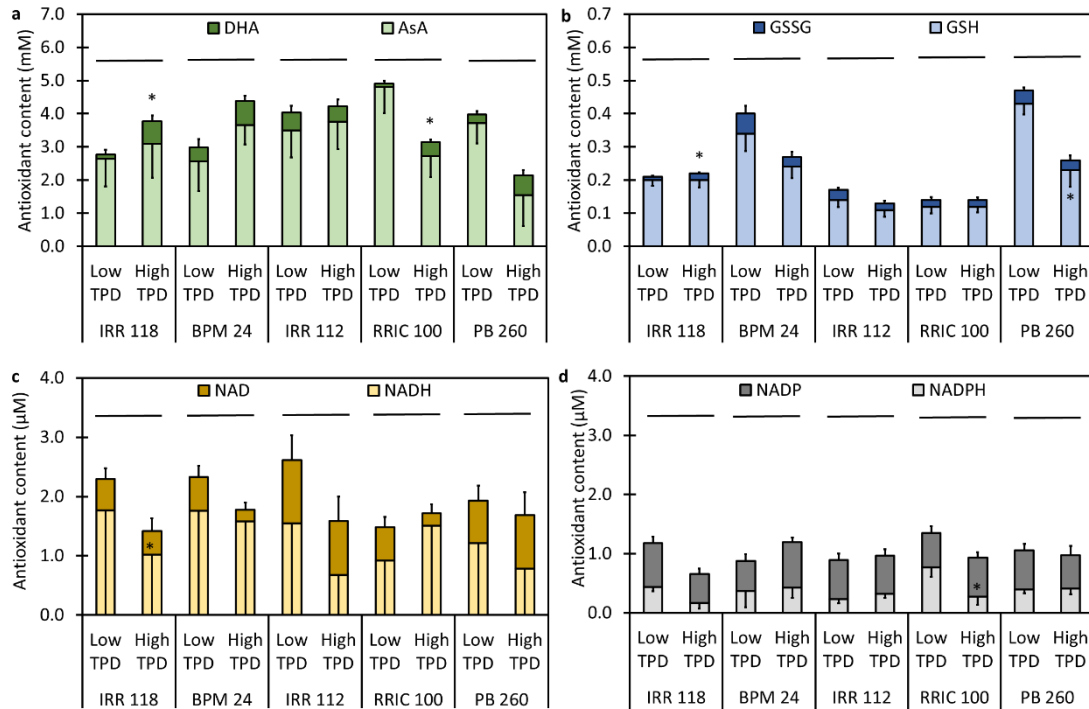


Figure 4.6. Effect of DCL severity on reduced and oxidised forms of antioxidant and cofactor contents. The effect of DCL severity was analysed separately by clone. Data are least-squares means \pm standard error. * indicated a significant difference from low DCL in the same clone according to Tukey Multiple Comparison Method at $\alpha = 0.05$

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 4.4.). The best model follows the equation:

$$\text{DCL} = 91.11 - (0.49 \times \text{Yield}) - (2.36 \times \text{Suc}) + (25.17 \times \text{DHA}) - (81.44 \times \text{GSH}) + (272.06 \times \text{GSSG}) - (0.08 \times \text{NADH}) - (0.05 \times \text{NAD})$$

Table 4.4. Prediction of best models for TPD percentage based on the 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

D. Alteration in the Ratio of Glutathione in the Reduced Thiols Content

The GSH/RSH ratio varied among clones, stress level, and TPD severity (Table 4.5.). For clone IRR 118, the GSH/RSH ratio was stable on d6 and d3 (0.47 and 0.43 respectively) then dropped on d1 (0.32) and ethephon stimulation (0.28). In BPM 24, the ratio increased from 0.19 on d6 to 0.24 on d3 and remained stable on 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, ranging from 0.45 to 0.64, for high DCL.

Table 4.5. The ratio of reduced glutathione content (GSH) to reduced thiols 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

E. Phenotypic Variability of Eight Recommended Rubber Clones

The antioxidant contents of eight recommended rubber clones showed a significant clonal variation in RSH, GSH, and NADH (Table 4.6.). The highest RSH was found in clone AVROS 2037 (1.00 mM), not significant to clone PB 260 (0.87 mM), and the lowest was IRR 39 (0.38 mM). The GSH content ranged from 0.48 mM (AVROS 2037) to 0.12 mM (IRR 39). The NADH content varied among assessed clones; ranged from 261.23 pmol/200 µl in clone SP 217 to 73.99 pmol/200 µl in clone PB 260.

Table 4.6. The antioxidant content in eight recommended rubber clones. Data are least-squares means + standard error. Different letters in the same column indicated significant differences according to Tukey Multiple Comparison Method at $\alpha = 0.05$

Clone	RSH (mM)	AsA (mM)	DHA (mM)	GSH (mM)	GSSG (mM)	NADH (pmol/200 μ l)	NAD (pmol/200 μ l)	NADPH (pmol/200 μ l)	NADP (pmol/200 μ l)
AVROS 2037	1.00 \pm 0.08 a	2.75 \pm 0.49 a	0.61 \pm 0.27 a	0.48 \pm 0.06 a	0.22 \pm 0.07 a	122.64 \pm 32.54 ab	136.67 \pm 26.53 a	113.84 \pm 52.28 a	128.00 \pm 26.58 a
GT 1	0.69 \pm 0.08 ab	0.69 \pm 0.49 a	1.19 \pm 0.27 a	0.34 \pm 0.06 ab	0.24 \pm 0.07 a	85.53 \pm 32.54 b	126.20 \pm 26.53 a	184.62 \pm 52.28 a	162.52 \pm 26.58 a
IRR 112	0.49 \pm 0.08 b	3.00 \pm 0.49 a	0.66 \pm 0.27 a	0.16 \pm 0.06 b	0.08 \pm 0.07 a	152.27 \pm 32.54 ab	78.76 \pm 26.53 a	249.54 \pm 52.28 a	121.00 \pm 26.58 a
IRR 39	0.38 \pm 0.08 b	2.61 \pm 0.49 a	0.69 \pm 0.27 a	0.12 \pm 0.06 b	0.07 \pm 0.07 a	157.80 \pm 32.54 ab	47.33 \pm 26.53 a	213.40 \pm 52.28 a	134.44 \pm 26.58 a
PB 260	0.87 \pm 0.08 a	2.74 \pm 0.49 a	0.67 \pm 0.27 a	0.38 \pm 0.06 ab	0.11 \pm 0.07 a	73.99 \pm 32.54 b	75.95 \pm 26.53 a	152.64 \pm 52.28 a	144.91 \pm 26.58 a
PR 261	0.48 \pm 0.09 b	1.75 \pm 0.55 a	1.22 \pm 0.30 a	0.24 \pm 0.07 ab	0.17 \pm 0.08 a	140.63 \pm 36.38 ab	94.78 \pm 29.66 a	196.00 \pm 58.46 a	83.65 \pm 29.71 a
RRIC 100	0.40 \pm 0.08 b	2.70 \pm 0.49 a	0.72 \pm 0.27 a	0.13 \pm 0.06 b	0.07 \pm 0.07 a	150.78 \pm 32.54 ab	39.86 \pm 26.53 a	190.29 \pm 52.28 a	93.34 \pm 26.58 a
SP 217	0.43 \pm 0.10 b	3.47 \pm 0.63 a	0.42 \pm 0.34 a	0.20 \pm 0.08 ab	0.06 \pm 0.10 a	261.23 \pm 42.01 a	19.81 \pm 34.25 a	235.43 \pm 67.50 a	76.02 \pm 34.31 a

F. Heritability of Some Antioxidants

RSH had the highest heritability at the tree and clone levels (Table 4.7.). The tree heritability from the highest to the lowest was RSH (0.423), ratio AsA/DHA (0.059), DHA (0.045), total AsA (0.021), and AsA (0.010). At the clone level, the highest heritability was encountered in RSH (0.687), followed by AsA/DHA (0.157), DHA (0.136), total AsA (0.059), and AsA (0.029).

