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Effect of Modifying EPDM on the Crosslink Distribution in NR/EPDM Blends

P.S. BROWN*# AND A.J. TINKER*

The swollen-state FT-NMR spectroscopic method of blend analysis[7] has been applied to blends of NR with two different EPDMs and chemically modified versions of these EPDMs. The crosslink densities in the EPDM phases of the blends with unmodified EPDM are very low, approximately 10 mol m^{-3}. The presence of the chemical modification to the EPDM has a dramatic effect on crosslinking in the EPDM phase, more than doubling it, but only a minor one on that in the NR phase. The overall crosslink density in the blend is, therefore, increased. Despite these changes there remains a large imbalance in the crosslink distribution in favour of the NR phase in both modified blends, yet the reported physical properties are good[3,4]. This suggests that there may be a threshold value for the crosslink density in the EPDM phase for a blend to achieve good physical properties.

Blends of NR with EPDM have long been recognised as suffering from cure rate incompatibility. This incompatibility arises from the difference in olefin concentration of the two polymers; NR is essentially 100 mol% olefin, whereas EPDM rarely comprises greater than 10% diene monomer by weight (2–3 mol% olefin). Cure rate incompatibility is thought to cause the inferior physical properties of NR/EPDM blends. Early attempts to improve the properties of EPDM/polydiene rubber blends involved changes in the cure system; either limiting the mobility of the cure system and cure intermediates by the use of a large metal counterion (*e.g.*, lead)[1], or by selecting curatives with a high solubility in the EPDM phase[2]. Several authors have used polymer modification as a route to reduce the cure rate incompatibility[3–5]. Hopper reacted a potential vulcanisation inhibitor with the EPDM olefin groups, producing PVI groups bound onto the EPDM. He postulated that these groups capture curatives during the early stages of vulcanisation[3] thus reducing the access of the NR to the cure system, improving the crosslink distribution and increasing tensile strength by 50% (15.2 MPa to 21.8 MPa). Coran reacted EPDM with maleic anhydride to introduce a potential metal chelating group onto the polymer backbone, thereby improving the physical

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properties of blends with NR[4], the tensile strength being increased from 14.8 MPa to 23.3 MPa. He postulates that zinc oxide from the sulphur-based cure system of the blend forms ionomeric crosslinks with these chelating groups on the modified EPDM. Morrissey halogenated the EPDM to increase the number of cure sites[5].

A previous paper reported the application of a ^{1}H CW-NMR spectroscopic technique to the study of crosslinking in the NR phase of blends with EPDM and a maleic anhydride modified EPDM[6]. Crosslinking in the EPDM phase could not be determined using this technique. This paper describes the application of the more recently developed technique of swollen-state FT-NMR spectroscopy[7] to the blends of the type described by both Coran and Hopper. The availability of ^{13}C NMR with this revised technique has permitted the study of crosslinking in both phases of the blends.

MATERIALS AND METHODS

The rubbers used in this study were *Nordel 1470* (E.I Dupont), *Intolan 155* (Enichem), and natural rubber (SMR 10, Malaysia). Modified *Intolan 155* was produced according to the method of Coran[4], whilst the PVI grafted EPDM [N-chlorothio-N-methyl-benzenesulphonamide grafted *Nordel 1470*, 2.9 g bound/100 g rubber (11.5 mmol/100 g)] was kindly supplied by Dr R.J. Hopper of the Goodyear Tire & Rubber Company, Akron, Ohio (USA). Rubber chemicals were standard commercial grade materials, and solvents were of AR grade except for the NMR solvents which were of spectroscopic grade [deuterochloroform ($CDCl_3$) and tetramethylsilane (TMS) Aldrich Chemical Company].

Compounding (*Tables 1* and *2*) was performed by using a BR size Banbury internal mixer or a two roll mill, the curatives being added on a two-roll mill. Test sheets (225 x 225 x 1 mm) were cured at 150°C to t_{max} as determined using Monsanto ODR or MDRE rheometers.

Samples of vulcanisate for NMR analysis were extracted for 4 h with methanol in a hot Soxhlet apparatus, dried to constant weight *in vacuo* and then stored *in vacuo* in the dark until required. Small slivers were swollen in $CDCl_3$, containing some TMS as an internal reference for 24–48 h before being trimmed so as to spin freely in an NMR tube containing fresh solvent.

