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RESEARCH ARTICLE

PRECISE SELECTION OF SUPERIOR *HEVEA* GENOTYPES FROM 2014 HAND-POLLINATED PROGENY

Madigasekara MMSK[1], Withanage SP[2*], Liyanage KK[2], Dharmarathne TTD[1] and Meegahakumbura MK[1*]

[1]Department of Export Agriculture, Faculty of Animal Science and Export Agriculture, Uva Wellassa University, Badulla 90000, Sri Lanka

[2]Genetics and Plant Breeding Division, Rubber Research Institute, Agalawatta 12200, Sri Lanka

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ABSTRACT

The hybridization and selection in rubber (*Hevea brasiliensis*) aim to produce superior genotypes. The perennial nature of *Hevea* is the major limitation in rubber breeding. Early selection is crucial for strengthening and shortening the breeding program. Therefore, 20 randomly selected individuals of the 2014 hand-pollinated progeny were used for the current morphological, anatomical, biochemical, and gene expression study to identify superior genotypes for future recommendations. The average yield, girth, and bark thickness were measured as morphological characteristics, while sucrose, inorganic phosphorus, thiol, and polyphenol were measured as biochemical characteristics. Dry rubber and total solid content were also measured as diagnostic indicators of latex. The bark anatomical analysis was done with a modified staining protocol. Gene expression analysis was performed using the Catalase (*HbCAT*) and Superoxide dismutase (*HbSOD*) genes to evaluate the proneness for Tapping Panel Dryness (TPD). Results indicated that girth (79.8%), bark thickness (60.8%), bark anatomical parameters such as the diameter of latex vessels (60.1%) and the number of latex vessels per unit area (density) (89.2%) positively correlated with rubber yield. The genotypes 2014HP-11, 2014HP-42, 2014HP-78, 2014HP-25, 2014HP-102, 2014HP-35, 2014HP-39, and 2014HP-21 showed comparatively higher morphological characteristics. The genotypes 2014HP-56 and 2014HP-57 showed potential for stimulation due to their high sucrose and low inorganic phosphorus contents. All other genotypes showed below-average performance for commercial recommendations. The optimized bark anatomical screening protocol of the current study clearly stained the secondary latex vessels. Genotypes 2014HP-21 and 2014HP-98 showed higher TPD traits morphologically. Therefore, expression of *HbCAT* and *HbSOD* genes was tested. The fold difference value was upregulated in the genotypes 2014HP-21 and 2014HP-98. As a result, these two genotypes are possibly prone to TPD. In conclusion, this study optimized the bark anatomical screening protocol, identified promising high-yielding genotypes and pinpointed the possible vulnerability of two genotypes (2014HP-21 and 2014HP-98) to TPD.

Keywords: Bark Anatomical Staining, Catalase (CAT), Gene Expression, Rubber Breeding, Super-oxide Dismutase (SOD), Tapping Panel Dryness (TPD)

INTRODUCTION

The Para rubber tree [*Hevea brasiliensis* (Willd. ex Adr. de Juss.) Muell-Arg.] was first introduced to Southeast Asia from Brazil by Sir Henry Wickham, the father of rubber plantations (Priyadarshan *et al*., 2009). Rubber is an industrial crop of the Euphorbiaceae family, mainly cultivated in tropical climates (Priyadarshan and Cle´ment-Demange, 2004). Rubber is Sri Lanka's third-largest plantation crop based on the area under cultivation (Ranasinghe *et al*., 2020). A rubber tree has an economic life span of around 30 years; latex is the most valuable component extracted. The rubber industry plays an important role in the economy of Sri Lanka; its contribution to GDP was 0.3% in 2020 (Central Bank of Sri Lanka, 2020).

Corresponding authors: pamuditharama@yahoo.co.uk, muditha@uwu.ac.lk

Perennial nature is the main challenge for *Hevea* breeding. Conventional breeding operations developed hybridized progenies and subsequently screened them to identify superior genotypes (Pethin *et al*., 2015). The *Hevea* breeding process begins with an annual hand pollination program to develop new genotypes. One breeding cycle takes at least 30 –35 years to complete. It takes at least 4 years from hand pollination to make selections at the mother plant nursery. The selected outstanding genotypes taken to the small-scale clonal trials (SSCTs) will require another 12-15 years to evaluate their genetic potential. The genotypes with good genetic potential are evaluated at the commercial level with Regional Plantation Companies (RPCs) as Estate Collaborative Trials (ECTs/RRI) for an additional 10–12 years. After the ECTs, selected genotypes with excellent genetic potential are recommended under group III clones (Withanage *et al*., 2017).

Hevea is a tall (25-30 m), deciduous, and perennial ligneous tree (Priyadarshan and Cle´ment-Demange, 2004). The trunk is conical or cylindrical, and the bark is the most important part of the rubber tree, containing latex-producing tissues. Rubber bark consists of pith, wood, and cortex separated from the wood by cambium (Verheye, 2010). In laticifers, latex is produced and stored in the inner cortex, making it a major component of cytoplasm. These are specialized vessels made up of chains of adjacent cells in the phloem arranged as concentric sheaths with bark (Archer and Audley, 1986). The quantity of latex that a plant produces is influenced by several physiological and biochemical parameters, as well as the number, diameter, and anatomical characteristics of the latex vessels (Gunasekara *et al*., 2013). In rubber estates, harvesting system recommendations to optimize latex production are based on Latex Diagnosis (LD).