Table 4.7. Heritability of some latex antioxidants

Parameter	Genetic variance	Residual variance	Tree heritability	Clone heritability
RSH	0.021	0.029	0.423	0.687
AsA	0.010	1.021	0.010	0.029
DHA	0.011	0.201	0.045	0.136
Total AsA	0.030	1.448	0.021	0.059
AsA/DHA	38.910	624.931	0.059	0.157

G. QTL Mapping of Latex Antioxidants-related Traits

The QTL mapping did not detect any QTL for each antioxidant parameter. For RSH, the highest LOD was 3.97 (LOD threshold was 4.40) in group 8 (Figure 4.7.A.). For AsA, the highest LOD was 3.59 (LOD threshold was 4.40) in group 9 (Figure 4.7B.). For DHA, the highest LOD was 3.19 (LOD threshold was 4.30) in group 7 (Figure 4.7C.). For total AsA, the highest LOD was 3.49 (LOD threshold was 4.30) in group 9 (Figure 4.7D.). For the ratio AsA/DHA, the highest LOD was 2.59 (LOD threshold was 4.40) in group 4 (Figure 4.7E.).

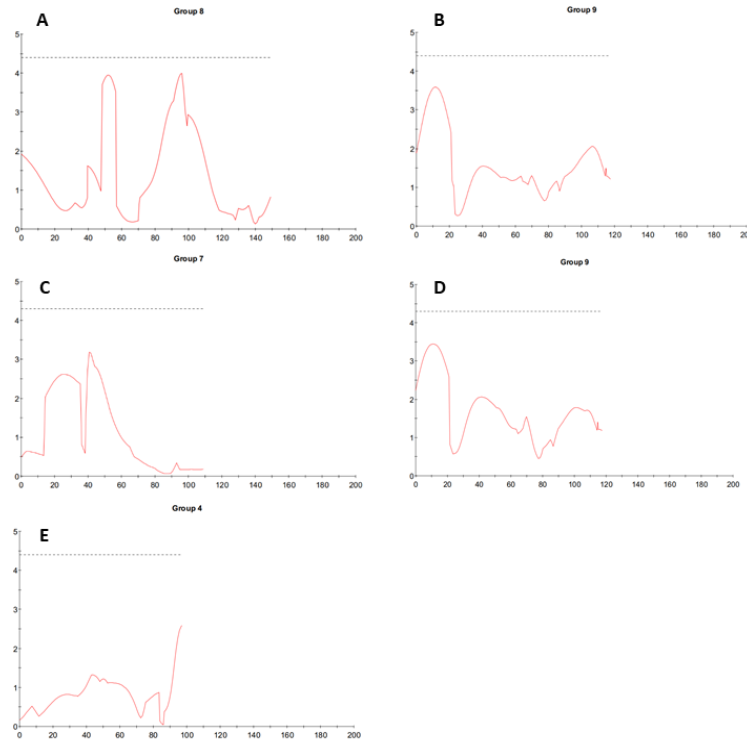


Figure 4.7. The highest LOD according to the Multiple QTL Model (MQM) for RSH (A), AsA (B), DHA (C), total Asa (D), and ratio AsA/DHA (E)

Kruskal-Wallis analysis showed that for RSH the highest probability of QTL is in group 8 locus g8A241 (Table 4.8.). For AsA, the highest possibility is group 4 locus g4SSH111 and group 14 locus g114T2611. For DHA, total AsA, and the ratio of AsA/DHA were group 9 locus g9Tas2222, group 9 locus g9A104, and group 3 locus 93T45 respectively.

Table 4.8. Kruskal-Wallis analysis result on analysed traits

Trait	Group	Position	Locus	Significance
RSH	8	48.396	g8A241	****
AsA	4	9.260	g4SSH111	***
AsA	14	42.447	g114T2611	***
DHA	9	0.00	g9Tas2222	***
Total AsA	9	50.974	g9A104	***
AsA/DHA	3	0.0000	93T45	***

V. DISCUSSION

The Ph.D. research is the first work combining yield, latex flow, LD, antioxidants, and cofactors in response to tapping frequency, ethephon stimulation, and TPD severity to have a better understanding of the regulation of latex antioxidants in response to harvesting stress and TPD occurrence. This is also the first report on the clonal variation and QTL mapping of the main antioxidants in the latex of *H. brasiliensis*. Here we discussed the evolution of oxidised and reduced forms of latex antioxidants, the role of RSH as a biomarker, and the interaction of RSH with other physiological parameters in response to stress type and duration. Finally, the variation in latex antioxidants from eight recommended rubber clones and the QTL mapping were also discussed.

A. Analysis of Oxidised and Reduced Antioxidants Improved the Knowledge of Antioxidant Regulation in *Hevea brasiliensis* Latex

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. H_2O_2 is neutralized into H_2O through electron transfer from AsA in the presence of APX. DHA is produced during the process and regenerated back to AsA using GSH as the electron donor, and the GSSG is transformed back to GSH through NADPH-dependent GR as an electron donor (Foyer and Halliwell, 1976; Noctor and Foyer, 1998).

In 1960, McMullen quantified thiols of low molecular weight in latex (Mc Mullen, 1960). He found that at least 93 % of the total -SH in latex is in the form of glutathione and cysteine in an 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 oxidised forms 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 are naturally part of plant defence against herbivory and pathogens (Konno, 2011; Ramos et al., 2019; Gracz-Bernaciak et al., 2021), are equipped with a 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 a 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.

B. 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 toxic oxygen molecules (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 a value ranging from 0.21 mM to 0.73 mM. In wheat, the inherent genetic variation in endogenous antioxidant 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 increases 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 statuses. These rubber clones showed a specific clonal response to ethephon stimulation with a significant increase in RSH for clone IRR 118 while it is stable for clone BPM 24. The clonal characteristic in response to harvesting systems is an essential parameter 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 the 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 in clone IRR 118 while it remains stable for clone BPM 24. These data were collected four months after the application of harvesting system treatments, which can be considered 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 compensation for the oxidative processes driven by tapping and stimulation. Although no significant alteration in GSH was noted, the tendency to increase GSH in response to tapping for both clones and to the ethephon stimulation for clone BPM 24 tends to confirm the hypothesis of Chrestin. In addition, another low molecular weight non-protein component of RSH not detected by the Ellman reagent

might play a crucial role in response to harvesting systems. In temporary stress, active biosynthesis of RSH is allowed due to sufficient energy availability, which is provided by carbohydrate substrate. These showed the relation between RSH, Suc, and Pi.

