**Seasonal Variation in the Enzymatic Activity of the Source-sink System of Rubber Plants in a Clonal Garden**


Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) is a key enzyme in the reactions of CO₂ assimilation in plants. Primary carbohydrates are converted into sucrose, which depends on the sucrose-P synthase (SPS) as a pivotal enzyme. In rubber tree, sucrose hydrolysis can occur intensively in the laticiferous cells of the stem and depends on the invertase (Inv) and sucrose synthase (SuSy) enzyme, producing reducing sugars, which are precursors of latex biosynthesis. The aim of this work was to evaluate seasonal changes in Rubisco, SPS, Inv and SuSy activities of rubber plants in a clonal garden (RRIM 600 and GT 1 clones), during warm/rainy and cold/dry seasons in Lavras, Minas Gerais, Brazil. The climatic differences in warm/rainy and cold/dry periods caused significant changes in all enzymatic activities evaluated. All enzymes had better performances in the warm/rainy period than in cold/dry. Enzymatic activities seemed to be more affected by temperature changes than by precipitation decrease.

**Keywords:** *Hevea brasiliensis*; enzyme activities; sucrose metabolism; seasonal variation

Seasonal variations in environment affect the photosynthetic apparatus and plant gas exchanges, but plant responses to these changes vary both among species and among genotypes of the same species¹. According to Miguel *et al.*² studies carried out about gas exchange and photochemical efficiency of photosystems indicate that photosynthetic variability is relatively usual in rubber tree [*Hevea brasiliensis* (Willd ex. Adr. of Juss.) Muell.-Arg].

Rubisco is a key enzyme in the reactions of CO₂ assimilation and primary synthesis of carbohydrates. In order to be exported from the source organs through the phloem, primary carbohydrates are converted into sucrose, which depends on the sucrose-P
synthase (SPS). Invertase (Inv) and sucrose synthase (SuSy) are enzymes which catalyse sucrose hydrolysis, producing reducing sugar, which are precursors of latex biosynthesis. At low temperatures, carbon export from leaves is always slower than the photosynthesis, resulting in carbohydrates storage, which may inhibit the sucrose synthesis. In the short run, sucrose synthesis also becomes lower at low temperatures because the SPS is highly sensitive to temperature variations.

Water stress or heat can cause change in carbon metabolism in cells, even before dehydration occurs, resulting in limitations to photosynthesis. Under heat stress, Rubisco is inactivated due to lower enzyme activation at higher temperature because of faster rates of Rubisco inactivation and slower rates of activase activity.

There are few studies about the environmental influences on enzymatic activity of Rubisco and enzymes of sucrose synthesis and hydrolysis in rubber trees. Yeang et al. observed that Inv activity in latex is lower in colder months. According to Devakumar et al. in cold and dry periods biochemical changes occur, which result in lower stability of lutoids, causing greater obstruction in latex flux and natural rubber production.

This study aimed to investigate the hypothesis that seasonal changes of climatic conditions affect in different magnitudes the activities of key enzymes involved in CO₂ assimilation and sucrose synthesis / hydrolysis in two rubber clones (RRIM 600 and GT 1), during warm/rainy and cold/dry seasons in Lavras, Minas Gerais, Brazil.

MATERIALS AND METHODS

In this experiment, RRIM 600 and GT 1 clones were evaluated. These clones have different performances in relation to latex production. RRIM 600 is more productive than GT1 in the edaphoclimatic conditions of Minas Gerais state. Plants were 18 years old and belonged to a clonal orchard of the experimental area of the Federal University of Lavras. The shoots of clonal plants were two years old from last pruning. From each clone, ten plants of 3m average height were selected.

The experimental design was completely randomised experimental with a 2 × 2 factorial (clones × periods) arrangement of treatments and ten replicates (plants). In order to encompass a broad representation of climatic characteristics of each period, data on the activity of each enzyme in each replicate was an average of six sampling dates during the warm and rainy period, and four dates during cold and dry period. In each period, plant material was collected every two weeks. Temperature and precipitation data indicated the warm−rainy season during January−March and the cold−dry season during June−July, respectively (Table 1). An ANOVA was performed with the data and for F significant values, means were compared using Tukey test (p ≤ 0.01).