Identification of the anatomy of the bark is vital to selecting high latex vessels containing superior genotypes. Different staining protocols were used to determine latex vessel characteristics. The Rubber Research Institute of Sri Lanka introduced a staining protocol for young *Hevea* tissues and older *Hevea* bark (Wimalaratna, 1973). In Addition, Sudan III (Johansen, 1940) or Sudan III with Osmic acid was used to determine latex vessel characteristics (Souza *et al*., 1995). Furthermore, Safranin and Fast green FCF were used to determine the general histology of *Hevea* (Thomas *et al*., 1995). Only "Oil-red O" was also used to stain *Hevea* bark samples in the past (Gireesh and Pravitha, 2009). These protocols had their weaknesses and needed further improvements. Consequently, Safranin (Mihiran, 2006; Kalpani *et al*., 2020) or Aqueous Safranin in combination with Astra Blue (Ramos *et al*., 2016) was also utilized to enhance the *Hevea* bark staining. However, an effective bark anatomical staining protocol for rubber is not available to date.

Tapping Panel Dryness (TPD) is a physiological disorder (Herlinawati *et al*., 2022), and this rubber syndrome is characterized by a reduction in latex flow upon tapping or, eventually, a complete cessation. *Hevea* clone's susceptibility to TPD was found to be correlated with a few biochemical characteristics, including high levels of inorganic phosphorus and low sucrose (Putranto *et al*., 2015). TPD occurred late in low latex metabolism clones and early in high latex metabolism clones when high tapping frequency and "ethephon stimulation" were applied. Compared with healthy trees, TPD-affected trees exhibited fewer laticifer vessels, which may indicate a change in cambial activity (Putranto *et al*., 2015). Some antioxidant substances, including thiols, ascorbic acid, various peroxidases, and superoxide dismutase (*HbSOD*), are reduced in TPD-affected rubber trees. In laticifer cells, excessive oxidative stress can destabilize cellular membranes and trigger lutoid rupture, leading to in situ latex coagulation (Li *et al*., 2016). Additionally, catalase (*HbCAT*) plays a role in the response to wintering stress in rubber trees (Amarasekara *et al*.,2020). Therefore, *HbCAT* and *HbSOD* were selected as primers for this study.

The annual breeding programs aim to increase the pool of breeding *Hevea*. In 2014, progeny

was established through hand pollination at the mother plant nursery, as individual trees were used for this study to select superior *Hevea* genotypes and shorten the breeding cycle by conducting bark anatomical, biochemical, and molecular analysis.

MATERIALS AND METHODS

This study was conducted at the Genetics and Plant Breeding Department of the Rubber Research Institute of Sri Lanka (RRISL), Nivithigalakale, Matugama, which is located at approximately 6°30' N latitude and 80°15' E longitude.

The study evaluated twenty randomly selected genotypes from the 2014 annual hand pollination program (2014HP) (Table 1). A single seedling tree represents each genotype. The genotypes were managed according to the recommendation of RRISL (Annual report of 2014 Rubber Research Institute of Sri Lanka), and nine-year-old seedlings were subjected to the experiments.

Table 1: Randomly Selected 2014HP Genotypes used for the current study with their parentage

Genotype	Parentage
2014HP-11	GP 44-24 × RRISL
2014HP-21	GP 44-24 × RRISL
2014HP-25	GP 44-24 × RRISL
2014HP-35	GP 44-24 × RRISL
2014HP-39	GP 44-24 × RRISL
2014HP-42	GP 44-24 × RRISL
2014HP-46	GP 44-24 × RRISL
2014HP-56	GP 44-24 × RRISL
2014HP-57	GP 44-24 × RRISL
2014HP- 78	GP 44-24 × RRISL
2014HP-80	GP 44-24 × RRISL
2014HP-86	GP 44-24 × RRISL
2014HP-90	GP 44-24 × RRISL
2014HP-98	GP 44-24 × RRISL
2014HP-102	GP 44-24 × RRISL
2014HP-110	GP 44-24 × RRISL
2014HP-118	GP 44-24 × RRISL 2001
2014HP-136	GP 44-24 × RRISL 2001
2014HP-139	GP 44-24 × RRISL 2100
2014HP-149	GP 44-24 × RRISL 2100

Sources: Annual Review of 2014 Rubber Research Institute of Sri Lanka

Yield data [Grams per tree per tapping (g/t/t)] were collected using the Hamaker-Morris-Mann (HMM) micro tapping method at 45 cm above ground level, employing the S2d3 tapping system.

Latex was individually collected from each plant into plastic cups. Cup coagulants were pressed and dried in a smokehouse until they reached a constant weight. The mean grams per tree per tapping (g/t/t) for a given year was calculated as follows.

g/t/t = Mean intake per tapper/ Task size (Nugawela, 1998)
Mean intake per taper: Average amount of latex/rubber collected by one tapper (g/day).
Task size: Number of trees assigned to one tapper per day (trees/day) (de Jonge and Westgarth,1962)

Girth (cm) was measured in each genotype at a height of 90 cm above ground level using a measuring tape. Additionally, bark thickness (cm) was recorded at the same height for each genotype, with data collected from three sides of the trunk using a bark gauge.

Bark Anatomy

Based on the cluster analysis of morphological characteristics (yield, girth, and bark thickness) shown in Figure 6, nine highest-performing genotypes and three lowest-performing genotypes were selected for bark anatomical analysis.

The number of latex vessels per unit area (Density of latex vessels) and diameter of latex vessels were studied as bark characters using existing protocols (Mihiran, 2018) with some modifications (Madigasekara *et al*., 2024).