Interestingly, several studies showed a drop in RSH after the long-term effect of high ethephon stimulation frequency (HSF) (Lacote et al., 2010; Traore et al., 2011; Dian et al., 2016, Dian et al., 2017) and high tapping frequency (HTF) (Obouayeba et al. (2009)), although in other study was stable (Sainoi et al., 2017). In long-term observation, RSH was generally lower for high-stress treatment than for lower-stress treatment and 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 significantly lower RSH content in the latex of TPD-affected trees. This suggests that the metabolic activity of laticifers has 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 of stress in laticifer. The RSH increased in temporary stress and then decreased in long-term stress and high TPD-affected trees (Table 5.1.).

Table 5.1. Yield, latex flow, antioxidants, and cofactors dynamic in different types and duration of 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 (μM)	-	-	-	Nd	Nd	-
NAD (μM)	-	-	-	Nd	Nd	-
NADPH (μM)	Nd	Nd	Nd	Nd	Nd	-
NADP (μM)	Nd	Nd	Nd	Nd	Nd	-

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

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 remains 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.

C. RSH Relationship with other Physiological Parameters

Tapping frequency and ethephon stimulation affected yield, latex flow, and physiological parameters (Figure 5.1.). In healthy trees, Suc significantly decreased in high tapping frequency due to 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 antioxidant biosynthesis, in particular RSH. However, the effect of RSH on luteoid 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.

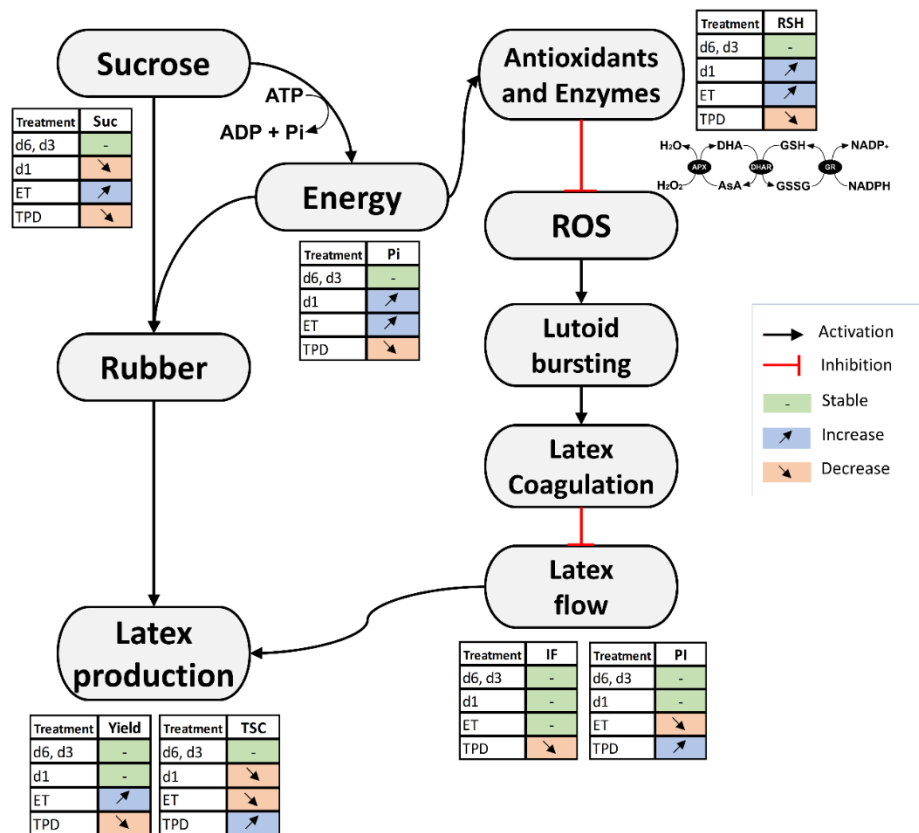


Figure 5.1. Working model of physiological and antioxidant dynamics in response to harvesting stress and TPD occurrence

Ethephon stimulation increased the 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 the maintenance of 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. Sucrose, the major precursor of rubber, penetrate the laticifer wall apoplastically. Then

sucrose is transported across the plasmalemma of the laticifer and used for rubber biosynthesis through the isoprenoid pathway (de Fay et al., 1989). 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 availability of carbohydrates and energy was 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 a lack of energy leading to luteoid bursting and latex coagulation, which is confirmed by a high PI. This phenomenon was also reported by Guo and collaborators (Guo et al., 2016).

Besides abiotic stress, tapping activity induces biotic stress in the form of fungal infections on tapped bark; for example, *Phytophthora palmivora* (Butl.) Butl. (black stripe disease) (Sdoodee, 2004) and *Ceratocystis fimbriata* Ell. & Halst. (moldy rot), and insect borers (Mathew and Gnanaharan, 2000; Hiremath and Prathapan, 2019). As ROS is produced at the earlier onset of cellular defense responses to biotic stress, the scavenging mechanism is also involved antioxidants and antioxidative enzymes (Das and Roychoudhury, 2014; Krishnan et al., 2019). Indeed, *H. brasiliensis* accumulates pathogenesis-related proteins such as chitinase and hevine in response to biotic stress. Nonetheless, latex antioxidants dynamic, including RSH, under biotic stress has never been reported. The study on this topic will broaden the knowledge of RSH regulation in response to environmental stress.

D. First Attempt on QTL Analysis on Latex Antioxidants-related Traits

Antioxidants quantification on young trees of eight recommended rubber clones showed that RSH, GSH, and NADH had clonal variation; while other parameters were comparable. A clonal variation on RSH was consistent with the previous observation in this project using ten years of mature trees. The GSH is part of the total thiols in latex. That is why these two parameters are always closely correlated in this thesis. Because RSH determination is easier to perform than GSH, RSH determination was chosen for the genetic analysis. The NADH has antioxidant power in the presence of enzymes and acts as the electron donor in the ascorbate-glutathione cycle. AsA showed a clonal variation in mature trees but not in young counterparts suggesting that the antioxidant level may develop along with age and the stress received by the plant. RSH, AsA, and DHA were selected for genetic analysis.

In this study, the QTL could not be detected for RSH, AsA, DHA, total AsA, and the ratio of AsA/DHA. Chandra and Pandey (2017) suggested that the heritability of the segregating genes and the size of the mapping population affect QTL mapping. In this study, the highest genotype heritability of the antioxidants trait was RSH 0.69, the other traits even lower than 0.2 indicating a low variability. It might be due to the low stress, tapped d3 since open tapping without stimulation, or not enough genotype involved in the analysis. Although the Kruskal-Wallis analysis gave an estimation of the locus position, another QTL analysis in high-stress conditions (d1 tapping frequency) and a larger population may bring more specific QTLs.

VI. CONCLUSION AND PERSPECTIVE

A. Conclusion

According to our study, some conclusions related to the research questions can be drawn as follows:

1. Ascorbate is the most concentrated antioxidant followed by glutathione. RSH and its related parameters (GSH and GSSG) varied among rubber clones. AsA was found in a much higher concentration than RSH, yet not significantly different among rubber clones as well as DHA and antioxidant cofactors (NADH, NAD, NADPH, and NADP).
2. RSH is influenced by many factors including genetics, harvesting system, and environment. RSH content tends to increase in response to temporary harvesting stress and then drop in long-term stress, usually accompanied by TPD occurrence. Knowing only the RSH value in one moment of tree production may mislead the interpretation.
3. This study revealed new insights into the regulation of antioxidants in laticifers in response to harvesting stress and TPD occurrence. It is also the first attempt to characterize the oxidation level of antioxidants in latex. The RSH synthesis is made possible by the capacity of laticifer metabolism to cope with harvesting stress, while a drop in RSH is a sign of long-term stress related to lower metabolic activity and TPD occurrence.
4. For better interpretation, reference of maximum RSH value under short-term high-stress and minimum value in long-term high-stress conditions are required as well

as information related to harvesting system history, plant age, TPD percentage, and other LD parameters data (TSC, Suc, and Pi). By combining this information, it can be determined whether the plant is under temporary stress, long-stress accumulation, or even already in an over-stress condition.

