Natural Rubber Research, **22** (1&2): 36-42, 2009

BIOCHEMICAL CHARACTERISATION OF RRII 400 SERIES CLONES OF *HEVEA BRASILIENSIS*

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Sreelatha, S., Mydin, K.K., Simon, S.P., Jacob, J. and Krishnakumar, R. (2009). Biochemical characterisation of RRII 400 series clones of *Hevea brasiliensis*. *Natural Rubber Research*, **22** (1&2): 36-42.

Five newly evolved RRII 400 series clones were biochemically characterised and compared with the popular clone RRII 105. Characterisation was done based on biochemical parameters associated with latex flow, flow cessation and latex regeneration. During the peak yielding season, clones RRII 422 and RRII 430 recorded significantly higher dry rubber yield than RRII 105. Biochemical parameters such as latex ATP, content, amount of thiols in C-serum and lutoid membrane ATPase were significantly higher in these clones than in RRII 105 while SOD levels were on par in these three clones. The highest ATP content was observed in clone RRII 429 followed by clones RRII 414, 422 and 430. Clones RRII 417 and 429 were characterised by low SOD in C-serum, and low ATPase and pyrophosphatase activities in the lutoid membrane. Sucrose, glutathione reductase activity, protein profiles of C-serum, hevein content and N-acetyl glucosaminidase activity in lutoids of all the 400 series clones were comparable to RRII 105. The significance of these biochemical parameters is discussed in relation to general metabolic activity of these clones.

Keywords: Antioxidant enzymes, C-serum, Latex regeneration, Rubber yield.

INTRODUCTION

Characterisation of *Hevea brasiliensis* clones based on physiological parameters and laticifer functioning has been well established by several researchers (Eschbach *et al.*, 1984; Serres *et al.*, 1988 ; Jacob *et al.*, 1985; Gohet *et al.*, 2003; Nair, 2003; Thanh and Thuy, 2003). RRII 105, the most popular clone was characterised as a metabolically active clone (Nair *et al.*, 2001) and the biochemical mechanism associated with its low yield during summer was also reported (Sreelatha *et al.*, 2007). The RRII 400 series clones are of recent development and some of them are now recommended for cultivation. Studies on the general metabolism of these newly developed clones are important to understand their response to different environmental conditions, tapping frequency, stimulation *etc.* Performance of these clones for yield and their morphological, molecular and physiological characterisation have already been reported (Licy *et al.*, 2003; Saraswathyamma *et al.*, 2006, Nair and Mydin, 2006; Mydin and Mercykutty, 2007). The present paper reports the characterisation of RRII 400 series clones based on their biochemical parameters and enzymes associated with latex regeneration, flow and cessation of latex flow.

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MATERIALS AND METHODS

Five *H. brasiliensis* clones of RRII 400 series (RRII 414, RRII 417, RRII 422, RRII 429 and RRII 430) were selected along with the popular check clone RRII 105. The trees were planted during 1993 at the Central Experiment Station of Rubber Research Institute of India, Chethackal. Nine trees per clone having comparable girth and yield were selected for the present study. The trees were in the fifth year of tapping under S/2 d3 6d/7 tapping system.

Dry rubber yield, parameters associated with rubber biosynthesis (sucrose and ATP), antioxidant enzymes and protective factors (superoxide dismutase, SOD; catalase, CAT; peroxidase, PX; thiols and glutathione reductase, GR, in C-serum) lutoid membrane enzymes, (H^+ ATPase and H^+ pyrophosphatase) involved in pH regulation; N-acetyl glucosaminidase and hevein in B-serum involved in cessation of flow; protein content and polypeptide profile of C-serum, were analysed during the peak yielding season (October-November) for two consecutive years.

The ATP concentration in latex was determined according to Amalou *et al.* (1992). Fresh latex samples were extracted with 2.5 per cent trichloroacetic acid (TCA). An aliquot was neutralised with 0.1N KOH and made up to a known volume with 30 mM Hepes (N-[2-Hydroxy ethyl] piperazine-N'-[2-ethane sulphonic acid]) -Tris (tris hydroxy methyl aminomethane) buffer pH 7.4, and quantified using bioluminescent kit (FL-AA-Sigma Chemical Company, USA), which contained luciferin-luciferase enzyme. Sucrose content in latex was measured after extraction with 2.5 per cent TCA, following the procedure of Scott and Melvin (1953).

For the assay of enzymes, latex samples were centrifuged at 23,000 rpm for 45 min at 4 ^{0}C. The clear C-serum was separated and directly used for enzyme assays. Fresh lutoids were used for the measurement of lutoid membrane ATPase and pyrophosphatase activities. For the preparation of B-serum, lutoids were washed in 0.4 M mannitol, centrifuged and subjected to repeated freezing and thawing and again centrifuged at 20,000 rpm for 30 min.

