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Protein Marker for Tapping Panel Dryness Identified as the Small Rubber Particle Protein (Hev b 3)

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Previous studies undertaken by different researchers using electrophoretic techniques have identified in Hevea *latex several putative protein markers that are linked to tapping panel dryness (TPD) A major marker that was frequently encountered was a latex protein of 22 kDa – 26 kDa. In the present study, analysis by Western blot and two-site ELISA showed this marker to be identical to the 22.4 kDa small rubber particle protein (SRPP), which is an allergenic peptide also known as Hev b 3. The SRPP level in C-serum of partially dry trees showed a significant increase over that of the normal yielding trees, confirming that the marker was SRPP. However, no similar increase was found in the SRPP levels when assayed in the whole latex. These results suggested that there was no* de novo *synthesis of SRPP with the onset of partial dryness and the protein is not therefore a direct marker for incipient tree dryness. The apparent increase of the protein observed in the C-serum was likely to be due to increased numbers of small rubber particles failing to separate out of the C-serum upon centrifugation. SRPP may nevertheless be* indirectly *associated with TPD by its being an indicator of latex stability.*

Various aspects on tapping panel dryness (TPD) in *Hevea*, also known as 'brown bast', have been examined intensively, all with the purpose of seeking early warning indicators of impending dryness. Earlier studies mainly concentrate on the understanding of physiological and biochemical development[1,2] of the malady afflicting the rubber trees. Histological and cytological symptoms[3–5] of TPD have also created interests in many workers. The influence of some agronomic practices on TPD[6] and nutritional status of the bark[7] have also been considered. Of late, electrophoretic techniques have been used as another means of securing molecular markers to characterise TPD and to forecast its impending incidence[8–12]. Certain protein markers have been identified in latex, a major marker reported in several studies being a protein of 22 kDa – 26 kDa[9–11].

This research examines the possibility that the 22 kDa – 26 kDa protein marker of TPD is a peptide known as the small rubber particle protein that is found on the surface of rubber particles. Latex from normal and partially dry trees are analysed by immunological techniques to verify the identity of the protein marker for TPD and to determine by quantitative measurements its effectiveness as an indicator for the development of dryness.

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MATERIALS AND METHODS

Experimental Trees

The trees used in the study were of the clone RRIM 600 trees grown in the Rubber Research Institute of Malaysia Experiment Station, Selangor, Malaysia. Ten partially dry trees and ten normal yielding control trees were selected. The degree of partial dryness was determined as the length of the tapping cut that was non-yielding expressed as a percentage of the total tapping cut length. The partially dry trees selected for the study were from 19% to 60% dry, with the average dryness being 39.9%.

Latex Collection and Preparation of Latex Sera

Latex from tapped trees was collected in ice-chilled containers and brought back to the laboratory for centrifugation and for the determination of total solids.

C-serum. Fresh latex was centrifuged at 44 000 g in a Sorvall RC 2B high-speed centrifuge at 4°C for 1 h and the aqueous serum phase was extracted.

SDS extracted fraction. Four percent sodium dodecyl sulphate (SDS) was added to fresh latex in the ratio 1:3 in order to obtain a final concentration of 1% (w/v) SDS in the latex. The mixture was left to stand for 1 h before centrifugation as above. The clear serum obtained following centrifugation was extracted.

Immuno-detection of SRPP

SDS-PAGE was carried out on 15% gel according to Laemmli[13] and the separated proteins were transferred electrophoretically on to nitrocellulose membrane. The resolved proteins were probed immunologically as previously described[14], but using the alkaline-phosphatase detection system. Briefly, the nitrocellulose membrane was blocked with 5% (w/v) non-fat milk and then incubated with USM/RC2, the monoclonal antibody against the small rubber particle protein, (SRPP). After washing in phosphate buffered saline (PBS), the membrane was incubated with rabbit anti-mouse IgG conjugated to alkaline phosphatase. An insoluble coloured product was generated by the enzyme substrate BCIP/NPT. A matching gel was stained with Coomassie brilliant blue.