5. The analysis of the reduced and oxidised forms of antioxidants could not give a clear direction of the oxidation, regeneration, and biosynthesis level of antioxidants. The high proportion of reduced antioxidant forms suggests that laticifers have a strong capacity to maintain reduction capacity. A further study is worth investigating.
6. A clonal variation on RSH in young rubber trees was consistent with the previous observation in this project using mature trees. The GSH, part of total thiols in latex, is always closely correlated with RSH.
7. RSH has high heritability; while ascorbate and its related parameters had lower values. The QTLs of latex antioxidants-related traits could not be detected in young trees under low harvesting stress using a small population of 102 genotypes.

B. Perspective

Our study reveals that RSH remains a good physiological marker if supported by reference data. Researchers or plantations need to establish a database of latex physiological values under a wide range of harvesting and environmental factors for better LD interpretation. It includes a clonal typology of response to stress according to the RSH parameter in addition to Suc and Pi. Other forms of thiols besides low

molecular non-protein thiols detected by the Ellman reagent might give a different response to harvesting systems and deserve investigation.

The analysis of reduced and oxidised forms of ascorbate and glutathione did not provide significant data to describe the oxidation, regeneration, and biosynthesis of antioxidants under applied treatments of tapping frequency, ethephon stimulation, and TPD occurrence. It leads to an assumption that laticifers are equipped with a powerful scavenging system that can neutralize stress at a certain level and duration. For a better understanding of the regulation of antioxidants in laticifers, studies under high and/or long harvesting stress as well as a larger panel of contrasting clones for latex metabolic activity, different plant tissues, and TPD severity as well as antioxidative enzyme activities are recommended. The effect of harvesting practice and TPD on biotic stresses including the accumulation of pathogenesis-related proteins is worth investigating.

The RSH, GSH, and NADH had clonal variation in eight young rubber clones. The analysis using 103 progenies of PB 260 x SP 217 showed that RSH has high heritability, while ascorbate and its related parameters were low. The QTLs of latex antioxidants-related traits could not be detected in young trees under low harvesting stress (d3 without stimulation). According to our work, RSH and antioxidants changed in response to harvesting stress. Other physiological traits that the use of a larger population of 200 genotypes led to better detection of QTLs (personal communication with Sigit Ismawanto). In addition, the application of high harvesting stress may also exacerbate to response between genotypes. Therefore, the analysis in higher stress, for

example in d1 tapping frequency, and larger populations may detect QTLs of latex antioxidants-related traits.

EXECUTIVE SUMMARY

Para rubber (*Hevea brasiliensis* Muell Arg.) is the main natural rubber-producing species. The tree is harvested through the incision of the bark to let the colloidal solution, called latex, flow out and be collected. The ethephon, an ethylene releaser, is widely used in rubber plantations to optimize yield. The studies of rubber physiological status explored the opportunity to support yield optimisation, through tapping and stimulation modification and investigate the stress-response mechanism, mainly for mitigation of the occurrence of Tapping Panel Dryness (TPD), a physiological syndrome induced by over-exploitation and over-stimulation.

Latex diagnosis (LD) is a method for estimating the plant's physiological status at one moment of tree production. It is performed by assessing 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. In relation to TPD prevention, RSH behaviour is still not fully understood. Theoretically, RSH decreases with the increase in harvesting stress due to rapid consumption in response to the accumulation of reactive oxygen species (ROS) production. However, in some studies, RSH content was reported to be stable or increased. The meta-analysis study, which was part of the project, revealed that RSH was poorly interpreted, only in 42.8 % of 131 scientific papers involved. The inconsistency of RSH values associated with the level of stress leads authors to limit the interpretation of their data. It calls for a comprehensive study of RSH dynamics in

different stress types and levels for a better understanding of RSH regulation in *H. brasiliensis* latex.

ROS scavenging system involves antioxidants and anti-oxidative enzymes. In latex, the main antioxidants are thiols (consisting of glutathione and cysteine), ascorbate (vitamin C isomer), and tocotrienol (vitamin E isomer). In addition, several enzymes are involved in neutralizing radical compounds including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), and ascorbate peroxidase (APX). Under conditions of low ROS concentrations, APX and GPX are very active, whereas CAT activity is increased under high ROS conditions. ROS detoxification occurs through a reduction-oxidation (redox) reaction. Copper-zinc superoxide dismutase (CuZnSOD) is the enzyme responsible for converting radical superoxide ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) in the cytosol. The detoxification of H_2O_2 has generally involved the ascorbate and glutathione cycle. H_2O_2 is neutralized into H_2O through electron transfer from reduced ascorbate (AsA) in the presence of APX. Oxidised ascorbate (DHA) is produced during the process and regenerated back to AsA using reduced glutathione (GSH) as the electron donor. The oxidised form of glutathione (GSSG) is produced and recycled back to GSH by reduced nicotinamide adenine dinucleotide phosphate (NADPH), catalysed by glutathione reductase (GR).

The increase in the oxidised form of antioxidants indicates active oxidation due to the presence of stress, while the increase in 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 oxidised forms of antioxidants in response to abiotic stress. This approach may give a better explanation of stress levels in *H. brasiliensis* than assessing RSH solely.

Studies on other plants revealed that the variability of antioxidant content could be detected in interspecific and intraspecific levels. In *H. brasiliensis*, several studies reported genotypic variation in antioxidants content and antioxidative enzyme activities such as a variation in tocotrienols and thiols content as well as the enzymatic activities. These variations could be exploited for the selection of oxidative stress-tolerant clones. The genetic analysis of RSH including genes underlying Quantitative Trait Locus (QTL) may be useful for identifying genetic markers, implementing marker-assisted selection, and developing TPD-tolerant clones (breeding for TPD-tolerant clones), especially in the context of climate change.

This dissertation research aims to complement the LD method, particularly in oxidative stress level and antioxidant capability estimation. This is the first work combining yield, latex flow, latex diagnosis, and antioxidant quantification in *H. brasiliensis* latex in order to have a better understanding of the regulation of latex antioxidants in response to harvesting stress and attempt to identify other potential redox parameters. This work is also the first attempt at genetic analysis to identify QTL related to latex's main antioxidant traits. The expected outcomes of this research include 1. better knowledge of the variation of antioxidants content in *H. brasiliensis* latex, 2. a better understanding of the evolution of RSH content in response to different stress of tapping, ethephon stimulation, and TPD occurrence, 3. working model of

physiological and antioxidant dynamics, including RSH, in different stress types and duration, 4. recommendation for better interpretation of RSH value in relation with harvesting stress and TPD occurrence, 5. new parameter of oxidative stress level based on antioxidants status, 6. significant antioxidant parameters for further genetic analysis, and 7. heritability and QTLs of latex main antioxidants traits. Based on the literature related to the research objectives, the hypotheses proposed are: 1. antioxidants content in latex varies among rubber clones, 2. antioxidants content, including RSH, alters in response to different stress of tapping, ethephon stimulation, and TPD occurrence, 3. the dynamic of physiological parameters and antioxidants under different stress types and duration will give a better understanding of antioxidants regulation in different stress types and duration, 4. the RSH regulation may provide better interpretation of RSH value in relation with harvesting stress and TPD occurrence, 5. the ratio of reduced to oxidised form of antioxidants may be better estimator of stress level in *H. brasiliensis* latex than RSH solely, 6. significant variation on some antioxidant parameters may be appropriate for further genetic analysis, and 7. the segregating population from TPD-susceptible and tolerant cross will provide the preliminary heritability and QTL data for latex antioxidants related traits.