FT-NMR spectra were obtained using a General Electric QE300 300 MHz Fourier Transform spectrometer fitted with a ^{13}C/^{1}H dual 5 mm probe, Nicolet 1280 processor and an Oxford Instruments 7 tesla super-conducting magnet. The acquisition conditions are given in *Table 3*. The FIDs were transferred to an Epson AX3S PC for manual phasing of the transformed FID, and the spectrum data were then transferred to a Prime minicomputer for further numerical analysis. Auto phasing was found not to cope well with these spectra which contain predominantly broad signals.

The width of the NR olefin peak in ^{1}H spectra of the blends was estimated by using the parameter H%[7], determined at a reference offset of 0.20 p.p.m. The crosslink density within the NR phase was interpolated from H% by using a

TABLE 1. FORMULATIONS I: NORDEL SINGLE POLYMER VULCANISATES

Compound	N1	N2	N3	N4	MN1	MN2	MN3	MN4
Nordel 1470	100	100	100	100				
Mod EPDM2[a]					106.6	106.6	106.6	106.6
Zinc oxide	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Stearic acid	1.75	1.75	1.75	1.75				
Wingstay L	1.00	1.00	1.00	1.00	0.50	0.50	0.50	0.50
Sulphur	0.50	1.00	1.50	2.00	0.50	1.00	1.50	2.00
MBS	0.25	0.50	0.75	1.00	0.25	0.50	0.75	1.00

[a] The modified EPDM2 is *Nordel 1470* with 0.5 p.p.h.r. *Wingstay L*, 3.5 p.p.h.r. Stearic acid and 2.9 g grafted sulphonamide.

TABLE 2. FORMULATIONS II: NR AND NR/EPDM BLENDS

Compound	Blend C1	Blend M1	Blend C2	Blend M2	NR1
SMR L	70	70	50	50	100
Intolan 155	30				
Mod EPDM1[a]		30			
Nordel 1470			50		
Mod EPDM2				53.30	
Zinc oxide	5.50	5.50	4.00	4.00	4.00
Stearic acid	2.00	2.00	1.75		1.75
Wingstay L			1.00	0.75	1.00
Sulphur	2.00	2.00	2.00	2.00	2.00
MBS	0.50	0.50	1.00	1.00	1.00

[a] The modified EPDM1 is *Intolan 155* modified with 2 p.p.h.r. maleic anhydride according to the method of Coran[4]

TABLE 3. NMR ACQUISITION PARAMETERS

Parameter	^{1}H	^{13}C
Frequency (MHz)	300.15	75.48
Sweep width (Hz)	6 024	20 000
Data size	16 384	32 768
Pulse width (μs)	3.0	6.0
Pulse width,°	3.0	60
Acquisition time (s)	1.36	0.819
Delay time (s)	10.0	3.0
Temperature (°C)	20 – 22	20 – 22
Spin rate (Hz)	18 – 20	18 – 20
Number of acquisitions	128	15 000, 40 000[a]

[a] 15 000 for single polymer vulcanisates, 40 000 for blends

previously obtained plot of H% against physical crosslink density[8]. The peak widths in ^{13}C NMR spectra of blends and single polymer vulcanisates were determined in two ways. Both of the NR olefin signals are single peaks (chemical shifts of 125 p.p.m. and 135 p.p.m.); as there is no overlap with other signals in the spectrum (*Figure 1*) peak width at half peak height ($W_{½}$), the conventional NMR measure, can be used as the line breadth measure. The $W_{½}$ data were converted to crosslink densities using the correlation presented in reference 7.

The ^{13}C NMR signals from the EPDM rubbers are more complicated. There is a considerable number of peaks in a narrow region of the spectra, and thus there is some degree of overlap. In addition, the two signals arising from the aliphatic NR backbone carbon atoms and that form the NR methyl group also lie in this region (*Figure 2*). Two sets of EPDM peaks show both a good variation of peak width with crosslink density and are sufficiently remote from the other signals in the region for easy analysis. These are the two peaks at 37 p.p.m. and the three peaks at 30 p.p.m. (*Figure 3*). Although the individual peaks within the two groups arise from different carbon atoms on the EPDM molecule, they all broaden with increasing EPDM cross-link density and are analysed as a group. Both sets of signals were analysed in the same way; a line broadening measure, H%, is calculated as the ratio of the signal intensity at the valley positions (a, a') to that of the highest peak in the group (b, b', *Figure 3*), correcting for the baseline intensity. The measurements were taken manually from large scale expansions of the spectrum.

The physical crosslink densities in the single polymer EPDM vulcanisates were determined by using stress-strain analyses according to the method of Chapman and Porter[9].