Quantitation of SRPP by Two-site ELISA

The wells of a microtitre plate (Costar, USA) were coated with 100 µl of the monoclonal antibody USM/RC2 (1:1 dilution in carbonate-bicarbonate buffer, pH 9.6). Coating was carried out for 3 h at room temperature and then overnight at 4°C. The plates were then washed with three cycles of PBS containing 0.05% (w/v) *Tween 20* (PBS-T). The two different latex fractions (C-serum and SDS-extracted latex serum) were diluted 1/100 in PBS containing 5% non-fat milk (PBS-M) and 100 µl was added to the wells and incubated for 1.5 h at room temperature. The wells were emptied and washed thrice in PBS-T. Polyclonal rabbit antiserum against SRPP diluted 1/1000 in PBS-M was then pipetted into the wells and incubated for 1.5 h at room temperature followed by similar washing cycles. Following this, 100 µl of the conjugate, goat anti-mouse IgG-alkaline phosphatase (1/1000 dilution in PBS-M, Sigma Chemical Co.) was added to the wells and incubated in the dark for 1 h at room temperature. After washing twice in PBS-T followed by one wash in 50 mM Tris-HCl buffer (pH 7.0), 100 µl of the enzyme substrate *p*-nitrophenyl phosphate (Sigma Chemical Co.) was added. The colour developed was read at 405 nm using an ELISA reader (Bio-Rad).

Estimation of the Bursting Index

The bursting index of lutoids was estimated based on the method of Ribaillier[15] and as described by Yeang[16]. Briefly, acid phosphatase activity in the C-serum (termed as 'free' acid phosphatase) and in SDS-extracted latex (termed as 'total acid phosphatase') was determined using *p*-nitrophenyl phosphate as the substrate, with the reaction carried out in 0.1 *M* citrate buffer. Change in optical density at 400 nm after 10 min was determined using a spectrophoto-meter. The bursting index was calculated as the acid phosphatase activity in the C-serum as a percentage of the total latex acid phosphatase activity, after correcting for variation in the total solids of the latex and dilution by the added SDS[16].

RESULTS AND DISCUSSION

The major proteinaceous marker for TPD described by Lacrotte *et al.*[9,11] appeared to be identical to that reported by Dian *et al.*[10] Lacrotte *et al.* referred to a predominant marker protein of 22 kDa that was correlated to the severity of TPD while Dian *et al.* considered the increase in a 26 kDa protein the most obvious change associated with the onset of dryness. The slight discrepancy in the size of the proteins reported by the two groups is not unusual and might have been due to the use of calibration markers from different suppliers. In a comparable situation, the small rubber particle protein (SRPP), an allergenic peptide located on the membrane of small rubber particles of *Hevea* latex, had similarly been ascribed varying molecular weights. Although SRPP (before it was identified as such) was initially described as being 27 kDa[17], subsequent reports revised this figure to 24 kDa[18] and 22 kDa[14,19] before the value of 22.4 kDa was established from its cDNA sequence[20].

There were various evidences in the literature to suggest that the major TPD marker protein described by Lacrotte *et al.* and by Dian *et al.* might in fact be the SRPP, also known under immunological nomenclature as Hev b 3. This is an allergenic latex protein that is especially reactive to latex-sensitised *spina bifida* patients[17,18]. Besides the similarity in size, there was also a similarity in isoelectric points of the proteins. The TPD marker protein had a reported pI of about 5.5 by iso-electric focussing[9]. A similar pI of about 5 was obtained for SRPP by iso-electric focussing[18] (although the calculated pI based on its amino acid composition[20] was 4.8).

There was another indication that the two proteins were the same. When C-serum proteins were separated by 2-D-electrophoresis, the marker protein had an unusual characteristic in sometimes appearing to 'diffuse' laterally on the 2D gel. This feature was shown clearly in *Figure 1* of Lacrotte *et al.'s* 1995 paper[9] and *Figure 6* of their 1997 paper[11]. The characteristic behaviour of the protein was again seen in *Figure 6* of the paper by Dian *et al*[10]. Co-incidentally, the same uncommon characteristic had earlier been observed for SRPP by Alenius *et al.*[17] who were looking into the allergenic properties of the protein. *Figure 3* in their paper depicted this clearly.