To achieve the research objectives, the study on the clonal variation of antioxidant content and the effect of tapping, stimulation, and TPD on the yield, latex flow, and antioxidant parameters was carried out, followed by a genetic analysis of the main antioxidant parameters in a segregating population. The project was started in February 2019. Following preparation and a series of laboratory training in 2019 – 2020, field

experiments were carried out from January to July 2021 at Sembawa Research Centre, Indonesian Rubber Research Institute, Palembang, Indonesia. Four field trials were established to study: 1. the clonal variation of antioxidants in five recommended rubber clones, 2. the effect of tapping and ethephon stimulation on the yield, latex flow, and antioxidants parameters, 3. the effect of TPD occurrence, and 4. genetic analysis of main antioxidant parameters.

Clonal variation in five recommended rubber clones i.e. IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260 (selected based on metabolic level) was performed in healthy trees of ten-year-old trees. The trees were tapped on the B0-1 panel every three days with one application of 2.5 % ethephon every month (harvesting system noted S/2 d3 ET2.5% 12/y). The effect of harvesting systems was carried out on clones IRR 118 and BPM 24. 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 replicates for each treatment. Latex sample collection was performed in April 2021, four months after treatments were applied. The effect of TPD was conducted 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 the B0-1 panel.

Phenotypic variability of main antioxidant parameters was carried out on five years old rubber trees to identify significant antioxidant parameters for further genetic analysis. Eight recommended rubber clones assessed included AVROS 2037, GT 1, IRR 112, IRR 39, PB 260, PR 261, RRIC 100, and SP 217. Five replications were used; each replication was the average of two individual trees. For heritability and QTL

analysis, 103 progenies of PB 260 (TPD susceptible clone) x SP 217 (TPD tolerant clone) were used.

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 oxidised 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 heritability and QTL analysis was performed on RSH, AsA, DHA, total AsA, and the ratio of AsA/DHA.

The interaction between Clones and the Harvesting system was analysed through 3-ways or 2-way analysis of variance (ANOVA). To study the effect of clonal variation on the antioxidants and the effect of tapping frequency, ethephon stimulation, and DCL severity, a one-way ANOVA followed by Tukey HSD test with $\alpha = 0.05$ was implemented. A supervised Linear Discriminant Analysis with DCL severity as a 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 the occurrence of TPD. Finally, to predict linearly the DCL percentage, a multiple stepwise regression analysis using the Akaike Information Criterion (AIC) was performed.

The result showed that AsA had a much higher concentration than GSH in *H. brasiliensis* latex suggesting that AsA may have an important role in the oxidative

stress scavenging process and calls for further investigation. The ratio of GSH/RSH varies from 0.19 to 0.64 according to the clone and the harvesting system is lower than the literature from McMullen (1960) that reported at least 93 % of the total -SH in latex is in the form of glutathione and cysteine.

The observation of five recommended rubber clones (IRR 118, BPM 24, IRR 112, RRIC 100, and PB 260) showed a clonal variation of RSH with a value ranging from 0.21 mM to 0.73 mM, lower than the literature that reported RSH content ranged from 0.5 to 0.9 mM (Jacob et al., 1984). Although RSH increases 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 rubber clones showed a specific clonal response to ethephon stimulation with a significant increase in RSH for clone IRR 118 while it is stable for clone BPM 24. The clonal characteristic in response to harvesting systems is an essential parameter to optimize the yield and prevent TPD.

These data were collected four months after the application of harvesting system treatments, which can be considered short-term or temporary stress. The increase in RSH was associated with greater latex metabolic activity associated with high Pi and active glutathione synthesis as compensation for the oxidative processes driven by tapping and stimulation. In addition, another low molecular weight non-protein component of RSH not detected by the Ellman reagent might play a crucial role in response to harvesting systems. In temporary stress, active biosynthesis of RSH is allowed due to sufficient energy availability, which is provided by carbohydrate

substrate. These showed the relation between RSH, Suc, and Pi. According to the literature, in long-term stress, RSH was generally lower for high-stress treatment than for lower-stress treatment and 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 suggesting that the metabolic activity of laticifers has 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. Literature showed that RSH was also determined by plant age, canopy condition, and environmental condition. It suggests that for RSH interpretation, information related to harvesting system history, plant age, TPD percentage, and other LD parameters data (TSC, Suc, and Pi) are required. 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.

RSH was the only antioxidant factor that significantly changed according to the level of stress in laticifer. 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 remains the appropriate antioxidant parameter to characterize latex. 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 are equipped with a powerful scavenging system that can neutralize stress at a certain level and duration. 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.

Our study provided a better understanding of antioxidant regulation, especially RSH, and its relation to other physiological parameters. In healthy trees, Suc significantly decreased in high tapping frequency due to rapid consumption as a consequence of active rubber biosynthesis to regenerate the expelled latex. 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 luteoid 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 significantly lowered PI, expressing a long latex flow. It might also result from high RSH content providing adequate protection of luteoid from harmful

free radicals. The high available energy reflected by high Pi enables the maintenance of 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 high TPD-affected trees, the yield dramatically dropped. The logical explanation is the reduction of metabolic activity in laticifers. In this condition, the availabilities of carbohydrates and energy were already low leading to limited Suc and water importations, reflected by low Suc and high TSC. In this condition, RSH biosynthesis was also disrupted due to a lack of energy leading to luteoid bursting and latex coagulation, which is confirmed by a high PI.

In conclusion, the study showed that RSH is a multifactorial parameter leading to difficulty to interpret. It tends to increase in response to temporary stress and then drop in long-term stress, usually accompanied by TPD occurrence. Using only the RSH value in one moment of the tree production may mislead the interpretation. For better interpretation, reference to maximum RSH value under no stress and minimum value in high-stress conditions are required as well as information related to harvesting system history, plant age, TPD percentage, and other LD parameters data. The analysis of the reduced and oxidised forms of antioxidants could not give a clear direction of the oxidation, regeneration, and biosynthesis level of antioxidants. However, further study is worth investigating.

In this study, the QTL could not be detected for RSH, AsA, DHA, total AsA, and the ratio of AsA/DHA. The data showed a low genotype heritability of the antioxidants

trait; the highest was RSH 0.69, and the other traits were even lower than 0.2 indicating a low variability in the 103 observed genotypes. It might be due to the low stress, tapped d3 since open tapping without stimulation, or not enough genotype involved in the analysis. The analysis in higher stress, for example, d1 tapping frequency, and larger population may give a better result.

