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The dynamics of non-structural carbohydrates and involved enzymes in relation to the latex yield of rubber trees

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Abstract

Rubber trees (*Hevea brasiliensis* Muell. Arg.) are a major crop of economic importance in Thailand. The main product harvested from the rubber trees is latex. Latex production depends on a biochemical process for synthesis, degradation, mobilization, and the storage of nonstructural carbohydrates (NSCs), such as starch and sucrose. The rubber trees demand carbohydrates and enzyme activities for growth, maintenance, and latex synthesis. Therefore, with the appropriate management of the taping system, the balance between growth and latex synthesis in the trees should be maintained since it is regarded as a balance between use and formation of NSCs, which is controlled by the involved enzymes. This research aimed at studying the effects of downward and upward tapping systems on NSCs content and on the activity of the enzymes in the wood and the bark of rubber tree trunks given that the two systems are related to latex yield. It was found that the latex yield was higher with the upward tapping system as compared to downward tapping. Furthermore, upward tapping had induced more sucrose synthase (SuSy) activity, which is involved with sucrose degradation, than downward tapping had. It was, therefore, concluded that the sucrose dynamic had depended on SuSy, which is the key enzyme located in bark with upward tapping. The sucrose had functioned as the local buffer in the bark, which is related to the balance system of high starch hydrolysis in the wood to produce higher rubber biosynthesis in latex cell metabolism with upward tapping.

Keywords: Non-structural carbohydrates (NSCs), Enzyme activities, Latex yield, *Hevea brasiliensis* Muell. Arg., Tapping methods

1. Introduction

Non-structural carbohydrates (NSCs), soluble sugars, and starch are the carbon source used for plant metabolism, growth, maintenance, and storage [1]. Sucrose plays a central role in the plant and is translocated via the phloem from the leaves (source tissue) to the stems, fruits, and the roots (sink tissues). Starch is stored in the chloroplast of the leaves or in the plates of parenchyma tissues called amyloplasts in the bark and wood. In addition, some plants store starch and/or sucrose in their fruits. The stored carbohydrates play an important role in plant metabolism during bud break and during early vegetative growth when they are the only source of carbohydrates [2,3]. They are also mobilized to face environmental stresses, such as water stress or pest infestation [4]. On the whole, they play a crucial role in a tree's long-term survival [5,6]. Rubber trees represent a singular and interesting case since they are intensively tapped to harvest latex, which is made of 40% dry rubber particles [7]. The regeneration of the exported latex, therefore, induces an artificial carbohydrate sink that competes with

the “natural” sinks (maintenance, growth, and reproduction). Hence, the dynamics of NSCs play a significant role in the long-term production of latex. Previous research has indicated that latex tapping enhances trunk starch reserve, while it decreases trunk radial development when compared to untapped trees. Starch plays a key role as a long-term reserve in wood and as a local buffer to keep the dynamic of balancing starch content in the bark. Soluble sugars, especially sucrose, function as an intermediary for latex production in the bark and for starch synthesis in the wood [5,6].

However, these results were established by comparing the carbohydrate contents in the wood and bark at various times. Yet, the results did not provide clues on when and where the starch and soluble sugars are synthesized or used. Indeed, the dynamics of NSCs during latex tapping periods are governed by enzymes that function as catalysts in several biochemical reactions.

Sucrose synthase (SuSy) and invertase (INV) are the primary enzymes involved in sucrose catalysis for the breakdown of sucrose, whereas sucrose phosphate synthase (SPS) is used in sucrose synthesis [8]. ADP-glucose phosphorylase (AGPase) and amylase (AMY) are used in starch synthesis and degradation, respectively [9].

Thus, the objective of this study was to explore the NSCs content and to determine the specific enzyme activities that are connected to long-term yield potentials in tapped rubber trees. To better understand the relationships between the NSCs content and the enzyme functions, the tapping systems, the locations on the trunks, and the seasons (low-yield and high-yield periods) in the bark, the wood, and the latex cells of rubber tree trunks were compared.

2. Materials and methods

2.1 Plant materials and experimental conditions

Rubber trees (*Hevea brasiliensis* Muell. Arg.), clone RRIM600, were studied at the Chachoengsao Rubber Research Center at the Rubber Authority of Thailand (CRRC-RAOT) in Eastern Thailand. The experimental design was a one-tree plot design with 3 trees per treatment. Two latex tapping methods were compared in the field as follows: 1) downward tapping in a half-spiral (S/2 d3, 1 of day tapping and 2 days of rest, a half spiral cut with 2.5% ethylene stimulation) and 2) upward tapping in a quarter-spiral (S/4U d3, 1day of tapping and 2 days of rest, a quarter spiral cut with 5% ethylene stimulation). These two tapping systems are generally employed throughout the economic life of the trees. For the first treatment, downward tapping was carried out for 6 years (4 years on panel A and then 2 years on panel B). For the second treatment, upward tapping was performed in the 11^{th} year of tapping, on panel C after 10 years of downward tapping (6 years of panel A and 4 years on panel B). This tapping panel management is the one of the most commonly applied in the field. Discrepancy in the age of trees at the time of sampling was due to the tapping sequence along the lifespan of the trees.