SRPP Presence in the C-serum of Partially Dry and Normal Yielding Trees

To determine whether the marker protein that was highly expressed in C-serum of latex obtained from partially dry trees was SRPP, C-serum proteins from normal yielding and partially dry trees were separated by electrophoresis and Western blotted on to nitrocellulose membrane. The presence of SRPP was detected using a monoclonal antibody specific to the protein. As expected, SRPP was detected in

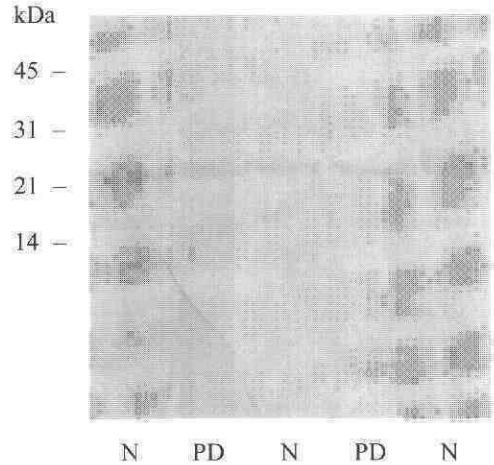

Figure 1. Detection of SRPP in C-serum from two normal yielding trees (N) and two partially dry trees (PD) by Western blot. Monoclonal antibody against SRPP was used as the primary antibody. A sample of SRPP (S) was run as the standard.

C-sera from both the partially dry and normal trees, as small rubber particles remain suspended in the C-serum after centrifugation[14]. The protein was nevertheless more prominently expressed in C-serum from partially dry trees *(Figure 1)*. As the monoclonal antibody that was used was specific for SRPP, this observation was consistent with the contention that TPD marker protein and SRPP were identical. Another band of about 14 kDa was recognised by the monoclonal antibody against SRPP in C-serum from partially dry trees, but this protein was absent in the controls. This minor band could be a fragment of SRPP as the protein is prone to fragmentation[18]. Dian *et al.*[10] has similarly reported a 14.5 kDa protein in trees afflicted with TPD.

Quantitation of SRPP in Latex from Partially Dry and Normal Yielding Trees

While the results from the Western blot studies point strongly to the TPD marker being SRPP, further investigations were still warranted. Firstly, the identification of the TPD marker as SRPP could be erroneous if the monoclonal antibody happened to cross-react with some other protein. Secondly, the Western blot did not allow for a more precise quantitation of the SRPP content in latex. To address the problem of cross-reactivity, a two-site ELISA employing a monoclonal antibody and a polyclonal antibody against SRPP was employed. The ELISA also enabled SRPP to be quantitated by colorimetry.

In the C-serum, the optical density (OD) values of SRPP from partially dry trees were much more variable than those from the normal yielding trees *(Figure 2)*. The mean OD for partially dry trees was significantly higher than that for the controls ($P<0.001$). This result was consistent with the observations made with the Western blot and confirmed that the major TPD marker reported in the literature was indeed SRPP. The marker is therefore an insoluble protein found on the membrane of small rubber particles that remain suspended in the C-serum[14].