RINGKASAN EKSEKUTIF

Tanaman karet (*Hevea brasiliensis* Muell Arg.) merupakan spesies penghasil utama karet alam. Tanaman karet dipanen dengan cara disadap pada untuk mendapatkan lateksnya. Etefon digunakan secara luas di perkebunan karet untuk mengoptimalkan produksi. Studi fisiologis lateks bertujuan menggali potensi untuk mendukung optimalisasi produksi melalui modifikasi sistem sadap dan stimulasi serta mitigasi Kering Alur Sadap (KAS), penyakit fisiologis yang dipicu oleh eksploitasi dan stimulasi yang berlebihan.

Diagnosis lateks (LD) adalah metode untuk memperkirakan status fisiologis tanaman karet pada satu titik produksi tertentu dengan cara menganalisa empat parameter utama yaitu *Total Solid Content* (TSC) yang menunjukkan regenerasi lateks di antara dua penyadapan, kadar sukrosa (Suc) yang menunjukkan ketersediaan karbohidrat, fosfat anorganik (Pi) yang mencerminkan aktivitas metabolisme, dan kadar tiol (RSH) yang menggambarkan kapasitas antioksidan dan tingkat cekaman. Terkait dengan mitigasi KAS, karakteristik RSH masih belum sepenuhnya dipahami. Secara teoritis, RSH menurun dengan meningkatnya cekaman akibat penggunaan yang tinggi sebagai respon terhadap akumulasi *Reactive Oxygen Species* (ROS). Namun demikian, beberapa literatur melaporkan bahwa RSH stabil atau bahkan meningkat dengan meningkatnya cekaman penyadapan. Studi meta-analisis, yang merupakan bagian dari penelitian disertasi ini, mengungkapkan bahwa interpretasi RSH lebih rendah dibanding parameter LD lainnya, hanya 42,8 % dari 131 artikel ilmiah yang diamati. Inkonsistensi nilai RSH menyebabkan kesulitan dalam menginterpretasikan

data RSH. Hal ini melatarbelakangi pentingnya studi yang komprehensif terhadap dinamika RSH pada jenis dan tingkat cekaman yang berbeda untuk mengetahui regulasi antioksidan, khususnya RSH, dalam lateks tanaman karet.

Detoksifikasi ROS melibatkan antioksidan dan enzim anti-oksidatif. Dalam lateks tanaman karet, antioksidan utama adalah tiol (terdiri dari glutathione dan sistein), askorbat (isomer vitamin C), dan tokotrienol (isomer vitamin E). Selain itu, beberapa enzim yang terlibat dalam menetralkan senyawa radikal di antaranya adalah superoksida dismutase (SOD), katalase (CAT), glutathione peroksidase (GPX), glutathione S-transferase (GST), dan askorbat peroksidase (APX). Dalam kondisi konsentrasi ROS rendah, APX dan GPX sangat aktif, sedangkan aktivitas CAT meningkat dalam kondisi ROS tinggi. Detoksifikasi ROS berlangsung melalui reaksi reduksi-oksidasi (redoks). Tembaga-seng superoksida dismutase (CuZnSOD) adalah enzim yang terlibat dalam konversi *radical superoxide* ($O_2^{\bullet-}$) menjadi hidrogen peroksida (H_2O_2) dalam sitosol. Detoksifikasi H_2O_2 umumnya melibatkan siklus askorbat dan glutathione. H_2O_2 dinetralkan menjadi H_2O melalui transfer elektron dari askorbat tereduksi (AsA) dengan adanya APX. Askorbat teroksidasi (DHA) diproduksi selama proses dan diregenerasi kembali menjadi AsA menggunakan glutathione tereduksi (GSH) sebagai donor elektron. Bentuk glutathione teroksidasi (GSSG) diproduksi dan didaur ulang kembali menjadi GSH oleh nicotinamide adenine dinucleotide phosphate (NADPH), dikatalisis oleh glutathione reductase (GR).

Peningkatan konsentrasi senyawa antioksidan teroksidasi menunjukkan proses oksidasi sebagai respons terhadap peningkatan cekaman, sedangkan peningkatan

senyawa antioksidan tereduksi menunjukkan *de novo* biosintesis atau aktivasi siklus askorbat-glutathione. Beberapa penelitian pada tanaman lain menunjukkan perubahan rasio senyawa antioksidan tereduksi dan teroksidasi akibat peningkatan cekaman abiotik. Pendekatan ini mungkin dapat menjelaskan tingkat cekaman dengan lebih baik daripada hanya menggunakan nilai RSH saja.

Literature menunjukkan bahwa variabilitas kadar antioksidan dapat dijumpai pada tingkat interspesifik dan intraspesifik. Pada *H. brasiliensis*, variasi genotipe dijumpai pada kandungan tokotrienol dan tiol serta aktivitas enzimatis. Variasi ini dapat digunakan dalam proses seleksi klon toleran terhadap cekaman oksidatif. Analisis *Quantitative Trait Locus* (QTL) bertujuan untuk mengidentifikasi penanda genetik dan dapat digunakan dalam mengembangkan klon toleran KAS.

Penelitian disertasi ini bertujuan untuk melengkapi metode LD, khususnya dalam pendugaan tingkat cekaman oksidatif dan kapasitas antioksidan. Penelitian ini adalah yang pertama kali menggabungkan parameter produksi, aliran lateks, LD, dan kuantifikasi antioksidan dalam lateks *H. brasiliensis* untuk mendapatkan pemahaman yang lebih baik tentang regulasi antioksidan lateks dalam tanggap cekaman penyadapan dan upaya untuk mengidentifikasi parameter redoks potensial lainnya. Penelitian ini ini juga merupakan upaya pertama dalam analisis genetik untuk mengidentifikasi QTL yang terkait dengan sifat antioksidan utama lateks. Luaran yang diharapkan dari penelitian ini antara lain: 1. pengetahuan yang lebih baik tentang variasi kadar antioksidan dalam lateks *H. brasiliensis*, 2. pemahaman yang lebih baik tentang evolusi kadar RSH dalam merespons cekaman penyadapan, stimulasi etefon,

dan kejadian KAS, 3. model dinamika fisiologis dan antioksidan, termasuk RSH, pada berbagai jenis dan durasi cekaman, 4. rekomendasi untuk interpretasi nilai RSH yang lebih baik dalam kaitannya dengan cekaman penyadapan dan kejadian KAS, 5. parameter baru tingkat cekaman oksidatif berdasarkan status antioksidan, 6. parameter antioksidan yang signifikan untuk analisis genetik lebih lanjut, dan 7. heritabilitas dan QTL dari sifat antioksidan utama lateks. Berdasarkan literatur yang terkait dengan tujuan penelitian, hipotesis yang diajukan adalah: 1. kandungan antioksidan dalam lateks bervariasi antar klon karet, 2. kandungan antioksidan, termasuk RSH, berubah dalam menanggapi cekaman penyadapan, stimulasi etefon, dan kejadian KAS, 3. dinamika parameter fisiologis dan antioksidan pada jenis dan durasi cekaman yang berbeda akan memberikan pemahaman yang lebih baik tentang regulasi antioksidan pada tipe dan durasi cekaman yang berbeda, 4. regulasi RSH dapat memberikan interpretasi nilai RSH yang lebih baik dalam kaitannya dengan cekaman panen dan kejadian KAS, 5. rasio antioksidan tereduksi menjadi teroksidasi kemungkinan merupakan penduga tingkat stres yang lebih baik pada lateks *H. brasiliensis* daripada nilai RSH, 6. variasi yang signifikan pada beberapa parameter antioksidan sesuai untuk analisis genetik lebih lanjut, dan 7. populasi segregasi dari persilangan klon yang rentan dan toleran KAS akan memberikan heritabilitas awal dan data QTL terkait sifat-sifat antioksidan lateks.