For determination of C-serum thiols (reduced glutathione), proteins present in the serum were removed by precipitation with 10 per cent TCA and centrifugation at 10, 000 rpm for 20 min. The supernatant was used for reduced glutathione estimation (Boyne and Ellman, 1972) using 5, 5' – dithiobis (2-nitrobenzoic acid). Glutathione reductase (Jacob *et al.*, 1984), SOD (Giannopolitis and Reis 1977), CAT (Chance and Mehly 1955) and PX (Guilbaut, 1976) in C-serum were assayed and expressed in relative units. One unit was defined as the change in O.D. per min per mg protein in the case of GR, PX and CAT. For SOD, one unit was defined as the amount of enzyme that produced 50 per cent reduction of NBT (nitroblue tetrazolium) under the assay conditions.

Activities of H^+ ATPase and pyrophosphatase were measured in intact lutoids (Gidrol *et al.*, 1988; Siswanto *et al.*, 1994). N-acetyl glucosaminidase activity was measured in B-serum according to Giordani *et al.* (1992). Hevein in B-serum was separated by gel filtration through sephadex G-25 column and eluted with 1.2 M acetic acid (Ukun *et al.*, 1999). Fractions (3 ml) were collected and the optical density was measured at 280 nm.

Proteins in C-serum were precipitated with 10 per cent TCA, the precipitate

dissolved in 0.1N NaOH and measured according to Lowry *et al.* (1951). SDS-PAGE profile of C-serum was done using 12.5 per cent acrylamide gel and stained with Coomassie Blue (Laemmli, 1970). The data on all the parameters were analysed statistically.

RESULTS AND DISCUSSION

Data on yield and biochemical parameters associated with rubber biosynthesis are presented in Table 1. Analysis of variance of the data showed a significantly high dry rubber yield in clones RRII 422 and RRII 430 during the peak yielding season (October-November) compared to the check clone RRII 105. The yield of the other 400 series clones was comparable to that of RRII 105. Superior yield and growth performance of RRII 400 series clones over RRII 105 was established in small-scale and large- scale trials (Mydin and Mercykutty, 2007).

Sucrose availability is a major factor that limits the yield of rubber. Positive and negative correlations between latex yield and sucrose have been reported (Eschbach *et al.*, 1984; Jacob *et al.*, 1985). High sucrose content in latex indicates good loading of sucrose to the laticiferous cells which leads to an active metabolism; however it could also indicate a low metabolic utilisation and low latex production. A sufficient sucrose loading is required by the laticiferous cells to sustain the latex regeneration (Gohet *et al.*, 2003). However, in the present study, no significant differences were found between the clones for sucrose.

The latex ATP was significantly higher in the RRII 400 series clones, registering the maximum in RRII 429, while RRII 417 and RRII 105 had comparable levels (Table 1).

Table 1. **Peak season yield and contents of ATP and sucrose in the latex**

Clone	Yield (peak season) (g/t/t)	ATP (μM)	Sucrose (mM)
RRII 414	104.1	286.9	5.7
RRII 417	107.7	270.1	7.8
RRII 422	116.6	284.8	6.9
RRII 429	100.5	290.9	7.7
RRII 430	114.6	277.4	6.1
RRII 105	93.3	257.2	6.5
CD (P = 0.05)	16.2	18.6	NS

Latex ATP is a very important regulator in rubber biosynthesis by its direct effect on metabolic pathways (supplying energy) and indirect effect mediated through lutoid membrane H^+ ATPase activity, which increases the latex pH to favourable levels and activates several pH dependent enzymes in the cytosol (Jacob *et al.*, 1985). Latex flow processes also require biochemical energy. An active metabolism generates sufficient intracellular energy which provides easy flow and thereby increases production. Significant positive correlation had been reported earlier between yield and latex ATP, lutoid membrane ATPase and C-serum pH (Amalou *et al.*, 1992; Sreelatha *et al.*, 2004).

Variations in the levels of antioxidant enzymes and thiols are presented in Table 2. RRII 105, RRII 414, RRII 422 and RRII 430 showed similar SOD and CAT activities. Clones RRII 417 and RRII 429 had significantly lower SOD and CAT activities than the other clones. All the clones of 400 series showed a lower PX activity than RRII 105. Peroxidases and NADH quinone reductase produce harmful quinones and toxic oxygen which are the major factors involved in lutoid membrane degradation which leads to early cessation of flow. The

factors that protect the membrane from these toxic effects are antioxidant enzymes such as SOD and CAT, and factors like reduced glutathione and ascorbic acid (Jacob *et al.*, 1985). Significantly high thiol content in C-serum was observed in all the RRII 400 series clones compared to RRII 105 (Table 2). High thiol content in these clones was also reported by Nair and Mydin (2006). Thiols inhibit peroxidases in the medium by preventing the formation of toxic oxygen and delay the degradation of lutoids. Thiols are also capable of activating the key enzymes in carbohydrate metabolism such as invertase and pyruvate kinase (Eschbach *et al.*, 1984). Levels of GR, which regenerates reduced thiol from its oxidised form, were similar in all the clones (Table 2). Thiol regeneration also requires a large amount of ATP.