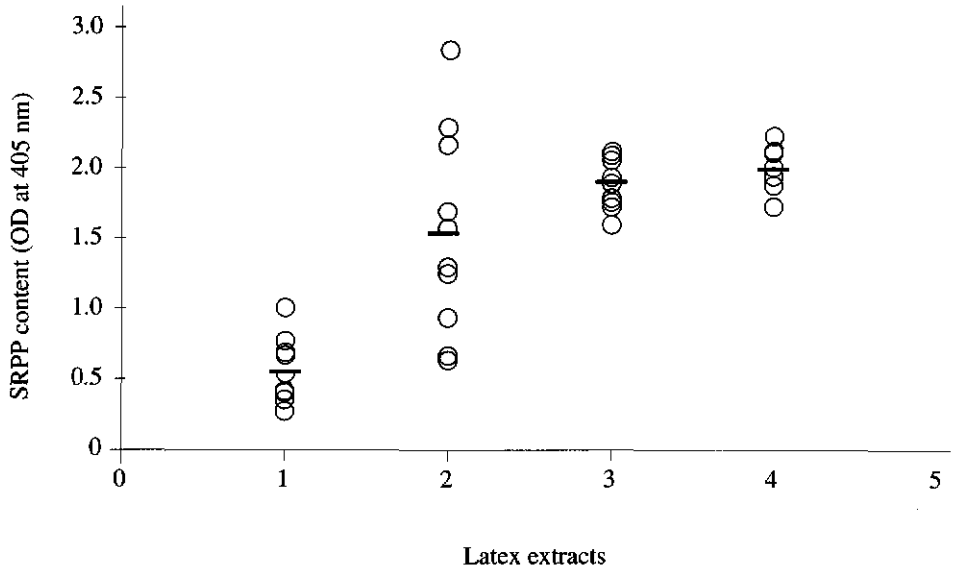

Figure 2. Variation in SRPP content in C-serum of normal trees (1) and partially dry trees (2), and SDS-extracted latex of normal trees (3) and partially dry trees (4). Mean values are indicated by bars.

The amount of SRPP present on small rubber particles suspended in the C-serum is only a very small proportion of the total SRPP in latex because most of the small rubber particles would have separated out in the rubber cream, especially in Moir's Zone 2, after centrifugation[18]. To determine if the total SRPP in whole latex actually increased with the onset of partial dryness, the latex was treated with SDS to solubilise all the SRPP bound to rubber particle membranes.

The OD values from SDS extracted latex also showed a slight increase in the partially dry trees over the normal trees, but this difference was not statistically significant *(Figure 2)*. The difference might have arisen from the fact that latex from partially dry trees had higher rubber content ($P = 0.053$, based on the latex total solids) and more SRPP could therefore be expected to be present for a given volume of the latex. When the SRPP content in the SDS extracted serum was corrected by dividing OD values by the total solids of individual trees (to give the relative amount of SRPP per unit of rubber), the difference between partially dry and normal trees was largely eliminated *(Figure 3)*. From these observations, it could be inferred that there was actually no increase in total latex SRPP in the partially dry trees where 19% to 60% of the tapping cut went dry.

The fact that SRPP was not increased with the incidence of dryness was not surprising. The protein is present in great abundance in whole latex although only a small amount of it is found in the C-serum on the suspended small rubber particles. In fact, after the rubber elongation factor, SRPP is the second most frequently expressed gene in *Hevea* latex[21]. The finding that SRPP in whole latex is unchanged implies that there was no significant

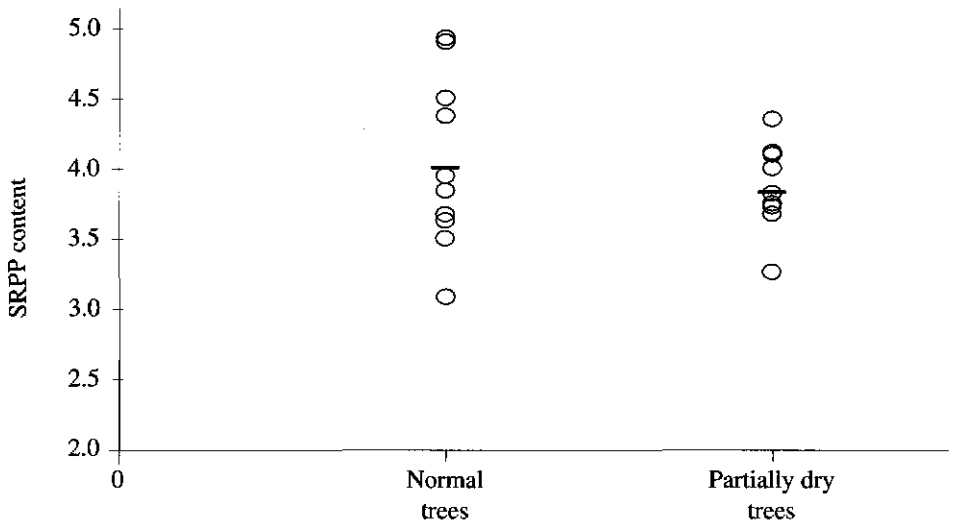

Figure 3. SRPP content in SDS extracted serum of normal and partially dry trees (corrected by dividing OD values by total solids of individual trees). Mean values are indicated by bars.

de novo synthesis of SRPP that might be *directly* associated with the development of TPD. If SRPP in the whole latex did not increase, there was need to explain why the content of this protein increased in the C-serum.