RESULTS AND DISCUSSION

N-chlorothio-sulphonamide Modified EPDM

Single polymer vulcanisates. Although the control and modified EPDM1 single polymer vulcanisates were similarly com-

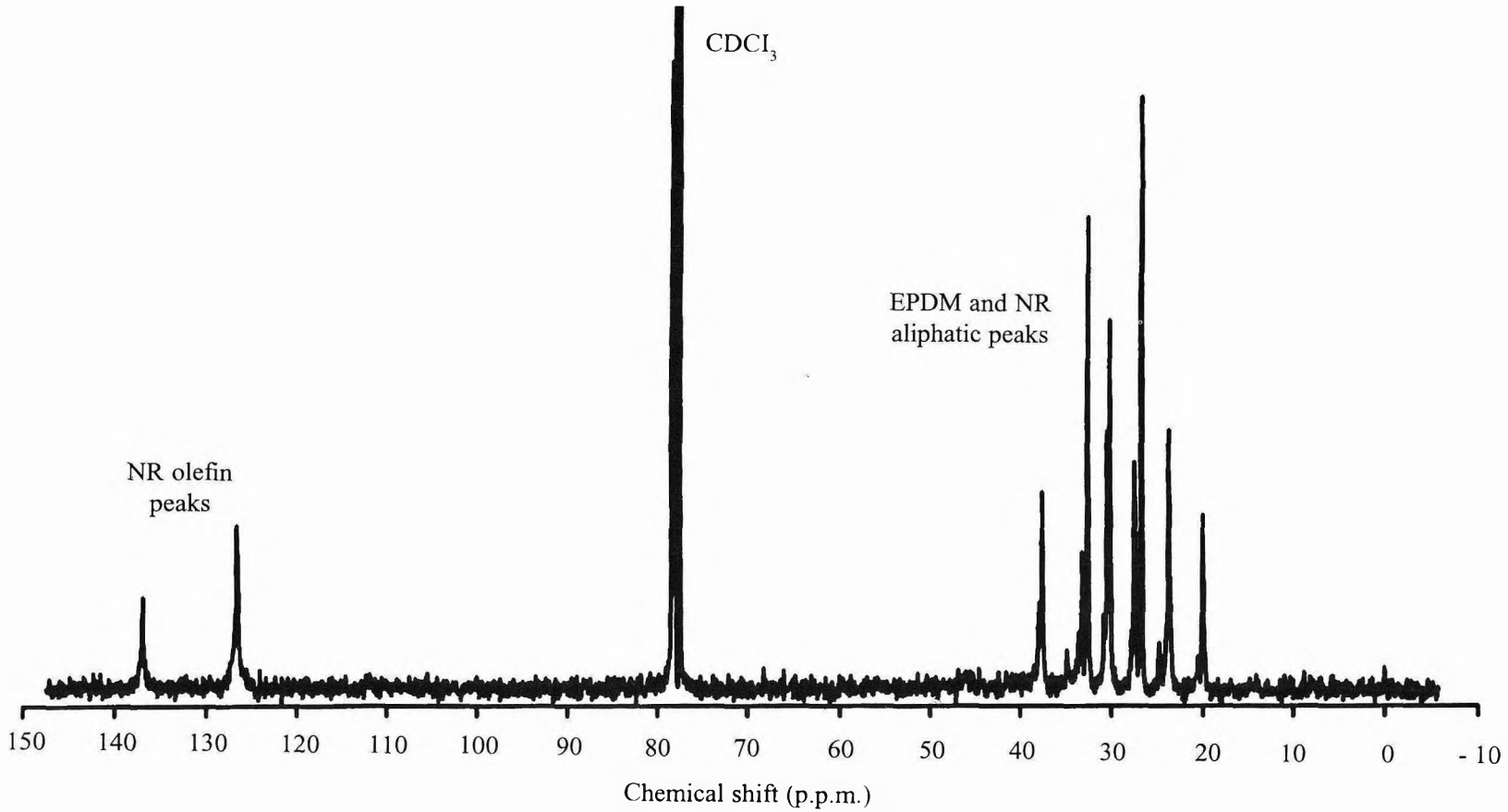

Figure 1. Full ^{13}C NMR spectrum of Blend C1 showing the two distinct regions of elastomer signals. The NR olefin peaks are labelled.