Variations in the enzymes associated with proton transport leading to pH regulation of the cytosol and cessation of latex flow are presented in Table 3. Significantly high lutoid H^+ ATPase was observed in clones RRII 414, RRII 422 and RRII 430 compared to RRII 105. Regulation of cytosolic pH is an important factor in rubber biosynthesis and is mediated by the action of ATPase and pyrophosphatase in the lutoid membrane. These enzymes use ATP and pyrophosphate (PPi) as energy sources to pump protons. Tonoplast-bound pyrophosphatase was characterised in latex by Siswanto *et al.* (1994). Cytosolic alkaline pyrophosphatase prevents the accumulation of PPi during intense metabolic activities during latex regeneration and inhibits its adverse effects and also enables the turnover of Pi for energy metabolism (Jacob *et al.*, 1986).

The lutoid ATPase activity also depends on the availability of ATP in the cytosol. RRII 429 showed a higher latex ATP but low ATPase and pyrophosphatase activity in lutoids, while in clones RRII 414 and RRII 105 a high ATPase and pyrophosphatase activities were observed. Correlations between yield and these enzyme activities were studied earlier in clone RRII 105 (Simon, 2003; Sreelatha, 2003).

No significant differences were observed in hevein content and N-acetyl glucosaminidase activity in B-serum between clones. Hevein, which is the major protein in lutoids, is a lectin - like protein involved in the coagulation of latex. Under normal condition, hevein does not interact with rubber particles because it is compartmentalised in lutoids. When lutoids

Table 2. **Biochemical parameters associated with antioxidant defence system in the latex of different clones**

Clone	SOD (units)	CAT (units)	PX (units)	GR (units)	Thiols (mg/ml)
RRII 414	0.89	0.075	0.26	1.28	0.28
RRII 417	0.66	0.047	0.23	1.26	0.33
RRII 422	0.91	0.051	0.25	1.21	0.32
RRII 429	0.65	0.055	0.24	1.25	0.28
RRII 430	0.95	0.064	0.22	1.20	0.31
RRII 105	0.93	0.060	0.32	1.30	0.25
CD (P = 0.05)	0.17	0.017	0.06	NS	0.02

Table 3. **Biochemical parameters associated with proton transport and cessation of flow of latex**

Clone	H^+ ATPase (μM Pi liberated/min/mg protein)	H^+ Pyrophosphatase (μM Pi liberated/min/mg protein)	N-acetyl glucosaminidase (μM p-nitrophenol liberated/min/mg protein)	Protein (C-serum) (mg/ml)
RRII 414	6.23	12.83	0.52	13.19
RRII 417	3.21	10.21	0.54	13.07
RRII 422	6.43	10.98	0.49	12.31
RRII 429	3.03	10.36	0.48	13.75
RRII 430	6.81	11.93	0.51	13.34
RRII 105	5.16	13.07	0.53	10.94
CD (P = 0.05)	1.02	1.23	NS	2.06

break during tapping, hevein and divalent cations are released from the lutoids. Hevein forms bridges between rubber particles by binding with N-acetyl glucosamine moiety of the 22 kD receptor protein present on the surface of rubber particles. Divalent cations provoke acidification of the medium which favours hevein binding (Gidrol *et al.*, 1994). N-acetyl glucosaminidase present in the lutoid serum inhibits this binding and delays coagulation. Recently a C-serum lectin-binding protein (CS-HLLBP) was identified with an anticoagulant role and a highly positive correlation between CS-HLLBP activity and rubber yield per tapping was reported (Wititsuwannakul *et al.*, 2008).

Total protein content in the C-serum of all clones except RRII 422 was significantly higher than that of RRII 105. This indicates the efficient protein biosynthetic capacity of these clones. Latex regeneration between two tappings involves not only rubber biosynthesis but also efficient biosynthesis of proteins that are removed through tapping. SDS-PAGE profile did not show any significant variation between clones.

In general, all the clones had an active metabolism during the peak yielding season and the present study profiled the biochemical characteristics of each clone compared to RRII 105. Among the RRII 400 series clones, higher thiols, SOD in C-serum and lutoid membrane ATPase were observed in clones RRII 422 and RRII 430 while RRII 429 had the highest latex ATP content. Clones RRII 417 and RRII 429 showed low ATPase, pyrophosphatase and SOD. This indicates that these two clones may respond well to stimulation. The increase in SOD, ATPase and pyrophosphatase was observed to be the initial biochemical changes induced by stimulation in low frequency tapped trees of clone RRII 105 (Simon, 2003; Sreelatha, 2003). Latex flow and regeneration factors analysed in this study showed variations among clones and this metabolic profiling would be useful for selecting suitable tapping systems and stimulation methods for these newly released clones. However, further studies are needed to establish the effect of stimulation on these parameters and latex production in these clones.

ACKNOWLEDGEMENT

The authors thank Mr. Ramesh B. Nair, Assistant Director (Statistics), RRII for the help rendered in statistical analysis of the data.

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