Bark samples were collected near the test tapping panel, with three blocks measuring 2 cm × 1 cm taken from each accession for initial preparation, and three slides prepared from each block. Bark samples were cut perpendicular to the axially oriented cells to avoid underestimating anatomical features when observing the laticiferous system. The bark samples were collected near the test

tapping panel with a chisel and hammer, ensuring minimal disturbance to the cambium of the plant (Figure 1). Subsequently, the samples were placed in the labelled Falcon tubes containing 70% alcohol to dehydrate the bark samples for 5 to 15 minutes (Shtein *et al*., 2023). A Formalin-Acetic Acid Alcohol (FAA) solution was used for the killing and fixation of bark samples. The standard properties of the FAA solution are as follows: 90 ml of Ethyl alcohol (70%), 5 ml of acetic acid, and 5 ml of formalin (Johansen, 1940; Shtein *et al*., 2023). Bark samples in Falcon tubes were filled with the FAA solution immediately after dehydration, and the minimum fixation time was 24 hours.

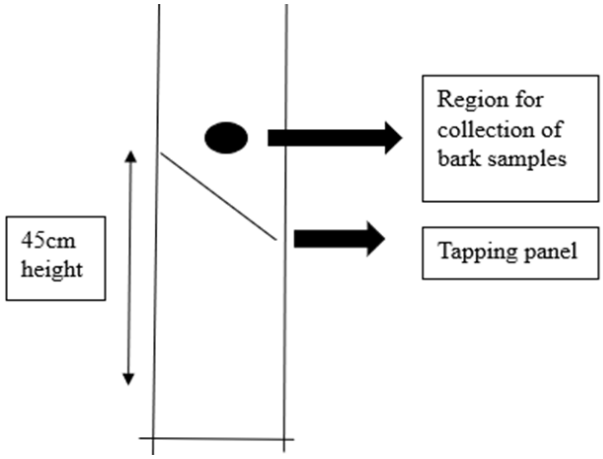


Figure 1: Sketch diagram of rubber stem showing the location to obtain bark samples

Paraffin was melted at a temperature of 60-65°C for embedding. The melted paraffin was poured into labelled blocks and left for a few minutes. Plastic ice cube molds were used to mould small, perfectly shaped paraffin blocks. Bark samples were soaked in 70% alcohol and cleaned with tissues. The bark samples were subsequently dipped into a liquid paraffin block using forceps, ensuring the soft bark faced downwards. The samples were kept at room temperature for half an hour to allow the paraffin to solidify. Following this, the paraffin block container was placed in the refrigerator for one hour to further harden the block, making it easier to remove it from the container.

The paraffin blocks were sharpened using a knife to fit the rotary microtome. A few sections of paraffin were cut off to expose the bark surface, and blocks were then placed in a beaker of cold water and refrigerated at 4°C for one hour.

Bark samples were prepared to a thickness of 10-15 µm in tangential longitudinal sections (TLS) using a rotary microtome (LAB-KITS SF-22580), and the sections containing soft bark were carefully separated using a smooth brush. Immediately after separation, the sections were placed into 70% alcohol.

The bark samples were stained using Sudan III for 20 to 30 minutes and samples were quickly washed with 50% alcohol (Johansen, 1940). The stained samples were mounted in glycerin and covered with a glass slide. The samples were visualized using a light microscope (Nikon Eclipse Ei). The microscopic images were analyzed with Image Focus 4 (Version 2.9) software.

Biochemical analysis

The standard method was used to measure sucrose, inorganic phosphorus, thiol, polyphenol, dry rubber, and total solids contents: The sucrose content of latex was measured using the method of Scott and Melvin (1953), inorganic phosphorus by Taussky and Shorr (1953), thiol content by Boyne and Ellman (1972), and polyphenol content following the procedure of Turkmen *et al*. (2006). Additionally, dry rubber content was determined using the standard method ISO 126:1995(E), while total solids content was measured according to ISO 124:1997(E) (International Standard Organization, 1984), as referenced by Anushka *et al*. (2019).

Clean, ice-chilled vessels were used to collect latex during the first 5 to 35 minutes after tapping. Approximately 2.5 g of latex was weighed using an analytical balance and coagulated with 2.5% trichloroacetic acid (TCA). The coagulant was squeezed, and the extract was collected in a 25 ml volumetric flask topped up with 2.5% TCA for the analysis of latex sucrose, inorganic phosphorus, thiol, and polyphenol contents.

Gene Expression Analysis

Total RNA Extraction

RNA extraction was performed using the TRIzol reagent according to the protocol described by Amarasekara *et al*. (2020). A volume of 250 µl of latex was collected into microfuge tubes containing TRIzol and mixed thoroughly. The samples were then transported to the laboratory, where the latex was crushed and subsequently stored at −20 ° C. After 20 minutes, samples were centrifuged, and the supernatant was transferred to a fresh microfuge tube. An equal volume (250 µl) of chloroform was added, followed by incubation at room temperature and a second round of centrifugation. The resulting aqueous phase was carefully transferred to a new microfuge tube, mixed with 400 µl of ice-cold isopropanol, and centrifuged again. The supernatant was discarded, and the resulting RNA pellet was washed with 95% cold ethanol and centrifuged. Finally, the ethanol was removed, the pellet was air-dried, and the RNA was resuspended in 25 µl of autoclaved diethyl pyrocarbonate (DEPC)-treated water.

Agarose Gel Electrophoresis to Visualize RNA

Agarose gel electrophoresis was performed to visualize the extracted RNA. The gel image was viewed using the "Infinity Vilaber Lourmattm" gel documentation system with "Vision Capture" software.

cDNA synthesis

cDNA was synthesized using high-capacity cDNA reverse transcription TaKaRa Prime Script™ RT Reagent kits, following the manufacturer's instructions. The reverse transcription mixture was prepared by mixing 2 µl of 10x RT buffer, 0.8 µl of 25x dNTP mix (100 mM), 2 µl of 10× RT random primers, 1 µl of MultiScribe TM reverse transcriptase, 10 µl of total RNA and 4.2 µl of nuclease-free water. The reaction was performed in a BIO-RAD Thermal cycler. The reaction mixture was incubated at 25°C for 10 minutes, followed by incubation at 37°C for 120 minutes. It was then annealed at 85°C for 5 minutes and subsequently held at 4°C. The cDNA samples were stored in a freezer at -20° C.