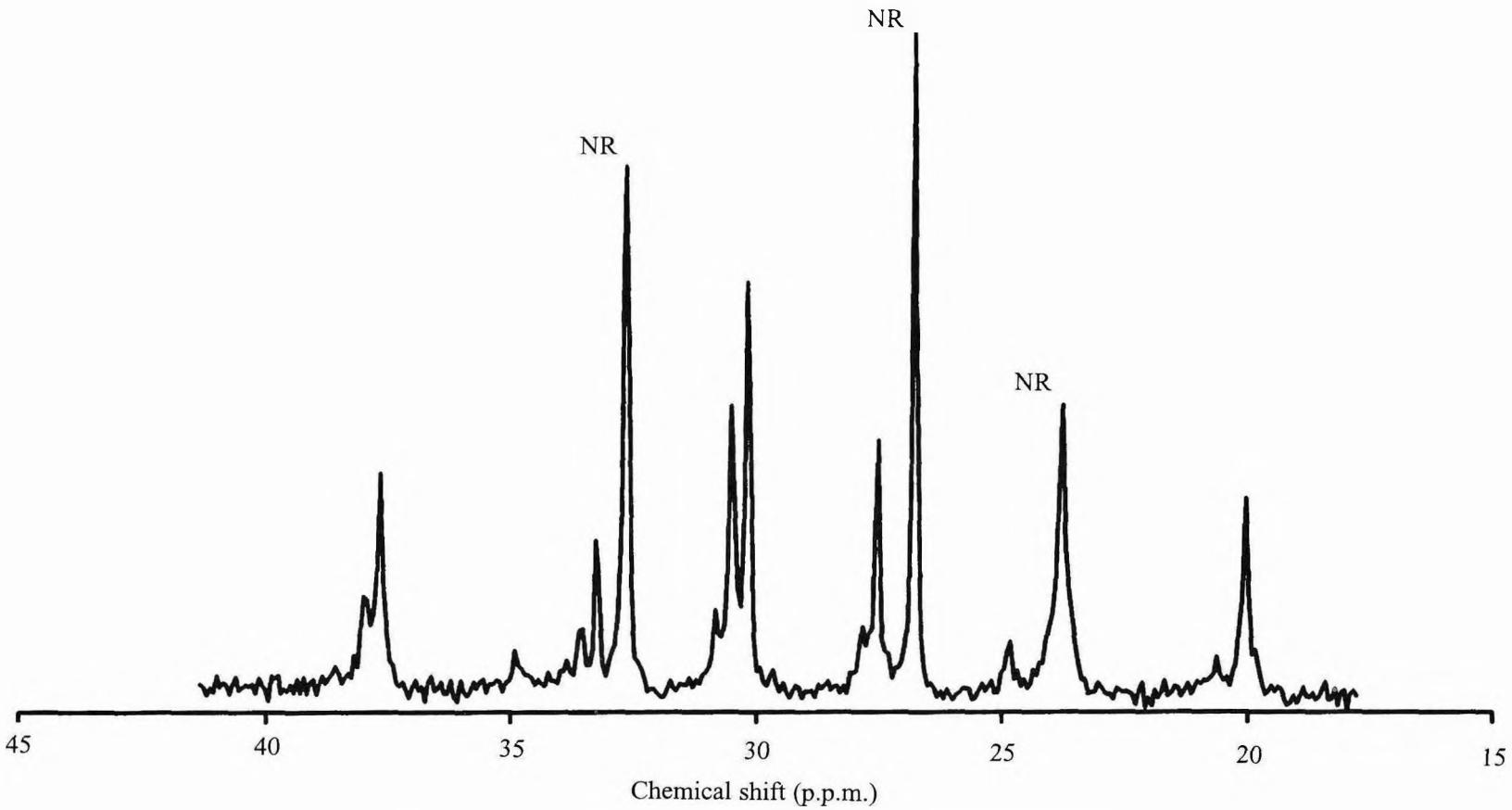

Figure 2. Aliphatic region (20–50 p.p.m.) of the ^{13}C NMR spectrum of Blend C1.