Samples of leaves and bark (eight discs 1 cm diameter) were collected at the same time (between 10:00 and 11:00). Fully expanded leaves (stage "D", 57 day-old) were selected and directly exposed to radiation towards the rising sun. Plant material was immersed immediately in liquid nitrogen, and then stored at -86°C for use in enzyme assays. The extraction of Rubisco and SPS from the leaves was done according to Sharkey et al. 500 μL of the supernatant were added to a 5 mL column of Sephadex G-25 diluted with water (1:1) and the eluate was used in enzyme assays.

The protocol described by Sharkey et al. was adapted for the determination of Rubisco activity. Two reactions were prepared to
evaluate Rubisco activity – initial (\(V_{\text{INITIAL}}\)) and total (\(V_{\text{TOTAL}}\)) velocities. The volume of reaction medium for \(V_{\text{INITIAL}}\) was 125 μL, and it was prepared on an ELISA, with bicine 100 mM pH 8.0, EDTA 1 mM, MgCl\(_2\) 15 mM, DTT 10 mM, NaHCO\(_3\) 9.2 mM, β-NADH 0.5 mM, ATP 2 mM, BSA 0.1%, creatine phosphate 5 mM, creatine phosphate kinase (PCK) 2 U mL\(^{-1}\), phosphoglycerate kinase (PGK) 40 U mL\(^{-1}\) and glyceraldehyde-3-P dehydrogenase (GAP-DH) 40 U mL\(^{-1}\). An aliquot of 20 μL of the desalted extract was added to reaction medium, plus 100 μL of water. Then, 5 μL RuBP 20.75 mM were added, and the total volume of the reaction medium was completed to 250 μL. The reaction medium for \(V_{\text{TOTAL}}\) was similar to that described for \(V_{\text{INITIAL}}\), except in relation to the concentrations of MgCl\(_2\) and NaHCO\(_3\), which were 20 and 25 mM, respectively. After the addition of RuBP to reaction medium, β-NADH oxidation took place for 30 minutes and reading was done at 340 nm. The results were shown in nmol CO\(_2\) g\(^{-1}\) fresh weight s\(^{-1}\).

Preparations by Isopp et al.\(^{11}\) used to determinate SPS activity were adopted. SPS activity was measured under saturating (\(V_{\text{MAX}}\)) and selective (\(V_{\text{SEL}}\)) substrate conditions as fructose-6-P-dependent formation of sucrose and sucrose-P from UDP-glucose. The reaction medium (70 μL) for \(V_{\text{MAX}}\) was 14 μL of HEPES-KOH 50 mM pH 7.4, MgCl\(_2\) 12 mM, DTT 1 mM, UDPG 12 mM; 2.8 μL of fructose 6-phosphate 12 mM, glucose-6-phosphate 36 mM; 30 μL of desalted enzyme extract and 23.2 μL water. Fructose-6-P and glucose-6-P were added last to initiate the reaction. The reaction medium for \(V_{\text{SEL}}\) was similar to that used for \(V_{\text{MAX}}\), except in relation to the addition of a Pi inhibitor (KH\(_2\)PO\(_4\)) 10 mM and to the fructose-6-phosphate and glucose-6-phosphate concentrations, which were 2 and 6 mM, respectively. \(V_{\text{SEL}}\) enzyme activity, that means SPS “activation state”, is a percentage of \(V_{\text{MAX}}\) enzyme activity, reflecting the degree of enzyme phosphorylation\(^{12}\). The reaction medium for SPS remained in a water bath at 25ºC for 10 minutes. KOH solution 70 μL 30% was added to each reaction medium, followed by boiling for 10 minutes. Then, anthrone solution 1 mL (40 mg anthrone, 1 mL water, 20 mL H\(_2\)SO\(_4\)) was added to each reaction medium, in a water bath at 40ºC for 20 minutes. Readings were taken at 620 nm\(^{13}\).

Inv and SuSy activities were determined in bark tissues because they represent a typical sink tissue in rubber tree. To evaluate the magnitude of sucrose hydrolysis, we