For each tapping system, 3 untapped trees were included as the internal controls. Samples were taken during the two seasons: at the start of the tapping season in May and during the high-yield period of the tapping season in October (Figure 1).

2.2 Sampling position on the rubber tree trunks

According to the tapping panel history of each system, the bark and wood on the trunks of the rubber trees were collected so that the sampling position could be either within or far away from the latex regeneration area or the drainage area (the bark area where the latex flows out after tapping) (Figure 1). Therefore, after tapping, the drainage area is located below the tapping cut in downward tapping and above of tapping cut in upward tapping. For the trees tapped in the downward manner, samples were then taken below the tapped panel, then in the latex regeneration area [5], or above the opposite panel, which had been untapped for 2 years at that time. Similarly, for the trees tapped in the upward manner, the samples were taken above the tapped panel, then in the regeneration area for that treatment, and on the opposite side of the tree, which had been untapped.

For the NSCs assays, the bark and wood were drilled with a borer (for the bark: diameter of 0.5 cm and a length of 1 cm and for the wood: diameter of 0.5 cm and a length of 4 cm). The bark was drilled by using an auger (with a diameter of 4 cm and a length of 1 cm) and wood was drilled by borer (with a diameter of 0.5 cm diameter and a length of 4 cm).

The samples were immediately immersed in liquid nitrogen. For the NSCs assays, samples were dried using a freeze dryer at 400 mTorr (-80°C for 24 h). The dried samples were milled with liquid nitrogen. Next, the powder samples were stored at -20°C. For the enzyme assays, the samples were milled with liquid nitrogen, and then they were stored at -80°C.

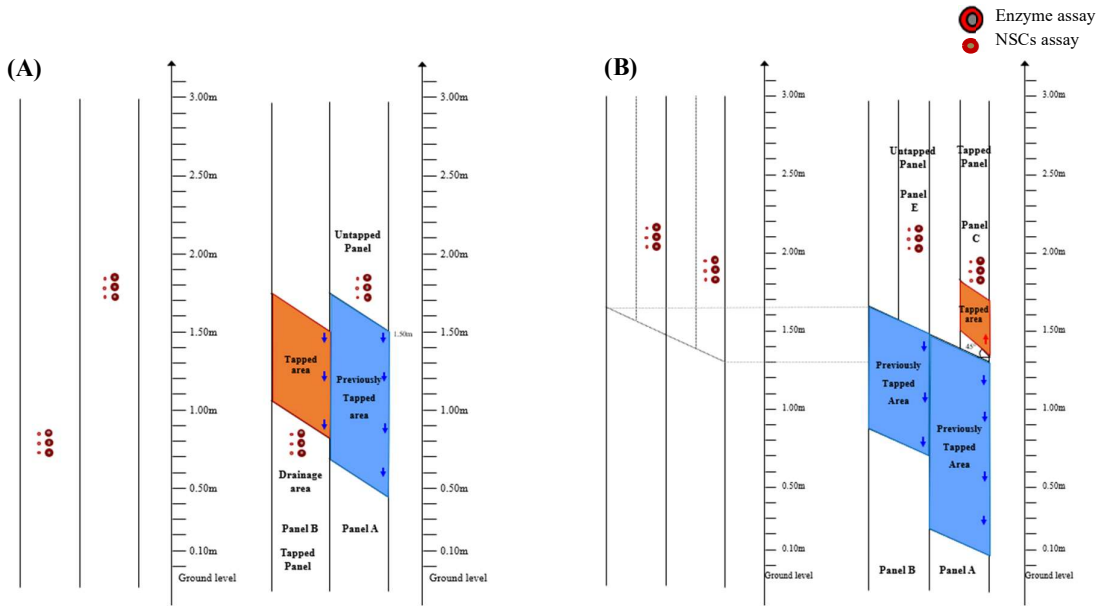

Figure 1 Sampling positions on the rubber tree trunk of untapped and tapped trees in downward tapping (A) on untapped panel (Panel A) and tapped panel (Panel B), and upward tapping (B) on untapped panels (Panel E) and tapped panel (Panel C) for the NSCs assay and the enzyme assay.

2.3 Biochemical analysis

2.3.1 The extraction of Non-structural carbohydrates (NSCs) and the assay

Samples were completely dried in an oven at 65°C for 2 h in order to extract the soluble sugars. At room temperature, the soluble sugar was extracted from a 20 mg sample using 1 ml of ethanol 80% twice for 30 min and ethanol 50% once for 30 min. Supernatants were mixed and filtered via mini-columns. The ethanol evaporated in an oven at 65°C for 24 h. The remaining pellet after sugar extraction was dried at 65°C for 2 h for starch analysis. After that, it was extracted and gelatinized at 90°C for 90 min with 0.02 N NaOH. α-Amyloglucosidase was used for starch hydrolysis at 50°C for 90 min to generate glucose. The soluble sugar and starch content were quantified by using hexokinase (HK), glucose-6-phosphate dehydrogenase (G6P-DH), and phosphoglucose isomerase (PGI) with adenosine triphosphate (ATP) and the oxidized form of nicotinamide adenine dinucleotide phosphate (NADP). The obtained product was NADPH (reduced form), which was followed by spectrophotometry at 340 nm [5].