Selection of primers

Forward and reverse primers for the *HbMnCAT, HbMnSOD,* and GAPDH genes were selected for expression analysis (Table 2).

Table 2: Genes and corresponding primers used for gene expression analysis

Gene	Enzyme	Primer sequence
HbMnCAT	Catalase	Forward 5'GGTATTGTGGTTCCTGGTAT 3' Reverse 5'ATGGTGATTGTTGTGATGAG3'
HbMnSOD	Superoxide dismutase	Forward 5'TGTGCTGTAATGTTGACCTA3' Reverse 5'GTTCACCTGTAAGTAGTATGC3'
HbGAPDH	Glyceraldyhyde-3-Phosphate dehydrogenase	Forward 5'TGTGTCCGTCGTGGATCCGA3' Reverse 5' GACGCCTTATCCTTGTCAGTGAAC3'

Source: Wang *et al*. (2014)

Real-time PCR for gene expression

Gene expression analysis was performed using a real-time PCR detection system (BIO-RAD CFX96™, USA). A 10 µl PCR reaction contained 2 µl of Evagreen™ qPCR mix with

buffer, 0.5 µl of 10 nM forward primer, 0.5 µl of 10 nM reverse primer, 2 µl of first-strand cDNA, and 5.4 µl of nuclease-free water. Real-time PCR was conducted for both *HbMnCAT* and *HbMnSOD* genes using annealing temperatures of 54.4°C and 54.5°C, respectively. Three PCR samples for the target gene and three PCR samples for the housekeeping gene (GAPDH) from each genotype were taken as replicates. Two samples of no DNA template (NTC) DNA were used as controls.

Molecular Data Analysis

The Livak method ($2^{-\Delta\Delta CT}$) was used to analyze the relative expression of *HbMnCAT* and *HbMnSOD* genes for RT PCR.

Δ Control = CT Control $_{\text{Target gene}}$ – CT Control Housekeeping gene

Δ Treatment = CT Treatment $_{\text{Target gene}}$ – CT Treatment $_{\text{Housekeeping gene}}$

$\Delta\Delta CT = \Delta$ Treatment – Δ Control

Fold difference = $2^{-\Delta\Delta CT}$

RESULTS AND DISCUSSION

Morphological characterization (yield, girth, bark thickness)

According to the mean g/t/t values, the genotype 2014HP-21 exhibited the highest mean yield (142.31 g). Genotypes 2014HP-11, 2014HP-42, 2014HP-78, 2014HP-25, 2014HP-39, 2014HP-35 and 2014HP-102 demonstrated moderately high yields (115.82g-77.19 g). The 2014HP-56, 2014HP-57, and 2014HP-139 recorded the lowest yields 1.22 g, 1.20 g, 1.03 g, respectively. In contrast, genotypes 2014HP-86, 2014HP-80, 2014HP-149, 2014HP-90, 2014HP-136, 2014HP-110, 2014HP-46, and 2014HP-118 showed no g/t/t values due to the lack of latex. The highest mean girth was observed in genotype 2014HP-25 (77 cm), while the lowest mean girth was recorded in genotype 2014HP-90 (10.8 cm). The highest mean bark thickness was observed in genotype 2014HP-102 (10.3 mm), whereas the lowest was recorded in genotype 2014HP-139 (3 mm). The 20 genotypes randomly selected for this experiment exhibited varying g/t/t values, girth and mean bark thickness. Despite the recommended fertilizer applications, standard management practices, and planting in a uniform block of land, the variation exhibited in the said parameters is possibly due to the genotypic effect. Twelve genotypes with g/t/t values were used for the biochemical and bark anatomical studies (Table 3).

Table 3: Mean values of g/t/t, Girth and Bark Thickness of selected 2014 Hand-Pollinated (HP) genotypes

Genotype	Mean g/t/t Value (g)	Mean Girth (cm)	Mean Bark Thickness (mm)
2014HP-21	142.31	63	7.3
2014HP-11	115.82	65.25	9
2014HP-42	106.31	62	10
2014HP-78	104.69	56.75	9.7
2014HP-25	100.08	77	9.7
2014HP-39	88.17	62.5	8.3
2014HP-35	77.81	52.75	7.7
2014HP-102	77.19	66.5	10.3
2014HP-98	27.23	44	7.3
2014HP-86	-	12	4.1
2014HP-80	-	11.5	5
2014HP-149	-	12.5	5
2014HP-90	-	10.8	4
2014HP-136	-	11	4
2014HP-110	-	14	4.7
2014HP-46	-	15	4
2014HP-118	-	14	3.3
2014HP-56	1.22	14	4
2014HP-57	1.20	16.25	5
2014HP-139	1.03	14	3

Pearson correlation analysis

Correlation results for girth, bark thickness, average dry rubber content, diameter of latex vessels, and number of latex vessels/unit area

The pairwise Pearson correlation analysis, performed using Minitab 19 software, revealed that variables such as girth, bark thickness, average dry rubber content, diameter of latex vessels, and the number of latex vessels per unit area were significantly correlated with other variables at the 0.05 significance level.

Correlation between yield and girth

There exists a strong positive correlation between yield and girth, with an R^2 of 79.8% (R^2 = Square of the regression coefficient of determination). This suggests that plant girth can be considered a significant positive predictor of latex yield (Figure 2).