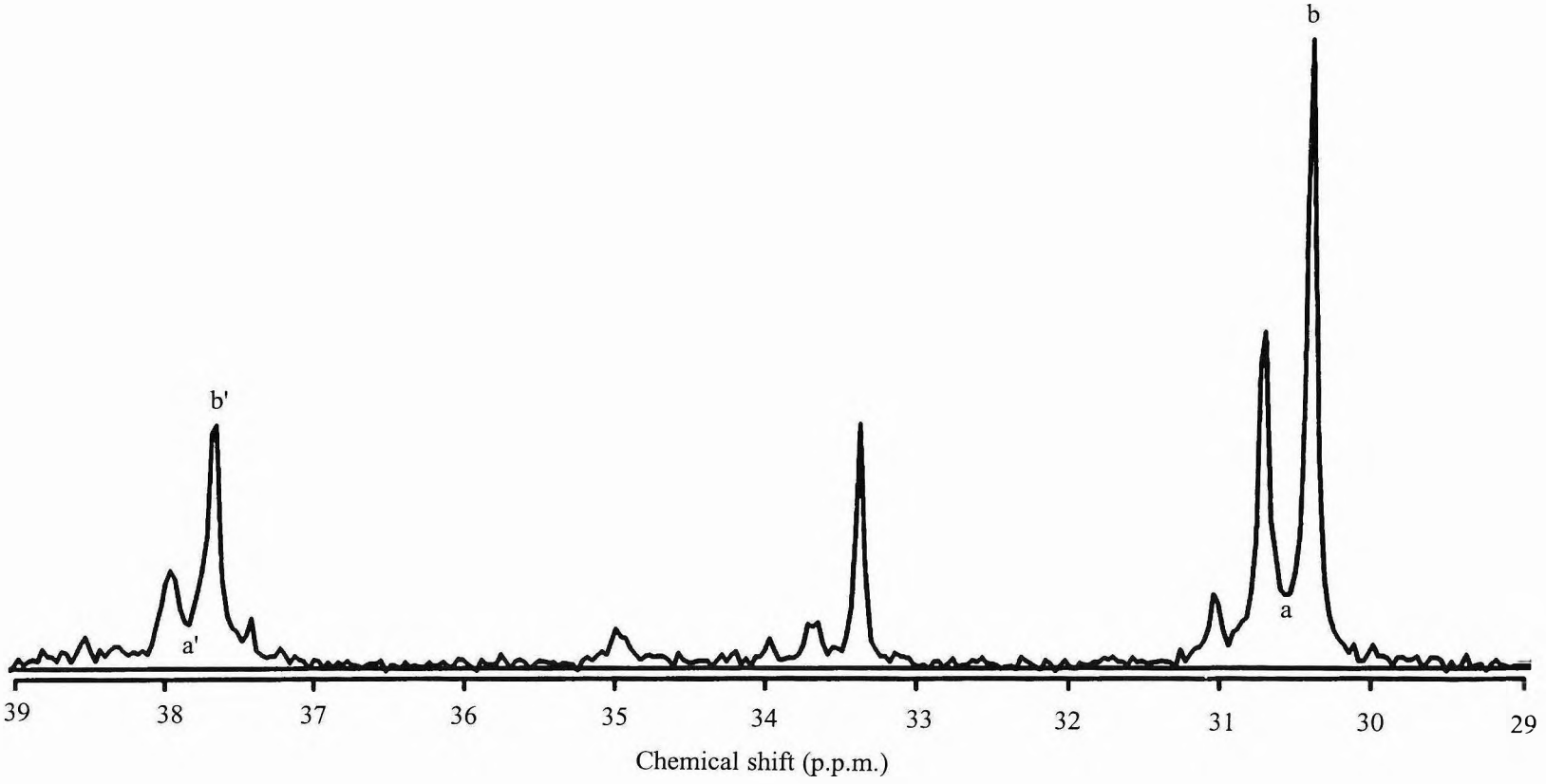

Figure 3. Expanded section of the ^{13}C NMR spectrum of single polymer vulcanisate N1 showing the triplet and doublet peaks at 30 and 37 p.p.m. together with the reference points used to determine the H% values (a and b and a' and b').
Note the similarity of the peaks with those of the Intolan 155 *blend in* Figure 2.

TABLE 4. NORDEL SINGLE POLYMER DATA

Vulcanisate	C1 (kPa)	½Mc (mol m^{-3})	H% (30)	H% (37)
N1	16.7	6.7	10.1	15.5
N2	60.8	24.7	39.9	45.5
N3	82.4	33.4	55.0	60.0
N4	91.3	37.0	62.0	64.0
MN1	82.4	33.4	58.1	62.9
MN2	107.9	43.8	62.5	68.9
MN3	121.6	49.4	67.7	72.0
MN4	125.6	51.0	72.0	81.0

pounded, (*Table 4*) their crosslink densities were very different. The values obtained with the control vulcanisates ranged from 6.7 to 37 mol m^{-3}, those using the modified material from 33.4 to 51 mol m^{-3} (*Table 5*). This increase of crosslinking in the presence of the modification was also reflected in the NMR spectra of the modified EPDM vulcanisates which were considerably broader. The two H% values (37 p.p.m. and 30 p.p.m.) were quite similar for both EPDMs, and H% was found to increase smoothly with crosslink density. These data appear to lie on a common curve, indicating that the modification does not greatly interfere with the analysis (*Figure 4*). This curve was used to interpolate the crosslink densities in the EPDM phases of the blends with NR (*Table 6*).