2.3.2 Enzyme activity extraction and assays

Polyvinyl polypyrrolidone (PVPP) was mixed with the fine powder of the sample in a 1:1 (w/w) ratio. The extraction buffer at 10 ml (50mM Hepes, 5mM $MgCl_2$, 1mM ethylene diamine tetra-acetic acid (EDTA), protease inhibitor cocktail, 5mM dithiothreitol (DTT), pH 7.0) was added to the sample and blended for 5 min. Next, the pellet sample was separated by centrifugation at 10,000 rpm at 4°C for 10 min. The pellet was re-extracted as previously explained. Supernatants from the first and second extractions were pooled. The dissolved enzymes in the supernatant were precipitated using $(NH_4)_2SO_4$ at between 20% to 80% saturation for protein precipitation. The protein pellet contained the enzymes to be studied, and their activities were measured using an enzyme assay method that had been modified for SuSy [10], INV [11], SPS [12], AGPase [13], and AMY [14].

2.3.3 Protein assay

The total protein concentration was measured using Bio-Rad protein assay kits, which had been referred to from the Bradford dye-binding method [15].

2.3.4 Latex biochemical parameters and latex yield

According to Jacob et al. [16-18], the biochemical ability of latex cells to produce rubber is based on their sucrose and inorganic phosphorus contents. On one hand, the sucrose content reflected the balance between the

sucrose consumption by the latex cells for energy production, latex biosynthesis, and the transfer of sucrose from the apoplast to the latex cells. On the other hand, the inorganic phosphorus content indicated the intensity of metabolic activity in the latex cells.

The latex was collected under the tapping cut (downward tapping) or above the tapping cut (upward tapping) in the drainage area (the area from which the latex comes from after tapping). Sucrose (Suc) and inorganic phosphorus (Pi) content were both measured by using the method developed by Centre de cooperation internationale en recherche agronomique pour le developpement (CIRAD) [16]. The sucrose and inorganic phosphorus contents were measured tree by tree.

The sucrose content was measured using the method from Ashwell et al [19], in which 0.4 mL. of 2.5% TCA and 3 ml of 5 mM anthrone were added to 0.1 ml of latex serum. The mixed solutions were incubated at 90 ºC for 10 min. The mixture was analyzed by spectrophotometer at the wavelength of 627 nm. Sucrose was used as the standard reagent in the range of 0.25 - 1.50 mM.

The Pi content was measured using the method from Taussky and Shorr et al [20] as follows: 1 ml of 2.5% TCA and 3 ml of vanadate/molybdate (2.56 mM ammonium metavanadate and 30.6 mM ammonium molybdate) were added in 0.5 ml of latex serum. The mixed solution was kept for 10 min. The mixture was analyzed by spectrophotometer at the wavelength of 410 nm. Potassium dihydrogen phosphate (KH_2PO_4) was used as the standard reagent in the range of 1 - 5 mM.

The latex yield was measured by weighing the cumulative coagulated rubber (cup lump) every month and was expressed as a gram of latex per tree per tapping per cm (g/t/t/cm) in both the downward and upward tapping methods.

2.3.5 Statistical analysis

The different treatments were analyzed for statistical significance by using Tukey's honestly significant difference (HSD) test with a *p*-value (less than 5%, $p < 0.05$). The statistical analyses of the biochemical parameters were expressed by using Xlstat software (2018.6 version, Addinsoft, 295 Paris, France).

3. Results and discussion

3.1 The contents of the non-structural carbohydrates (NSCs) and enzyme activities in the bark and the wood of the rubber tree trunks tapped with downward and upward tapping methods

The results of NSCs showed that the starch content had been significantly higher in the wood than in the bark, whereas the soluble sugar contents (sucrose and hexose) had been significantly higher in the bark than in the wood with both tapping methods (Figure 2A and 2B). This was consistent with results from Chantuma, et al [5,21] indicating that starch had been used as a carbon reserve in the wood compartment (parenchymatous tissues) and that the sucrose had acted as an intermediary for latex production in latex cells of bark.

The enzymes involved in sucrose metabolism, INV and especially SuSy, had presented a higher trend of enzyme activities in the bark than in the wood. The latex cells are located in the bark, so a high degree of activity of these enzymes is likely linked to the use of sucrose for rubber biosynthesis. It is well-known that latex sucrose content sharply decreases in tapped trees as compared to untapped ones [22]. Moreover, the SuSy activity in the bark had been higher with upward than with the downward tapping method. Since the latex yield was higher with the upward method [23], this is consistent with a higher demand for sucrose degradation in this treatment. Thereby, SuSy located in bark could be the main enzyme that produces reducing sugars (fructose and UDP-glucose) and the intermediary (glucose-6-phosphate), which were used for latex biosynthesis [24]. SPS, which is involved in sucrose synthesis showed a higher activity in the wood than in bark (Figure 2E, 2F, and 2G). AGPase and AMY, which are involved with starch accumulation and hydrolysis, showed higher activities in the wood than in the bark (Figure 2C and 2D). Consequently, AGPase played the main role in starch synthesis by accumulating carbon in the wood sink tissue, more precisely in the amyloplasts [25]. It is possible that when the rubber trees need carbon for latex regeneration, the stored starch in wood will be hydrolyzed by AMY to produce soluble sugars, which will then be transferred through the vascular ray from the parenchyma cells to the latex cells [26].