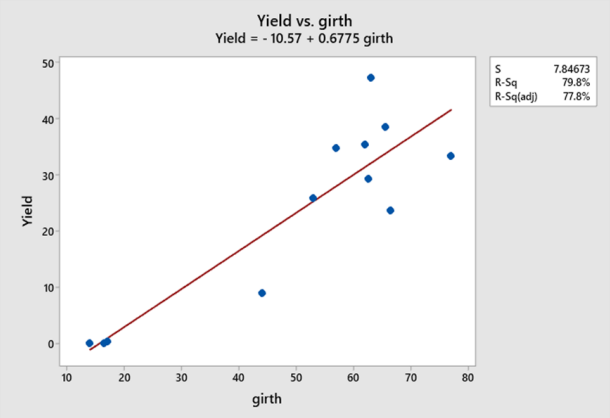


Figure 2: Variation of yield with girth
The correspondence regression equation is Yield= - 10.57+0.6775 girth.

The yield of the rubber trees can vary depending on the age of the tree, environmental conditions, and biochemical factors, and this pattern is genotype-specific (Nguyen *et al*., 2016). Consequently, this study demonstrated a strong positive correlation between latex yield and girth. The previous study conducted by Karunarathna in 2006, using RRIC 100, RRIC 121 and PB 86 clones, also demonstrated a positive correlation between yields and girth. Additionally, Vijayakumar *et al*. (2000) and Karim (2007) also observed a positive correlation between latex yield and plant girth. The increasing girth enhanced the average latex yield; however, this can vary due to climatic conditions and agronomic practices (Karunaratne *et al.,* 2006; Sant' *et al*., 2020). Furthermore, according to the 1998 hand-pollinated progeny, 65 new genotypes were assessed along with control clones such as RRIC 121, RRIC 130 and RRISL 205. Of these, 63% of the genotypes were recorded as having significantly higher or similar average yields compared to the control clones, while 82% exhibited significantly higher or similar mean girth compared to controls (Anushka *et al*., 2019). In contrast, with the clones PB 260, GT 1, PB 217, and AF 261, it was discovered that yield and girth had a negative relationship (Lacote *et al*., 2004).

Correlation between yield and bark thickness

There is a moderate positive correlation between yield and bark thickness, with an R^2 of 60.8%. This indicates that bark thickness has a positive influence on yield (Figure 3). In the current study, a moderate positive correlation was reported between yield and bark thickness. Similarly, Mihiran (2006) also reported a moderate positive correlation between yield and bark thickness. Additionally, Sankarimal *et al.* (2009) observed significant clonal differences in bark thickness, with the values ranging from 5.23 mm to 8.30 mm.

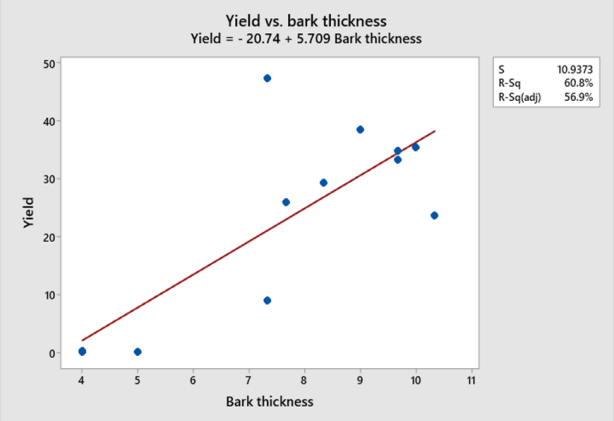


Figure 3: Variation of yield with bark thickness
The correspondence regression equation is Yield= - 20.74 +5.709 bark thickness.

Correlation between yield and diameter of latex vessels

The diameter of latex vessels positively correlates with latex yield, as demonstrated by a 60.1% R-squared value. This indicates that there is a moderate positive correlation between latex yield and vessel diameter (Figure 4). When considering the relationship between yield and the diameter of latex vessels, this study demonstrated a moderate positive correlation. Mihiran (2018) indicated that the yield and diameter of latex vessels have a negative correlation with mean diameter negatively affecting latex yield. Tan *et al*. (2019) also suggested that laticifer diameter have a negative correlation with rubber yield. This indicates that the diameter of latex vessels does not affect latex yield, contrary to what was observed in this study. However, in the current study, we were able to observe and measure the latex vessel diameter very clearly as a result of our

modified staining protocol. This is possibly instrumental in giving the moderate positive correlation observed between yield and latex vessel diameter in the current study.

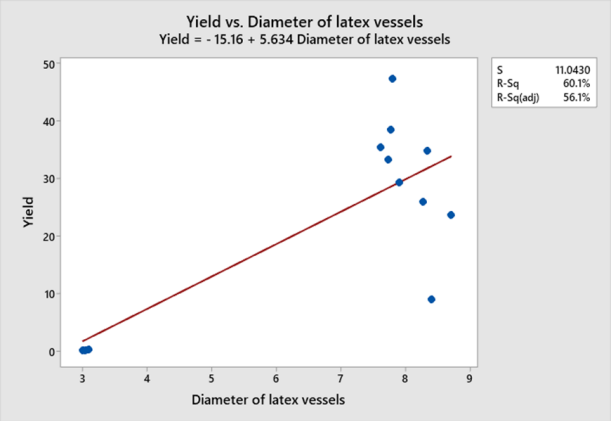


Figure 4: Variation of yield with diameter of latex vessels
The correspondence regression equation is Yield= - 15.16 + 5.634 bark thickness.