NR/EPDM blends. ^{1}H NMR spectroscopy of the blends can only give information regarding the NR phase. H% was found to be similar in both blends, with that in the modified blend having a slightly lower value (*Table 5*). The difference is similar to the scatter expected in the NMR measurements, so it may not be significant, but it is equivalent to a fall of about 5% in the crosslink density in the NR phase (85 to 81 mol m^{-3}). A similar small reduction in the NR crosslink density was observed in the ^{13}C NMR spectra of the blends, 95 to 92 mol m^{-3}. These values are a little higher than those determined from ^{1}H NMR spectroscopy, but not so great as to cause concern over the reliability of the technique. That both methods indicate that the modification effects a small reduction in the NR crosslink density suggests that it is a real observation and not just scatter in the data.

In both blends, the crosslink density in the NR phase is considerably higher than in the single polymer analogue (NR1 in *Table 5*). These increases in cross linking in the NR phases of the blends (25–30 mol m^{-3}) represent a considerable capture of the curatives by that phase, even in the presence of the vulcanisation inhibitor bound to the EPDM.

Despite the minimal effect on crosslinking in the NR phase, this modification does cause a considerable increase in the crosslink density in the EPDM phase of

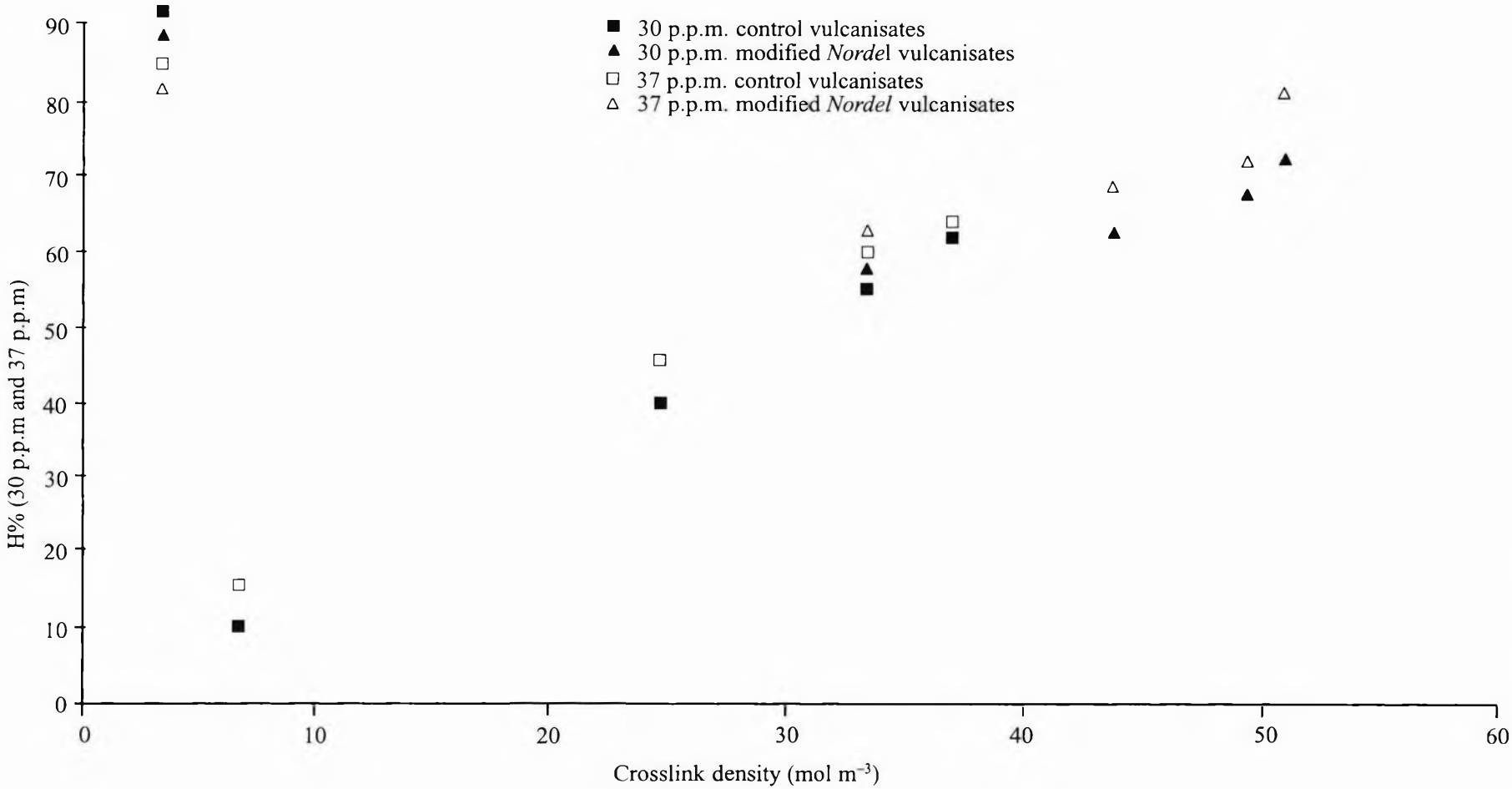

Figure 4. H% (^{13}C spectra 30 p.p.m. peaks) versus *crosslink density for single polymer* Nordel *Vulcanisates.*

TABLE 5. N-CHLOROTHIO-SULPHONAMIDE EPDM BLEND DATA

	Blend C2	Blend M2	NR1
EPDM H% (30 p.p.m.)	10.9	42	
EPDM H% (37 p.p.m.)	15.2	46.2	
EPDM ½Mc, mol m^{-3} (30, 37 p.p.m.)	7.0, 6.5	26.3, 25.0	
NR H% (^{1}H NMR)	84	81	70
NR ½Mc, mol m^{-3} (^{1}H NMR)	85	82	57
NR $W_{½}$, Hz (^{13}C NMR)	57, 57	54, 54	35, 35
NR ½Mc, mol m^{-3} (^{13}C NMR)	95	92	65

the blend, raising it by a factor of 3.5 (*Table 5*). The modification is present at about 11 mmol/100 g in the EPDM[3], or 5.5 mmol of modification in the blend, whilst 1 g of MBS is 4.4 mmol of the accelerator. If all of the compounded accelerator is preferentially captured by the polymer-bound PVI and a crosslink in the EPDM is produced upon its release, a crosslink density of about 90 mol m^{-3} would result. That level is not observed, however crosslinking in the EPDM does increase by about 18 mol m^{-3} to a level of 26 mol m^{-3},a significant improvement over that in the control. This indicates that only 20–30% of the polymer bound PVI is acting in the manner suggested by Hopper[3].