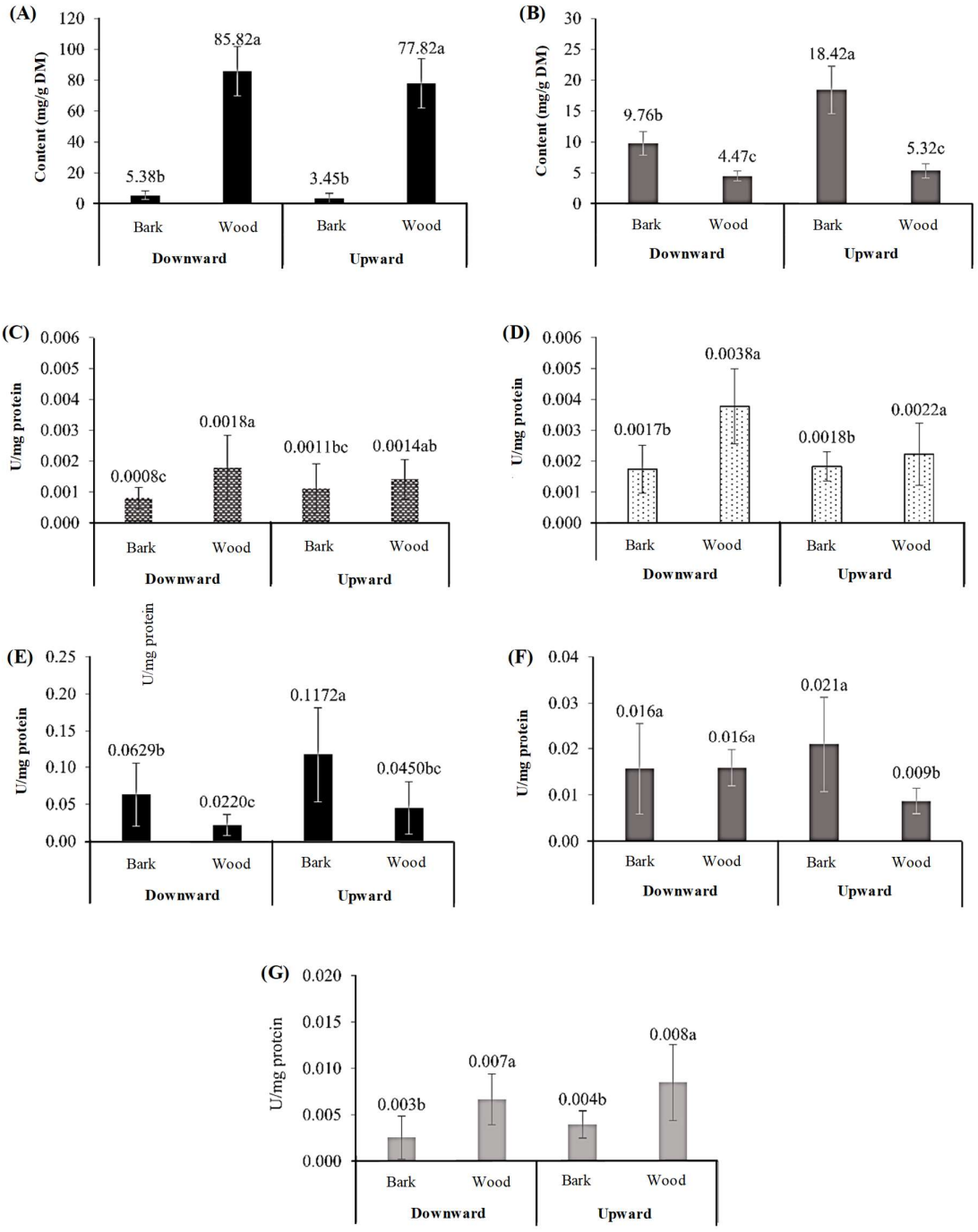

Figure 2 The content of the NSCs and enzyme activities in the bark and wood of rubber trees (Clone RRIM600) tapped by downward and upward methods: (A) Starch and (B) SS content, (C) AGPase and (D) AMY activities related starch metabolism, (E) SuSy, (F) INV, and (G) SPS activities related with sucrose metabolism. The data was averaged from all parameters consisting of trees (tapped and untapped trees), panels (tapped and resting (untapped) panels) and tapping periods (May and October). The different letters indicate a statistically significant difference between the treatments at $p < 0.05$.

3.2 The influence of tapping methods on the NSCs dynamics and involved enzymes

3.2.1 In the bark: The NSCs and the involved enzymes

Regardless of the trees (untapped or tapped trees), the panels (tapped or untapped panels) or the seasons (May or October), the sucrose content of the rubber tree trunks was found to be higher with upward tapping than with downward tapping (Figure 3A and 3B). This is consistent with the fact that with upward tapping, the bark of the trees is directly linked to the canopy [27]. Silpi et al [28] showed an increasing bottom-up gradient in the content of sucrose of the rubber tree trunks. This has actually been frequently observed in trees [29] and is likely due to the proximity of the sucrose source (leaves) in the upper part of the trunk [27].