Correlation between yield and number of latex vessels/ unit area (Density)

According to the analysis of yield and latex vessel density, latex vessel density is positively correlated with latex yield, demonstrating an R-squared value of 89.2%. This indicates a strong positive correlation between yield and latex vessel density (Figure 5). Wycherley (1969) and Gomez (1982) showed a positive correlation between the latex vessel density and latex yield. This study obtained results similar to those of Wycherley and Gomez.

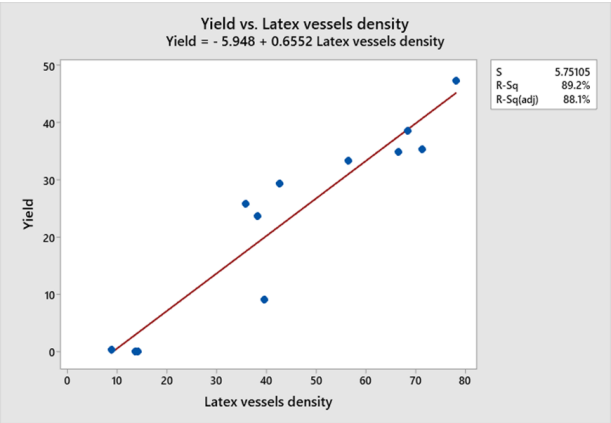


Figure 5: Variation of yield with the number of latex vessels per unit area (Density).
The correspondence regression equation is Yield= - 5.948+ 0.6552 latex vessels density.

Cluster analysis for yield, girth, bark thickness, diameter of latex vessels, and number of latex vessels/unit area

This includes a cluster analysis of all characteristics assessed, such as yield, girth, bark thickness, the diameter of latex vessels, and the number of latex vessels per unit area. Genotypes 2014HP-11, 2014HP-42, 2014HP-78, 2014HP-25, 2014HP-21, 2014HP-35, 2014HP-39, 2014HP-102 and 2014HP-98 were included in cluster one, while genotypes 2014HP-56, 2014HP-139, and 2014HP-57 were included in cluster two (Figure 6).

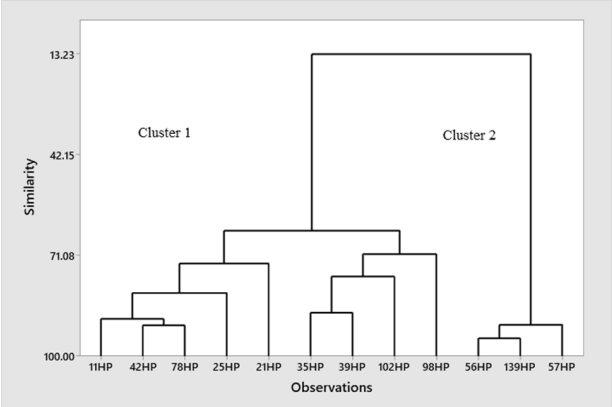
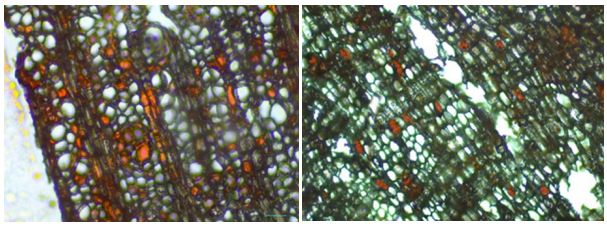


Figure 6: Yield, girth, bark thickness, the diameter of latex vessels, and the number of latex vessels/units' area-based cluster analysis.
Cluster 1:2014HP-11, 2014HP-42, 2014HP-78, 2014HP-25, 2014HP-21, 2014HP-35, 2014HP-39,2014HP-102, 2014HP-98 Cluster 2: 2014HP-56, 2014HP-139, 2014HP-57

Light Microscopy Images of Latex Vessels of *Hevea* Bark Cross Sections

The structural organization of *Hevea* bark was observed before the measurements were taken. The latex vessels are arranged in concentric cylinders throughout the phloem tissue. In cross-section, these cylinders present as rings of latex vessels. The smooth, thick, and straight cell surfaces featuring secondary articulated laticifers are distinguished from the vascular cambium. They are arranged in rings parallel to the vascular cambium within the secondary phloem of the trunk. In the cross-section of bark, laticifer vessels appear circular or slightly irregular in shape, closely opposed to the neighboring parenchyma. The cells that appear orange in color in cross sections of the

following high-yielding (plate 1.a) and low-yielding (plate 1.b) genotypes are latex vessels.



(a)- 2014HP-21 (b)- 2014HP-139

Plate 1: Cross section of high-yielding (a) and low-yielding (b) bark samples

According to bark anatomical analysis, Mihiran (2006) and Kalpani *et al*. (2020) conducted their *Hevea* bark anatomical studies using a coloring agent, Safranin. However, every lignified, cutinized, suberized and chitinized structure can be stained with safranin in addition to latex vessels. Therefore, latex vessels cannot be clearly identified. In contrast, Sudan III can be used to stain lecithin, resins, latex, wax, and cuticles, while chloroplasts display a dull red color (Johansen, 1940). Three lipid classes exist in *Hevea,* including neutral lipids, phospholipids and glycolipids. These lipids are distributed non-homogeneously within latex fractions, with neutral lipids being the predominant species (49-68%) among the total lipids in *Hevea* latex (Bottier, 2020). Sudan III is a fat-soluble dye that primarily reacts with neutral lipids. Therefore, Sudan III can be used as the best stain to observe latex vessels. Despite Premakumari *et al*. (1985) and Vinod and Thomas (2006) conducted bark anatomical studies using Sudan III as a coloring agent, no clear and precise protocol is available to date. Therefore, this study focused on optimizing the *Hevea* bark anatomical protocol, and the newly modified protocol presented in the methodology section enables more precise observation of secondary latex vessels compared to earlier protocols.