It is interesting to note that the crosslink density in the EPDM phase of control blend C2 is comparable to that in the single polymer EPDM vulcanisate N1, but the crosslink density in the EPDM phase of the modified blend M2 is lower than that observed in the single polymer vulcanisate MN1, the modified EPDM analogue of vulcanisate N1. Thus it appears that additional crosslinking, which the modification produces in the single polymer EPDM vulcanisates, is reduced upon blending with NR. The crosslink density determined for the NR phase of blend M2 shows that this phase is still capturing a significant proportion of the curatives even in the presence of the PVI bound to the EPDM. The reduced effect of the PVI modifier in boosting crosslinking in the EPDM phase in the blend is a consequence of the curative capture by the NR phase.

Blends with Maleic Anhydride Modified EPDM

The crosslink densities in the NR components of NR/maleic anhydride modified EPDM blend and its control blend were reported in an earlier publication[6]. The NR was found to have a crosslink density considerably higher than expected from the compounding. The observed level was equivalent to that expected if 80–90% of the curatives in the blend were utilised by this polymer alone, *i.e.* equivalent to a single polymer NR vulcanisate compounded with almost twice the level of curatives used in the blend. The maleic acid modification of the EPDM had only a minor effect on the crosslinking in the NR phase[6]. In this current study, the use of ^{13}C NMR

TABLE 6. MALEIC ANHYDRIDE EPDM BLEND DATA

	Blend C1	Blend M1
EPDM H% (30, 37 p.p.m.)	17.9, 21.4	43.3, 45.6
EPDM crosslink density, mol m^{-3} (30, 37 p.p.m.)	11.4, 10.5	26.4, 24.7
NR $W_{½}$, Hz (125, 135 p.p.m.)	30, 30	24, 24
NR crosslink density, mol m^{-3}	61	52
NR crosslink density by ^{1}H NMR[6], mol m^{-3}	56	53

spectroscopy allows the study of crosslinking in the EPDM phase. In the absence of a series of single polymer *Intolan 155* vulcanisates to create the necessary crosslink density H% correlation plot, a fully quantitative analysis of the data is not possible. However, the similarity in the ethylene:propylene ratio of the two EPDMs means that the crosslink density-H% correlation produced for *Nordel 1470* should be applicable to these blends without too great an error, thus the analysis of crosslinking in the EPDM phase can be qualitative or even semi quantitative.