SRPP as a Marker for Latex Instability

Tree dryness is frequently associated with the instability of the latex within laticifers of the affected trees. A principal cause of latex instability is the de-stabilisation of the lutoids present in the latex[1,22,23]. In trees suffering from TPD, the lutoid membranes (the tonoplast) becomes degraded, leading to the lutoids rupturing and releasing their contents, the B-serum, which de-stabilise latex[1,22,23]. Lutoid damage is most commonly quantified by the bursting index of lutoids[15,16], and in the present study, tree dryness was indeed characterised by significantly increased bursting index (53.0 in partially dry trees as compared with 33.3 in control trees; $P = 0.009$). Lutoid damage, as reflected in the bursting index of lutoids, has previously been shown to be correlated with the onset and development of dryness[23]. Again in the present study, partial dryness was correlated with the bursting index ($r = 0.578$, $P = 0.008$, *Table 1*), indicating that it could serve as a marker for the onset of tree dryness. One possible weakness of the bursting index of lutoids, however, was that its determination depended on the lutoids actually breaking to release their contents. In fact, degraded and unstable lutoids would exhibit a de-polarisation of their membranes even before they actually break[24]. Hence, the bursting index of lutoids may not be a sufficiently sensitive indicator of lutoid de-stabilisation where the lutoids are degraded, but are not broken.

Small rubber particles are generally less dense than the C-serum, whereas the lutoids are more so. The former have been shown to adhere

TABLE 1. CORRELATION OF BARK DRYNESS (%) WITH THE BURSTING INDEX OF LUTOIDS, C-SERUM SRPP AND TOTAL LATEX SRPP

Variable	Regression coefficient (r)	Statistical significance
Bursting index of lutoid	0.578	P = 0.008
C-serum SRPP	0.712	P < 0.001
Total latex SRPP	0.396	P = 0.084
Total latex SRPP, corrected for total solids	–0.016	P = 0.951

to de-stabilised intact lutoids where their membranes have presumably de-polarised[25]. Such agglomerations of rubber particles and unstable lutoids could have a net specific gravity close to that of the C-serum and they may remain suspended in the C-serum after latex centrifugation. Hence, the significant increase in SRPP that was observed in the C-serum of partially dry trees could have been due to small rubber particles adhering to the increased unstable lutoids present in the latex. By this rationale, SRPP could itself serve as a suitable indicator of lutoid and whole latex instability. Indeed, logarithm-transformed C-serum SRPP was found to be significantly correlated to the bursting index of lutoids (r = 0.553, P = 0.011, *Figure 4*). When C-serum SRPP was correlated against partial dryness, the correlation coefficient, r = 0.712, P<0.001 *(Figure 5, Table 1)*, was even better than the correlation obtained with the bursting index of lutoids (r = 0.578, as mentioned above).

The fact that total latex SRPP did not increase with tree dryness has been mentioned above. As shown in *Table 1*, total latex SRPP was poorly correlated to tree dryness (r = 0.396), this correlation disappearing altogether when SRPP values were corrected for the rubber content (measured by the latex total solids).

In addition to the correlation data presented in *Table 1*, the relative usefulness of C-serum SRPP and the bursting index of lutoids as a marker for TPD can be gauged from the partial correlation coefficients between dryness, SRPP and bursting index, when the variation in either SRPP or bursting index was kept constant. A partial correlation coefficient of 0.596 (P<0.01) was obtained between dryness and SRPP even with the bursting index held constant. However, the partial correlation coefficient between dryness and bursting index (r = 0.349) was no longer significant once the variation in SRPP was taken into account. These results showed that the relationship between dryness and bursting index could be largely explained by the relationship between the bursting index and SRPP. This was confirmed in the multiple regression between dryness with SRPP and the bursting index. The regression equation obtained was:

Partial dryness = –13.475 + 17.37 (C-serum SRPP) + 0.360 (bursting index).