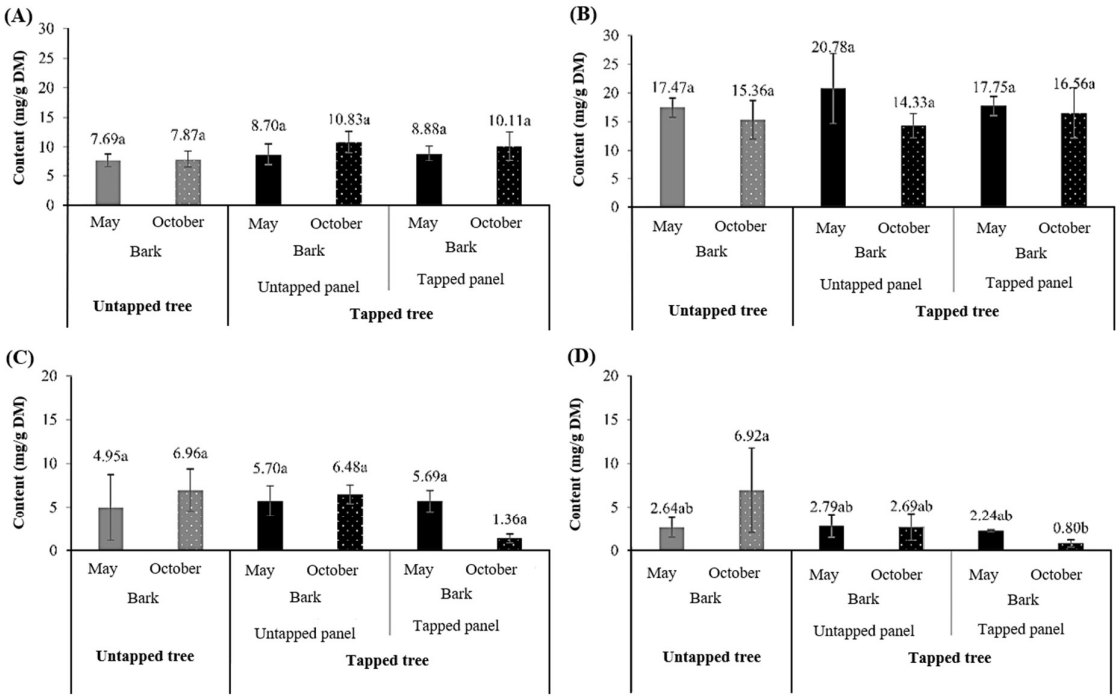

Figure 3 The sucrose and starch contents in the bark of Clone RRIM600 tapped by downward (A and C) and upward (B and D) tapping methods during May to October. The different letters indicate a statistically significant difference between the treatments within a plot at $p < 0.05$.

Considering the enzyme activities in bark, SuSy activity had increased during the high-yield season of October and had been significantly higher in tapping panels with upward tapping method. Conversely, INV activity was not found to have significantly differed between the two tapping methods (Figure 4A and 4B). This could mean that upward tapping had induced high sucrose catalysis by SuSy, which is located in bark, and in order to compensate for the latex loss from the harvesting, glucose-6-phosphate could have been produced as the precursor for rubber regeneration. Moreover, previous research found that SuSy and INV were related to sucrose utilization in the latex of tapped trees [30,31].

The AGPase and AMY activities in bark had not been significantly different between tapping periods (May and October) with both tapping methods. Nevertheless, with both tapping methods, AGPase and AMY showed an increasing trend in their activity during high yield in October (Figure 4C and 4D). It is possible that this result was related to a significant decrease of starch metabolism on tapped panels (Figure 3C and 3D).