Untuk mencapai tujuan penelitian, dilakukan studi variasi klonal kandungan antioksidan dan pengaruh penyadapan, stimulasi etefon dan KAS terhadap parameter produksi, aliran lateks, dan kadar antioksidan yang dilanjutkan dengan analisis genetik

parameter antioksidan utama pada populasi segregasi. Proyek ini dimulai pada Februari 2019. Setelah persiapan dan serangkaian pelatihan laboratorium pada 2019 – 2020, percobaan lapangan dilakukan pada Januari hingga Juli 2021 di Pusat Penelitian Sembawa, Pusat Penelitian Karet, Palembang, Indonesia. Empat uji coba lapangan dilakukan untuk mempelajari: 1. variasi klon terhadap kadar antioksidan pada lima klon karet yang direkomendasikan, 2. pengaruh penyadapan dan stimulasi etefon terhadap parameter hasil, aliran lateks dan antioksidan, 3. pengaruh kejadian KAS, dan 4. analisis genetik parameter antioksidan utama.

Variasi klon pada lima klon karet yang dipilih berdasarkan tingkat metabolisme lateks yaitu IRR 118, BPM 24, IRR 112, RRIC 100, dan PB 260 dilakukan pada pohon sehat berumur sepuluh tahun. Pohon sampel disadap pada panel B0-1 setiap tiga hari dengan satu aplikasi etefon 2,5 % setiap bulan (sistem panen S/2 d3 ET2,5% 12/y). Pengaruh sistem sadap diamati pada klon IRR 118 dan BPM 24. Empat sistem pemanenan (S/2 d6, S/2 d3, S/2 d1, dan S/2 d3 ET2.5% 12/y) dibandingkan menggunakan lima pohon sebagai ulangan untuk setiap perlakuan. Pengambilan sampel lateks dilakukan pada bulan April 2021, empat bulan setelah perlakuan diterapkan. Pengaruh KAS dilakukan pada lima klon yang direkomendasikan yaitu IRR 118, BPM 24, IRR 112, RRIC 100, dan PB 260 yang disadap dengan sistem panen S/2 d3 ET 2,5% 12/th pada panel B0-1.

Variabilitas fenotipik parameter antioksidan utama dilakukan pada pohon karet berumur lima tahun untuk mengidentifikasi parameter antioksidan yang signifikan dan analisis genetik lebih lanjut. Delapan klon karet direkomendasikan yang diamati

meliputi AVROS 2037, GT 1, IRR 112, IRR 39, PB 260, PR 261, RRIC 100, dan SP 217 menggunakan lima ulangan. Setiap ulangan adalah rata-rata dari dua individu pohon. Untuk analisis heritabilitas dan QTL, digunakan 103 progeni dari PB 260 (klon rentan TPD) x SP 217 (klon toleran TPD).

Parameter yang diamati terkait dengan produksi dan aliran lateks meliputi produksi lateks, aliran awal (IF), indeks penyumbatan (PI), dan *bursting index* (BI). Untuk diagnosis lateks, parameter yang diamati adalah TSC, Suc, Pi, dan RSH. Parameter antioksidan yang diamati meliputi kadar antioksidan tereduksi (AsA dan GSH) dan teroksidasi (DHA dan GSSG). Kofaktor yang terkait dengan regenerasi antioksidan diamati dalam bentuk teroksidasi dan tereduksi meliputi nikotinamida adenin dinukleotida (NAD, NADH), dan nikotinamida adenin dinukleotida fosfat (NADP, NADPH). Analisis heritabilitas dan QTL dilakukan terhadap RSH, AsA, DHA, AsA total, dan rasio AsA/DHA.

Interaksi antara klon dan sistem sadap dianalisis melalui Analisis Sidik Ragam (ANOVA) 3 arah atau 2 arah. Untuk mengetahui pengaruh variasi klonal terhadap kadar antioksidan dan pengaruh frekuensi penyadapan, stimulasi ethephon, dan KAS, dilakukan uji ANOVA satu arah yang dilanjutkan dengan uji Tukey HSD dengan $\alpha = 0,05$. Analisis Diskriminan Linier dengan tingkat keparahan KAS sebagai variabel terikat dilakukan dengan melibatkan parameter produksi, aliran lateks, diagnosis lateks, antioksidan, dan kofaktor untuk mendapatkan kombinasi yang dapat menjelaskan tingkat keparahan KAS. Selanjutnya, untuk memprediksi persentase KAS

secara linier dilakukan analisis regresi berganda menggunakan Akaike Information Criterion (AIC).

Hasil penelitian menunjukkan bahwa AsA memiliki konsentrasi yang lebih tinggi dalam lateks dibandingkan dengan GSH yang mengindikasikan bahwa AsA kemungkinan memiliki peran yang penting dalam proses netralisasi radikal bebas sehingga perlu diselidiki lebih lanjut. Rasio GSH/RSH bervariasi dari 0,19 hingga 0,64 berdasarkan jenis klon dan sistem sadap, lebih rendah dari literatur yang melaporkan setidaknya 93 % dari total -SH dalam lateks dalam bentuk glutathione dan cysteine (McMullen, 1960).

Pengamatan terhadap lima klon karet yaitu IRR 118, BPM 24, IRR 112, RRIC 100, dan PB 260 menunjukkan variasi nilai RSH antara 0,21 mM hingga 0,73 mM, lebih rendah dari literatur yang melaporkan kandungan RSH berkisar dari 0,5 hingga 0,9 mM (Jacob et al., 1984). Meskipun RSH meningkat pada semua klon yang diuji pada perlakuan frekuensi penyadapan tinggi (d1), variasi klonal dijumpai pada perlakuan stimulasi etefon. Klon IRR 118 lebih toleran terhadap KAS dibandingkan dengan klon BPM 24. Klon yang diuji menunjukkan respon klon yang spesifik terhadap stimulasi etefon dengan peningkatan RSH yang signifikan pada klon IRR 118 sementara pada klon BPM 14 stabil. Karakteristik klon dalam tanggap sistem sadap dan stimulasi merupakan parameter penting untuk mengoptimalkan hasil dan mencegah KAS.

Pada penelitian ini, data dikumpulkan empat bulan setelah penerapan perlakuan sistem sadap yang dapat dianggap sebagai cekaman jangka pendek atau sementara.

Peningkatan RSH disebabkan aktivitas metabolisme lateks yang lebih tinggi, tercermin dari kadar Pi yang tinggi, dan aktivasi sintesis glutathione sebagai respons terhadap akumulasi ROS. Komponen tiol lainnya yang tidak terdeteksi oleh reagen Ellman kemungkinan memiliki peran penting dalam tanggap sistem sadap sehingga perlu diselidiki. Dalam kondisi cekaman jangka pendek, biosintesis RSH berlangsung secara aktif karena ketersediaan karbohidrat dan energi yang memadai. Hal ini menunjukkan hubungan yang erat antara RSH, Suc, dan Pi. Menurut literatur, dalam kondisi stres jangka panjang, RSH umumnya lebih rendah pada perlakuan cekaman yang tinggi dibanding perlakuan cekaman rendah. Kondisi ini menunjukkan rendahnya kemampuan biosintesis RSH karena kurangnya substrat energi. Perbedaan nilai RSH pada kondisi stress temporer dan stress jangka panjang menunjukkan bahwa RSH dipengaruhi oleh durasi cekaman.