Physiological and Biochemical Parameters

Biochemical parameters were evaluated as presented in Table 4. Among them, the genotypes 2014HP-21, 2014HP-11, 2014HP-42, 2014HP-78, 2014HP-25, 2014HP-39, 2014HP-35, 2014HP-102 and 2014HP-98 exhibited comparable values for latex physiological and biochemical parameters. These results align with the recorded higher yields. Only the 2014HP-56 and 2014HP-57 genotypes differ, as they exhibit elevated levels of sucrose and comparatively low inorganic phosphorus, which may potentially allow for stimulation. A rubber clone has a low latex metabolism and a high latex production potential if it has high sucrose and low inorganic phosphorus contents. The use of ethephon is necessary to activate the latex metabolism for this type of clone. In contrast, a rubber clone has active latex metabolism if it has low sucrose and high inorganic phosphorus contents. Low metabolism clones are more TPD -tolerant, and active metabolism clones are usually more susceptible to TPD (Putranto *et al*., 2015).

Table 4: Results of the latex physiological and biochemical parameter evaluation study

Genotype	Sucrose content (Mm)	Thiol content (mM)	Inorganic Phosphorus (Mm)	Polyphenol (Mm)	DRC%	TSC%	Total volume (Mm)
2014HP-11	2.88	0.39	4.80	0.14	44.51	47.49	50
2014HP- 21	3.14	0.37	9.39	0.14	38.91	41.74	135
2014HP-25	4.10	0.31	4.36	0.17	45.49	48.79	17
2014HP-35	1.93	0.37	12.80	0.16	36.30	39.22	25
2014HP-56	10.23	0.69	0.71	0.21	20.63	-	4
2014HP-57	12.97	0.36	4.36	0.21	33.23	-	4
2014HP-78	6.92	0.33	6.23	0.14	38.46	41.58	139
2014HP-102	1.00	0.37	4.67	0.14	41.81	44.34	82
2014HP-39	6.95	0.28	5.13	1.66	50.22	53.40	13
2014HP-42	2.52	0.28	18.76	1.21	47.40	49.34	12
2014HP-98	2.11	0.36	3.73	1.35	39.11	-	4
2014HP-139	-	0.76	-	2.20	29.56	-	1

Proneness to Tapping Panel Dryness (TPD)

Genotype 2014HP-21 was the highest-yielding genotype and had a longer duration of latex flow compared to the other genotypes. Natural rubber clones with generally high yields are often susceptible to tapping panel dryness. When the harvesting of a rubber tree exceeds its physiological capacity of regeneration, it usually leads to TPD (Hiriwala and Nugawela, 2016). The genotype 2014HP-98 exhibited visible symptoms of tapping panel dryness during the micro-tapping period (Plate 2). However, as the present study was conducted at the mother plant nursery stage with approximately nine-year-old trees, gene expression analysis should be repeated on these two genotypes, as further investigation is required to confirm their proneness to TPD characteristics.

Plate 2: Visual observation of tapping panel dryness-prone 2014HP-98 genotype

Gene expression study

Agarose Gel Electrophoresis

After the extraction of RNA from 2014HP-21, 2014HP-98 and 2014HP-102 genotypes, agarose gel electrophoresis was used to confirm the presence of RNA (Figure 7). The 2014 HP-102 genotype, which exhibited medium morphological performance, was used as the control. The presence of RNA from 2014HP-21, 2014HP-102 and 2014HP-98 genotypes was confirmed as the bands corresponding to all genotypes are present in the agarose gel.

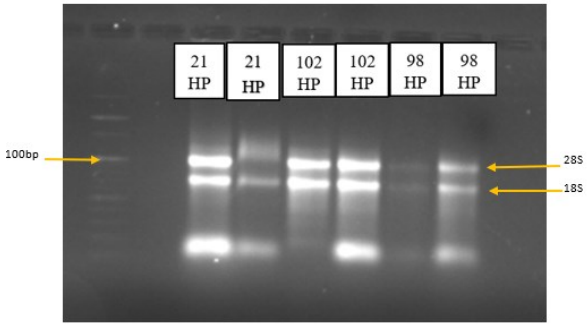


Figure 7: Agarose gel electrophoresis of RNA related to 2014HP-21, 2014HP-102 and 2014HP-98

Quantitative Real-Time PCR for the *HbMnCAT* and *HbMnSOD* Gene Expression

Reactive oxygen species (ROS) scavenging systems are involved in various kinds of biotic and abiotic stresses. These excessive environmental and harvesting stress ROS-scavenging systems cause an overproduction of ROS that cannot be overcome (Leclercq *et al*., 2012). ROS are involved in the coagulation of rubber particles, which reduces natural rubber production. High ROS production in latex cells triggers oxidative stress and damages cellular membranes, especially lutoids, which contain coagulant factors involved in the aggregation of rubber particles in latex cells, resulting latex flow is either partial or complete stopped. That is, due to lutoid decompartmentalization, it releases various proteins that can join rubber particles together. A lectin-like protein that bridges between rubber particles is most likely involved in the mechanism of latex coagulation. In extreme circumstances, as ROS induce senescence, it leads to the formation of brown bast in the rubber bark tissues. These two symptoms can be called tapping panel dryness (Leclercq *et al*., 2012; Montoro *et al*., 2018; Putranto *et al*., 2015).