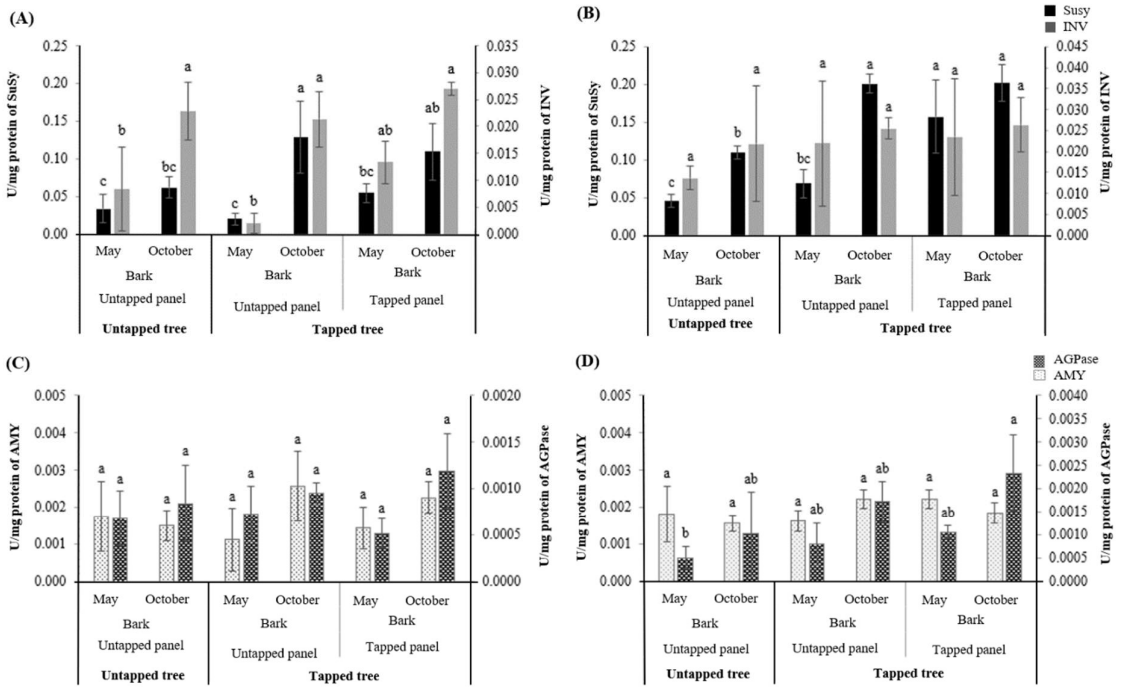

Figure 4 Enzyme activities of SuSy, INV, AGPase, and AMY in bark tapped by downward (A and C) and upward (B and D) tapping, in tapped and untapped trees, and in tapped and untapped panels during May to October (The different letters indicate a statistically significant difference between the treatments for a given enzyme activity at $p < 0.05$).

3.2.2 In the wood: NSCs and involved enzymes

Starch content in the wood had been slightly higher with downward tapping than with upward tapping, and was usually persistent with the decreasing starch gradient bottom-up, which was found in the trees [21]. The starch contents in the wood had not significantly differed between tapped and untapped trees. However, with the downward tapping, there was an increasing trend on untapped panels during the high-yield period in October, as compared to low-yield period. Similarly to the previous study by Chantuma et al. [5] and Silpi et al. [6], this confirmed that downward tapping had created a sink in the carbon storage in the wood. Moreover, it clearly appeared that intensifying the tapping intensity by, for example, using the double cut alternate system [5,21] had even led to starch accumulation in wood on both the untapped and tapped panels. This clearly showed the effects that the downward tapping system had had on the starch accumulation in wood. On the contrary, starch content showed a decreasing trend on both panels of the tapped trees, on which upward tapping had been employed, but it was not determined to be significant (Figure 5A and 5B). The sucrose content in the wood was not found to be significantly different with either of the tapping methods. Nonetheless, the trend of increasing sucrose was discovered on tapped panels with both tapping methods (Figure 5C and 5D). Possibly, with downward tapping, more starch was reserved rather than hydrolyzed. In contrast with upward tapping, more starch hydrolysis than synthesis would have been induced. On one hand, the sucrose in the wood may have served as the carbon source for starch synthesis, while on the other hand, it may have been transported to sustain latex regeneration in the bark.

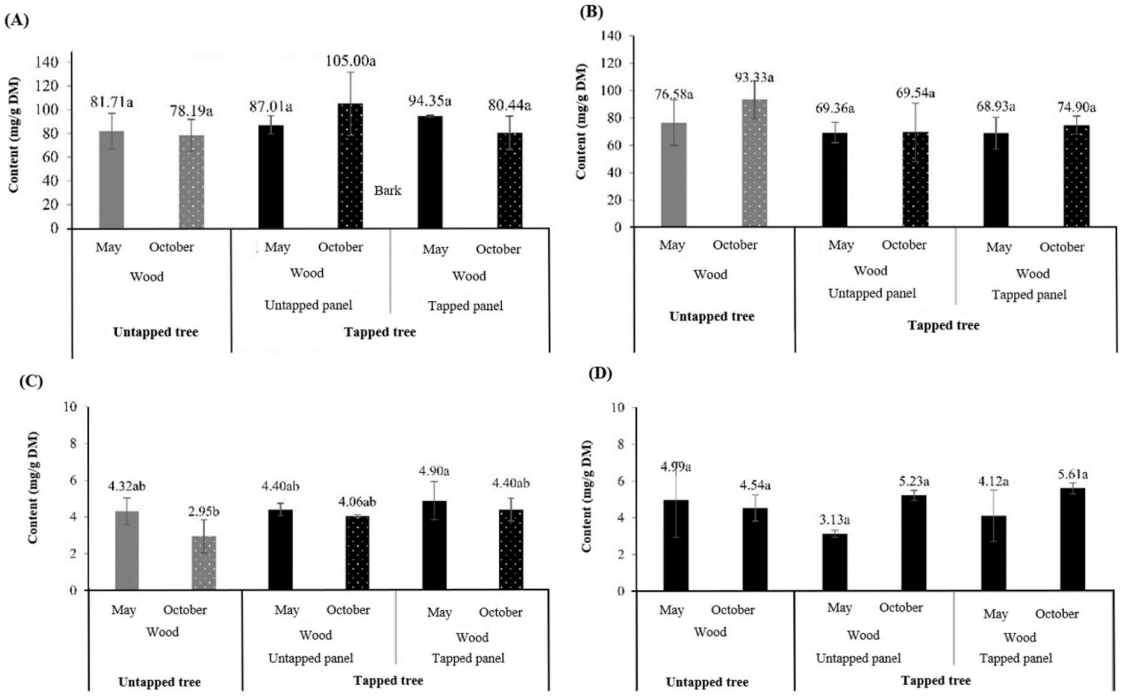

Figure 5 The starch and sucrose contents in the wood tapped by downward (A and C) and upward (B and D) tapping methods, in tapped and untapped (control) trees, and in tapped and untapped panels during May to October (The different letters indicate a statistically significant difference between treatments at $p < 0.05$.).