Pada kondisi intensitas KAS tinggi, RSH turun bersamaan dengan parameter fisiologis lainnya yang menunjukkan bahwa aktivitas metabolisme latisifer secara keseluruhan sangat rendah. Ketersediaan energi yang minim, tercermin dari kadar Pi yang rendah, menyebabkan biosintesis antioksidan termasuk RSH sangat terbatas. Secara umum, hasil penelitian ini menunjukkan bahwa RSH dipengaruhi oleh klon, jenis, dan durasi cekaman. Hal ini dapat menjelaskan mengapa RSH sulit diinterpretasikan dengan hanya mengetahui nilainya dalam kondisi tertentu. Data mengenai dinamika RSH dalam kondisi tanpa cekaman dan tingkat cekaman tinggi pada setiap klon dapat membantu untuk mengetahui tingkat cekaman yang dialami tanaman. Penelitian lain menunjukkan bahwa RSH juga ditentukan oleh umur tanaman,

kondisi tajuk, dan kondisi lingkungan. Hal ini menunjukkan bahwa untuk interpretasi RSH yang lebih baik diperlukan informasi sejarah sistem sadap, umur tanaman, persentase TPD, dan data parameter LD lainnya (TSC, Suc, dan Pi). Dengan menggabungkan faktor-faktor tersebut, dapat ditentukan apakah tanaman dalam kondisi cekaman sementara, cekaman jangka panjang, atau bahkan sudah dalam kondisi cekaman yang berlebihan.

RSH merupakan satu-satunya faktor antioksidan yang berubah secara signifikan seiring tingkat cekaman pada latisifer. Tidak ada perubahan signifikan pada antioksidan lain (AsA, DHA, GSH, dan GSSG) serta kofaktor (NADH, NAD, NADPH, dan NADP). Hal ini menunjukkan bahwa RSH tetap menjadi parameter antioksidan yang penting untuk mengkarakterisasi lateks *H. brasiliensis*. Rasio AsA/DHA dan GSH/GSSG akibat cekaman penyadapan masing-masing berkisar antara 1,09 hingga 2,64 dan dari 1,11 hingga 2,65. Hasil pengamatan belum memberikan data yang signifikan untuk menggambarkan tingkat oksidasi, regenerasi, dan biosintesis antioksidan pada kondisi cekaman penyadapan. Hal ini kemungkinan disebabkan tingkat dan durasi cekaman tidak cukup kuat untuk menginduksi perubahan metabolisme askorbat dan glutathione yang signifikan. Hal ini juga menimbulkan asumsi bahwa latisifer dilengkapi dengan sistem detoksifikasi yang kuat dalam menetralkan cekaman pada tingkat dan durasi tertentu. Penelitian lebih lanjut dengan jumlah ulangan yang lebih besar dan jangka waktu yang lebih lama diperlukan untuk mengetahui pengaruh fenologi tanaman dan jangka waktu cekaman penyadapan.

Hasil penelitian ini memberikan pemahaman yang lebih baik tentang regulasi antioksidan, terutama RSH, dan hubungannya dengan parameter fisiologis lainnya. Pada pohon yang sehat, kadar sukrosa menurun secara signifikan pada frekuensi sadap yang tinggi karena konsumsi yang cepat sebagai konsekuensi dari aktivasi biosintesis karet untuk meregenerasi lateks yang dikeluarkan. Kadar Pi yang tinggi menunjukkan tingkat aktivitas metabolisme yang tinggi dalam latisifer, memungkinkan peningkatan ketersediaan energi untuk biosintesis karet dan juga antioksidan termasuk RSH. Namun demikian, efek RSH pada stabilitas lutoid tidak signifikan terlihat dari IF dan PI yang tetap stabil. Penurunan TSC menunjukkan aktivitas transport air ke dalam latisifer yang menimbulkan efek pengenceran dan penurunan kadar padatan total dalam lateks. Hal ini juga menunjukkan regenerasi karet yang terbatas antara dua sadapan menyebabkan efek terhadap produksi yang tidak signifikan. Stimulasi etefon secara signifikan menurunkan PI dan meningkatkan lama aliran lateks. Hal ini menunjukkan efek kandungan RSH yang tinggi dapat memberikan perlindungan lutoid terhadap radikal bebas. Ketersediaan energi tercermin dari tingginya kadar Pi yang memungkinkan untuk mempertahankan tingkat biosintesis RSH. Peningkatan kadar Suc dalam lateks menunjukkan impor sukrosa secara aktif ke dalam latisifer sebagai kompensasi laju biosintesis karet. Efek pengenceran kandungan karet juga dijumpai pada perlakuan stimulasi etefon. Pada pohon yang mengalami KAS tinggi, produksi lateks menurun drastis. Dalam kondisi ini, ketersediaan karbohidrat dan energi sudah sangat rendah sehingga impor Suc dan air terbatas, tercermin dari kadar Suc yang rendah dan TSC

yang tinggi. Biosintesis RSH juga terganggu akibat keterbatasan energi menyebabkan terjadinya *luteoid bursting* dan koagulasi lateks, yang dikonfirmasi oleh PI yang tinggi.

Sebagai kesimpulan, penelitian ini menunjukkan bahwa RSH adalah parameter yang dipengaruhi multi faktor yang menyebabkan kesulitan dalam penafsiran datanya. RSH cenderung meningkat pada cekaman jangka pendek kemudian menurun pada stres jangka panjang, biasanya disertai dengan peningkatan kejadian KAS. Nilai RSH dalam satu titik produksi pohon dapat menimbulkan kekeliruan dalam interpretasi. Untuk interpretasi yang lebih baik, diperlukan referensi nilai RSH maksimum dalam kondisi tanpa cekaman dan nilai minimum dalam kondisi cekaman yang tinggi serta informasi terkait riwayat sistem sadap, umur tanaman, persentase KAS, dan data parameter LD lainnya. Analisis antioksidan tereduksi dan teroksidasi belum memberikan gambaran yang terkait tingkat oksidasi, regenerasi, dan biosintesis antioksidan. Penelitian lebih lanjut diperlukan dengan cekaman yang lebih ekstrim dan jangka waktu yang lebih panjang untuk mengetahui efek terhadap kadar antioksidan tereduksi dan teroksidasi.

Pada penelitian ini, QTL untuk kadar RSH, AsA, DHA, total AsA, dan rasio AsA/DHA tidak dapat dideteksi. Data menunjukkan heritabilitas tertinggi pada RSH 0,69 sedangkan heritabilitas sifat-sifat lainnya lebih rendah dari 0,2. Hal ini kemungkinan disebabkan cekaman yang rendah karena tanaman disadap d3 tanpa stimulan sejak buka sadap. Faktor lain adalah jumlah genotipe yang digunakan dalam analisis tidak mencukupi. Analisis pada cekaman yang lebih tinggi dan populasi yang lebih besar kemungkinan dapat memberikan hasil yang lebih baik.

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