<table>
<thead>
<tr>
<th>Month</th>
<th>Minimum temperature (°C)</th>
<th>Mean temperature (°C)</th>
<th>Maximum temperature (°C)</th>
<th>Precipitation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan</td>
<td>18.3</td>
<td>23.0</td>
<td>29.4</td>
<td>149.9</td>
</tr>
<tr>
<td>Feb</td>
<td>18.6</td>
<td>23.1</td>
<td>30.0</td>
<td>284.9</td>
</tr>
<tr>
<td>Mar</td>
<td>18.0</td>
<td>22.4</td>
<td>29.3</td>
<td>281.5</td>
</tr>
<tr>
<td>Apr</td>
<td>16.2</td>
<td>20.9</td>
<td>27.8</td>
<td>17.4</td>
</tr>
<tr>
<td>May</td>
<td>12.1</td>
<td>17.4</td>
<td>25.3</td>
<td>34.5</td>
</tr>
<tr>
<td>Jun</td>
<td>11.2</td>
<td>16.5</td>
<td>24.7</td>
<td>11.6</td>
</tr>
<tr>
<td>Jul</td>
<td>10.7</td>
<td>17.3</td>
<td>26.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Data provided by the meteorological station of Lavras.
considered that reducing sugars production was derived from the sum of Inv and SuSy activities. Reaction medium for assays were prepared in vivo, as suggested by Cairo et al. Assays for Inv activity were performed in two pH levels in order to accommodate the neutral and acidic enzyme isoforms. Reducing sugars were determined according to Miller and readings were performed at 540 nm.

RESULTS AND DISCUSSION

Enzymatic activities were significantly affected by the seasons but interactions among clones x periods and effects of the clones on enzymatic activities were both nonsignificant (Table 2). Thus, the enzymatic activities of the source-sink system, in each season, were represented by the mean of RRIM 600 and GT 1 clones (Figures 1-3).

The decline in Rubisco activity during cold and dry season expressed in both \( V_{\text{INITIAL}} \) and \( V_{\text{TOTAL}} \) (Figure 1), was consistent with the behaviour of this enzyme, as observed for other species at low temperatures. Sucrose biosynthesis is decreased at low temperatures, thereby restricting phosphate recycling and photophosphorylation. As the maximum capacity to utilise trioses-P depends on the temperature, chloroplast demand for phosphate tends to become higher in cold periods. Restrictions to triose-P use can trigger feedback mechanisms that reduce photosynthetic electron transport, which limits ATP supply and RuBP regeneration. The lower RuBP's capacity of regeneration at low temperature becomes limiting for photosynthesis especially with respect to regeneration of RuBP components, associated with regeneration of inorganic phosphate during starch and sucrose synthesis. These developments limit the action of the Rubisco which loses some of its carboxylation efficiency, reducing the rate of photosynthetic carbon assimilation.

The weekly weather conditions associated with optimum yields were maximum temperature of 30.4°C, minimum temperature of 22.8°C, 5.9 h sunshine and 72 mm of rainfall. Kositsup et al. reported that rubber saplings acclimate their photosynthetic characteristics in response to increasing temperature, and higher temperatures caused an enhanced photosynthetic capacity in the leaves as well as larger activation energy for photosynthesis. At low temperatures, net photosynthesis is lower due to the decline in

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Rubisco Mean Squares</th>
<th>SPS Mean Squares</th>
<th>Inv + Susy Mean Squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( V_{\text{INITIAL}} )</td>
<td>( V_{\text{TOTAL}} )</td>
<td>( V_{\text{SEL}} )</td>
</tr>
<tr>
<td>Clone (C)</td>
<td>1</td>
<td>0.004</td>
<td>0.084</td>
<td>0.012</td>
</tr>
<tr>
<td>Period (P)</td>
<td>1</td>
<td>100.475**</td>
<td>237.029**</td>
<td>83.293**</td>
</tr>
<tr>
<td>C ( \times ) P</td>
<td>1</td>
<td>0.138</td>
<td>0.622</td>
<td>0.114</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>0.151</td>
<td>0.384</td>
<td>0.167</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td>11.7</td>
<td>12.4</td>
<td>10.9</td>
</tr>
</tbody>
</table>

** F significant values \( p \leq 0.01 \)
chlorophyll fluorescence and carboxylation efficiency. Besides, the stomatal factor does not affect seasonal variations on net photosynthesis because stomatal conductance remains unchanged\(^2\). In our study, the lower Rubisco activity during the cold and dry season seemed to cause a decreased carboxylation efficiency and would influence seasonal variations on net photosynthesis.