Consequently, the gene expression study was conducted using the stress tolerance genes *HbMnCAT* and *HbMnSOD* to confirm whether these two genotypes were more prone to tapping panel dryness. To determine the expression level of the *HbMnCAT* and

HbMnSOD genes, GAPDH was used as the housekeeping gene. GAPDH is one of the widely used housekeeping/ reference genes which normalizes fold changes (Luke *et al*., 2015).

In rubber trees, the up-regulation of several TPD-related genes may lead to programmed cell death, while down-regulation of the genes that encode a translocase of the mitochondrial outer membrane also occurs in TPD trees. This may affect mitochondrial metabolism and impair biosynthesis (Venkatachalam *et al*., 2007).

The *HbMnCAT* and *HbMnSOD* genes were successfully amplified during the Quantitative Real-Time PCR (qRT-PCR). For the 2014HP -21 and 2014HP-98 genotypes, the fold difference value for the *HbMnCAT* gene was 1.80 and 1.17 respectively (Table 5). The *CAT* gene (*HbMnCAT*) expression is usually used to screen the stress responsiveness in rubber clones. Amarasekara *et al*. (2020) reported that the fold difference of the *CAT* gene was significantly upregulated in clones RRISL203 and RRISL Centennial 3 during the wintering period. These two clones can be considered stress-susceptible. Additionally, the *CAT* gene exhibited downregulation in the *Hevea* clones RRIM 600, RRII 105, RRII 414 and RRII 208 under drought conditions. It means all these clones were drought-tolerant (Luke *et al*., 2015). Previous studies also show that *CAT* gene expression was upregulated in Tapping Panel Dryness-affected rubber trees (Bandara *et al*., 2018). Irulappan *et al*. (2023) recently reported that TPD-affected clones (RRII105, PB285, PB217, and PB235) show an upregulation of *CAT* gene expression in Kerala, India. Interestingly, both genotypes, 2014HP-21 and 2014HP-98 showed slight upregulation in *CAT* gene expression in the current study (Table 5).

The fold difference values for the *HbMnSOD* gene were 1.48 and 1.37 in the 2014HP-21 and 2014HP-98 genotypes, respectively (Table 6). The fold difference values of both *HbMnCAT* and *HbMnSOD* gene expressions were greater than 1, indicating that the 2014HP-21 and 2014HP-98 genotypes were possibly upregulated. However, it is better to repeat this experiment before giving solid conclusions.

Superoxide dismutase (SOD) is a vital metalloenzyme associated with drought tolerance in plants. Yet, regular *SOD* genes are not available in rubber. However, nine *HbMnSOD* genes have been identified in the rubber genome (Yu *et al*., 2022). According to Yu's study, most *HbMnSOD* genes were found to be significantly up-regulated during drought stress. In addition, *HbSOD* genes have also been studied in TPD studies of rubber. For instance, Leclercq *et al*. (2012) reported cytosolic *HbCuZnSOD* gene was expressed at a lower level in the TPD-resistant clones PB235 and GT1 when compared to the TPD-susceptible clone RRIM 600. In this study, the expression of the *HbMnSOD* gene was slightly upregulated in the genotypes 2014HP-21 and 2014HP-98 (Table 6).

Table 5: Fold Difference of *HbMnCAT* Gene for the Selected Genotypes of 2014HP Progeny

Genotype	Ct mean *CAT*	GAPDH	ΔCT	ΔΔ CT	$2^{-\Delta\Delta C}{}_T$
2014HP-21	21.24	14.09	7.155	Treatment-(-0.85) Control- 8.005	1.80
2014HP-98	20.285	12.51	7.775	Treatment-(-0.23) Control- 8.005	1.17

Table 6: Fold Difference of *HbMnSOD* Gene for the Selected Genotypes of 2014HP Progeny

Genotype	Ct mean *SOD*	GAPDH	ΔCT	ΔΔ CT	$2^{-\Delta\Delta C}{}_T$
2014HP-21	22.68	19.26	3.42	Treatment- (-0.57) Control- 3.99	1.48
2014HP-98	19.24	15.71	3.53	Treatment- (-0.46) Control- 3.99	1.37

Therefore, gene expression analysis of *HbMnCAT* and *HbMnSOD* indicates that both genotypes 2014HP-21, and 2014HP-98 are possibly susceptible to tapping panel dryness. However, as the fold difference reported in the current study is less than 2, further studies are needed to confirm these results.

CONCLUSION

Girth, bark thickness, and average yield data were collected from 20 randomly selected genotypes of the 2014HP progeny, and 8 genotypes that reported low yields were excluded from the analysis. The selected 12 genotypes were also included for an anatomical analysis of the bark. The results of the regression analysis showed strong positive correlations between rubber yield and variables such as girth and latex vessel density. Additionally, latex diagnosis was performed by assessing biochemical parameters. High-performing genotypes (2014HP-11, 2014HP-25, 2014HP-78, and 2014HP-42) identified in the current study using multiple analysis could be moved directly to ECT evaluation without prior assessment under SSCT levels, allowing the breeding cycle to be reduced by 12 to 15 years. The expression of *HbMnCAT* and *HbMnSOD* genes indicated that the genotypes 2014HP-21 and 2014HP-98 are possibly prone to tapping panel dryness. However, these two genotypes require further testing on a small scale to verify the gene expression results before considering their removal from the breeding pool or recommending an alternative tapping system. The modified bark anatomical staining protocol presented in the current study could be effectively used in future.

AUTHOR CONTRIBUTION

SPW conceptualized and designed the study. MMSKM performed the experiment and analyzed the data. KKL assisted the bark anatomical staining. MKM, SPW and TTD supervised the study. MMSKM wrote the first draft. MKM, SPW, TTDD and KKL critically revised the manuscript.

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