While the regression coefficient for SRPP was significant (P = 0.007), that for the bursting index was not (P = 0.143). This showed that the bursting index did not

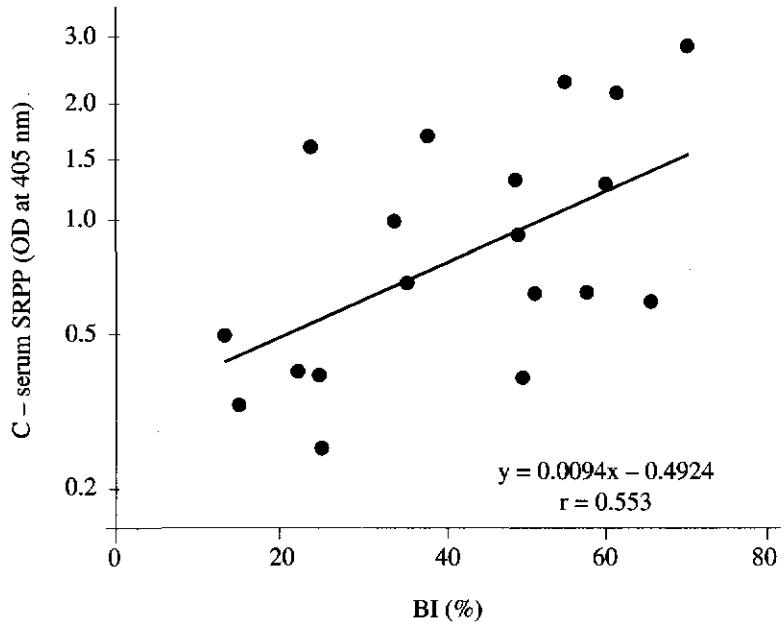

Figure 4. Regression of C-serum SRPP (logarithm-transformed scale) on bursting index of lutoids (BI).

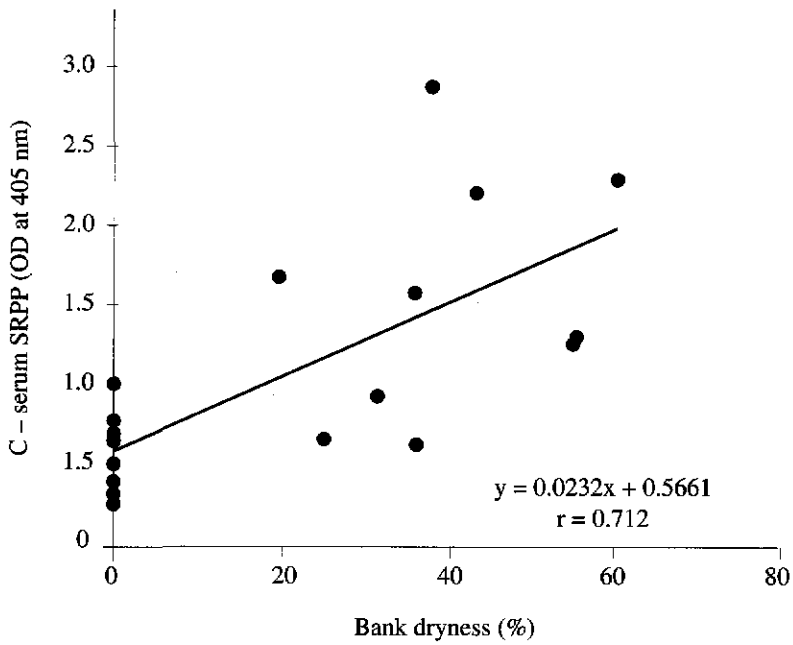

Figure 5. Regression of C-serum SRPP (OD at 405 nm) on bark partial dryness.

contribute further in explaining the variation in dryness by SRPP alone

From these results, SRPP might be a good *indirect indicator* for the onset of TPD by its being a sensitive indicator of latex instability

CONCLUSION

- The 22 kDa – 26 kDa protein marker for TPD is identified as the small rubber particle protein (SRPP), also known as Hev b 3 It is an insoluble protein found on the membrane of small rubber particles
- SRPP content rises in the C-serum of latex from partially dry trees possibly because of the increase in small rubber particles that remain suspended in the serum of de-stabilised latex after centrifugation
- There is no *de novo* synthesis of SRPP associated with TPD and the protein is not therefore a *direct* marker for incipient tree dryness
- SRPP may be *indirectly* associated with TPD by its being an indicator of latex instability It is a better indicator than the bursting index of lutoids in explaining the variation in partial dryness

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