It is unlikely that decreased Rubisco activity during cold and dry period was caused by soil water deficit. The plants had showed youthful appearance due to the shoots (3 m height average), but the root system was probably already well developed. It is unlikely that lack of rain has caused stress to the plants to the point of affecting Rubisco activity. Rubisco activation varied from 66% during warm and wet period to 76% in dry and cold period. It was considered high in both periods, but was significantly lower in warm and rainy period. Rubisco activation under moderate to high temperatures may be limited due to a lower regeneration capacity of the RuBP\(^25\). Thus, ATP:ADP levels and redox potential of the chloroplasts declined, leading to a lower Rubisco activation. The lower Rubisco activation under higher temperatures may be more related to limitations in electron transport efficiency than the direct effect of heat on the integrity of the Rubisco activase enzyme\(^17\).

The SPS activity declined significantly in cold and dry period (Figure 2). As discussed previously, temperature seemed to be the major environmental influence on decreased SPS activity instead of soil moisture due to the root system, probably, was well developed. Temperature effects on SPS activity are variable. Guy \textit{et al.}\(^{26}\) and Holaday \textit{et al.}\(^{27}\) observed an increased SPS activity when spinach plants were transferred from 24–25ºC to 5–10ºC. Conversely, in potato leaves, temperature increase from 17–19ºC to 29–31ºC results in higher SPS activity\(^{28}\). In soybean leaves, SPS activity is lower at 14–18ºC than at 22–26ºC\(^{29}\). It is unlikely that

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**Figure 1. Rubisco activity in** \(V_{\text{INITIAL}}\) (light columns) and \(V_{\text{TOTAL}}\) (dark columns) in RRIM 600 and GT 1 clones (average of clones) in both periods (warm/rainy and cold/dry). The bars in each column indicate the standard deviation of 20 replications. The lowercase letters compare \(V_{\text{INITIAL}}\) means, and capital letters compare \(V_{\text{TOTAL}}\) means (Tukey test; \(p \leq 0.01\)).
the low temperature alters kinetic properties of pre-existing enzymes, such as the SPS, resulting in lower enzymatic activity\textsuperscript{28}. The decline in SPS activity in our research was due to the lower demand for carbohydrates in sink tissues (bark). This response was due to low latex production during this period. However, our data is not sufficient to explain the causes of decline in SPS activity when the temperature becomes lower.

SPS activations in RRIM 600 and GT 1 clones in warm and rainy period were 77\% and 67\% respectively, and became higher in dry and cold period (86\% and 87\%, respectively). Environmental conditions in cold and dry period may have caused severe restrictions to SPS activity in $V_{\text{MAX}}$, given that its decline was more pronounced than that in $V_{\text{SEL}}$. Considering that $V_{\text{SEL}}/V_{\text{MAX}}$ ratio indicates the level of SPS activation, changes in these two parameters may have been caused by increased SPS activation in dry and cold period. The increase of SPS activation in both clones, despite the decline of SPS activity in $V_{\text{SEL}}$ and $V_{\text{MAX}}$ was a similar result to what Zrenner and Stitt\textsuperscript{30} and Castrillo\textsuperscript{31} observed on spinach and beans, respectively, subjected to low temperatures and water restrictions.

Sucrose hydrolysis and increase in reducing sugar levels, derived from sum of Inv (vacuolar acidic and neutral) and SuSy activities were higher in warm and rainy period than in dry and cold (Figure 3). Yeang \textit{et al}.\textsuperscript{8} observed a decline in Inv activity in the “serum C” component of latex in the cooler months. As discussed earlier, temperature seemed to be the major environmental influence on lower levels of reducing sugars during cold and dry period. Temperature decrease can affect enzymes activity of sucrose hydrolysis, changing the levels of reducing sugars\textsuperscript{32}. Low temperatures can also cause instability in lutoids, reducing the flow of latex and natural rubber production\textsuperscript{9}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{SPS activity in $V_{\text{SEL}}$ (light columns) and $V_{\text{MAX}}$ (dark columns) in RRIM 600 and GT 1 clones in both periods (warm/rainy and cold/dry). The bars in each column indicate the standard deviation of 20 replications. Lowercase letters compare $V_{\text{SEL}}$ means, and capital letters compare $V_{\text{MAX}}$ means (Tukey test; \textit{p} \leq 0.01).}
\end{figure}
CONCLUSION

The climatic differences in warm/rainy and cold/dry periods cause significant changes in all enzymatic activities of the source-sink system of the rubber clones studied. All enzymes evaluated have better performance in the warm/rainy period than in cold/dry. Enzymatic activities seem to be more affected by temperature changes than by precipitation decrease.

Date of receipt: September 2014
Date of acceptance: April 2015

REFERENCES