AGPase activity in wood, involved in starch accumulation, showed a slight increase from the tapped trees in October with both downward and upward taping.

The AMY in the wood showed higher activity in tapped trees with downward tapping than in the untapped trees. This did not occur with upward tapping. With both tapping systems, the AMY activity had been higher in October than in May (Figure 6A and 6B). The increase in AMY activity in the wood during the high-yield period could have been related to higher starch hydrolysis with downward tapping (Figure 5A). Conversely, during the same period, the AGPase activity, which was higher in October, could have helped to balance the starch content. The two enzyme's activities in October could be an indicator of the global metabolic activity of the rubber trees during the high-yield period. This indicated that in wood, the dynamics of the metabolism of starch is related to both AGPase and Amy activities. This can create a balance for starch content during the high-yield period of October even though the starch contents tend to be lower in tapped trees with upward tapping.

SuSy activity in wood was significantly higher on the tapped panels of tapped trees during the high-yield period of October with downward tapping. It was not found to differ with upward tapping. When comparing downward and upward tapping, the INV and SPS activities were not found to differ on both panels of the tree trunks (Figure 6C and 6D). Thus, downward tapping had mainly induced SuSy activity in the wood to catalyze the sucrose and to produce NDP-glucose and fructose, which are used as the precursors for starch synthesis in the amyloplasts (Figure 7).

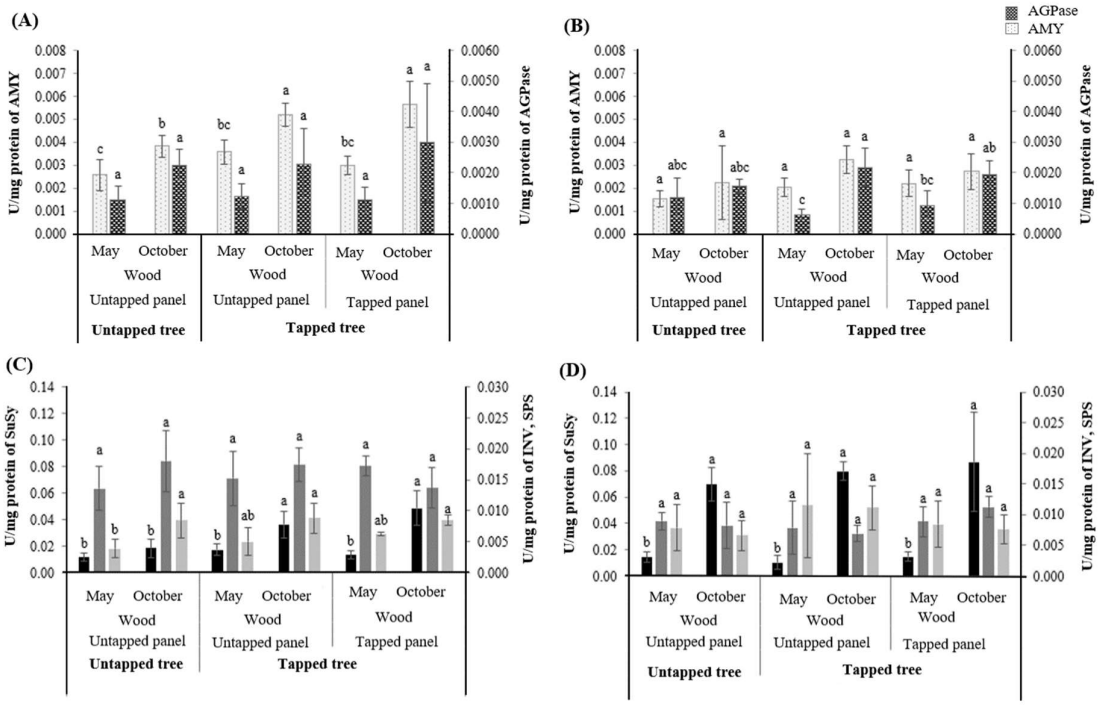

Figure 6 Enzyme activities of AGPase, AMY, SuSy, INV, and SPS in wood tapped by the downward (A and C) and upward (B and D) tapping methods, in tapped and untapped (control) trees, and in tapped and untapped panels during May to October. The different letters indicate a statistically significant difference between treatments for a given enzyme activity at $p < 0.05$.

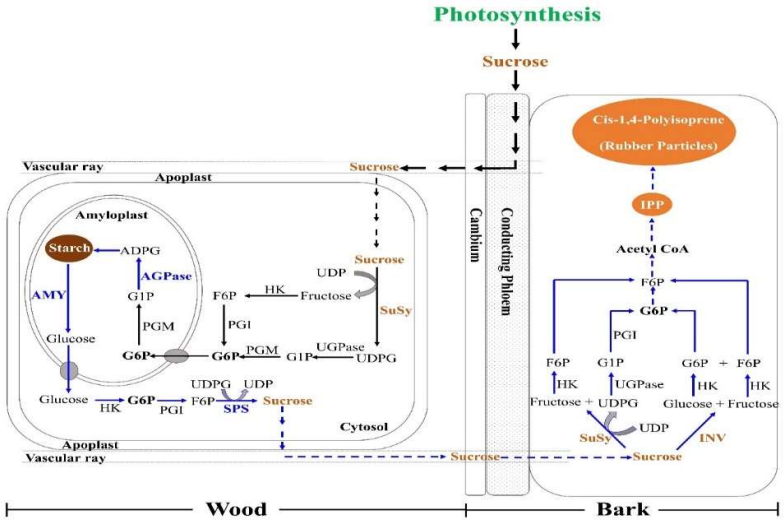

Figure 7 The schematic pathway of the enzyme mechanism and NSCs metabolism model in the wood and bark of rubber tree trunks.

3.3 The influence of tapping method on latex production

We compared the latex yield, the Suc and the Pi contents of the latex cells (latex diagnosis) between downward and upward tapping. The results showed that the yield with upward tapping had been significantly higher (1.58 g/t/t/cm) than with downward tapping (0.58 g/t/t/cm) in October. According to the measurement of the latex biochemical parameters, the Suc content had been significantly higher with upward tapping than with the downward tapping method during each season, and with upward tapping, it had decreased from May to October with 10.07 and 7.06 mM, respectively. Pi contents had not differed (Table 1). Suc and Pi had not differed between

seasons with downward tapping in contrast to upward tapping. This meant that upward tapping had induced more sucrose catalysis in the latex cells than downward tapping to sustain the higher biosynthesis. Furthermore, it was discovered that upward tapping had yielded a higher productivity than downward tapping.

Table 1 Latex production and latex diagnosis (Suc and Pi) during low-yield period (May) and the high-yield period (October) for downward and upward tapping.

Taping methods	Month	Latex		
		Sucrose (Suc, mM)	Inorganic phosphorus (Pi, mM)	Yield (g/t/t/cm)
Downward	May	3.98 ± 1.26^{bc}	14.14 ± 0.80^{a}	0.28 ± 0.03^{b}
	October	3.90 ± 1.66^{c}	14.88 ± 1.07^{a}	0.58 ± 0.13^{b}
Upward	May	10.07 ± 0.74^{a}	18.94 ± 4.23^{a}	0.37 ± 0.007^{b}
	October	7.06 ± 0.85^{ab}	14.61 ± 3.84^{a}	1.58 ± 0.33^{a}

The different letters indicate a statistically significant difference between the treatments at $p < 0.05$.

Finally, these results indicated that the latex cell metabolism and biosynthesis had been mainly related to the SuSy enzyme located in bark, which had catalyzed sucrose hydrolysis to synthesize intermediary compounds, which were transferred to the laticifer cells, whereas SuSy in the wood had catalyzed sucrose synthesis to support starch accumulation. Several reports showed that SuSy is the main enzyme, which is responsible for sucrose catalysis to synthesize starch in the sink organs instead of INV. Some examples are potato tubers, cassava roots, bean seeds, tomato fruit, and woody tissue [8,33,34]. From our results and the previous ones on the dynamics of NSCs and involved enzymes, we were able to propose the schematic pathway in the wood and bark that is shown in Figure 7. Possibly, carbon from the leaf sources would be transferred into the wood (large pool) for carbohydrate storage after the rubber trees had been tapped, and the stored carbohydrates would be used for latex regeneration [35].

In upward tapping, reserved starch in wood would be hydrolyzed at a high rate to support the high sucrose content in the bark. Sucrose seemed to be balanced and to function as a local buffer in the bark, which was hydrolyzed by the main enzyme SuSy to produce the precursors that would be used for high latex synthesis in latex cells. Thus, there was higher starch hydrolysis in the wood found with upward tapping than with downward tapping. The high sucrose content obtained from hydrolyzed starch in the wood was transferred to the bark and was linked to the high catalytic activity of the enzyme SuSy. Consequently, this would be able to sustain the higher latex yield from the upward tapping.

4. Conclusion

The study addressed the dynamics of the NSCs and the involved enzymes, which were related to latex production in rubber trees, by comparing the downward and upward tapping latex harvesting methods. It was found that the upward tapping method had had a higher effect than the downward tapping on the dynamics of NSCs and had induced higher activities of the involved enzyme, especially SuSy. This was in accordance with the result of having a higher latex yield in October with upward tapping than with downward tapping. The dynamics of sucrose had depended mainly on SuSy, which was found to be more active under upward tapping in bark at the upper position (in accordance with the sucrose gradient). Starch was accumulated on the untapped positions by the action of AGPase, and it was hydrolyzed by AMY with downward tapping. Furthermore, sucrose seemed to have played a buffering role in the bark.

5. Acknowledgements

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