The peak width data are given in *Table 6*. The data for the NR olefin peaks are in agreement with the earlier ^{1}H data in that there is a small reduction in peak width in the presence of the modification ($W_{½}$ falls from 30 to 24 Hz). The EPDM multiple peaks at 30 and 37 p.p.m. both show considerable increases in H%, the values roughly doubling (*Table 6*).

The ^{13}C NMR peak width data confirm the finding of the earlier study; the NR crosslink density is only slightly reduced (from 61 to 52 mol m^{-3}). These data are in remarkably good agreement with the earlier ^{1}H results (*Table 6*), again suggesting that the reduction is real. While it is strictly incorrect to use the crosslink density/H% correlations produced for the other EPDM in this study to estimate the EPDM crosslink density in these blends, its use will provide a reasonable estimate of the crosslink density in the EPDM phase. Such an analysis suggests that the EPDM crosslink density in the modified blend is about two and a half times that in the control blend (25 mol m^{-3}, up from 11 mol m^{-3}). It is worth noting that this value is similar to that found in the blends with N-chlorothiosulphonamide modified EPDM.

CONCLUSIONS

These two different approaches to solving the problem of cure incompatibility between NR and EPDM elastomers result in broadly similar changes in the crosslink distribution. The poor properties of the control blends are certainly a consequence of the very low crosslink density in the EPDM phases of these blends. Both modifications cause a significant increase in the crosslink density within the EPDM phase without causing a great change in the NR phase of the blend, yet in both cases the crosslink distributions still show a marked bias in favour of the NR phases. The improved physical properties that arise from these modifications[3,4,6] are probably due to a combination of effects. The increase in overall crosslink density and the reduction in the difference

in the moduli of the two phases must contribute to the improvement. Reducing the imbalance of crosslinking may also lead to improved interfacial crosslinking[10] which would have a marked effect on ultimate properties.

The NMR results show that it is not necessary to create an even crosslink distribution in the blends to produce good physical properties. Merely to have a sufficient level of crosslinking in the EPDM phase of the blend 20-25 mol m^{-3} would appear to be enough for this blend system. This is an important result. It is unlikely that any simple process could be found to effect even crosslinking in these blends, however, a crosslink concentration of 25 mol m^{-3} in the EPDM only requires a two to three fold increase, an achievable target. There may also be implications for other blend systems, although the optimum crosslink density in the softer phase may not be that found in these studies.

ACKNOWLEDGEMENT

The authors would like to express their sincere thanks to Dr R.J. Hopper of Good-year Tire & Rubber Company, Akron, Ohio for the kind gift of the N-chlorothio-sulphonamide modified *Nordel 1470*, without which this work would have been impossible. The authors would also like to thank the Board of the Tun Abdul Razak Research Centre for permission to publish this work.

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Latex Protein Allergy: A Prevalence Study of Factory Workers

M.R. AZIZAH[*], M. SHAHNAZ[*], H. HASMA[**#], K.L. MOK[**], ESAH YIP[**] AND B.A. NASURUDDIN[*]

This paper concerns the study of prevalence of Type I latex protein allergy among workers in various latex glove factories in Malaysia. A total of 149 subjects (108 females and 41 males) with a mean age of 30.6 years were examined both by questionnaires and by skin prick test (SPT). In the absence of a standardised SPT latex allergen mixture, the clinical test was carried out using six glove extracts with extractable protein content varying from 0.02 mg/g to 0.75 mg/g of gloves (or 20 μg/g to 750 μg/g), as measured by the RRIM modified Lowry microassay.

Only three subjects were found to show wheal size ranging from 2– 4 mm when tested with glove extracts with extractable protein content of > 0.6 mg/g. Such reaction, was however not detected in all cases when protein levels were at 0.1 mg/g or lower. This prevalence as compared to those reported in the West is relatively low.

Natural rubber products have been used widely for over a hundred years. Although their use has been known to be associated with Type IV allergy in some users for more than sixty years[1], no serious incidence has occurred through their usage. While this type of allergy is brought about by some residual chemicals[2,3], added to the latex during processing, the Type I allergy reported recently[4–7] is caused by the presence of some residual soluble proteins in latex products[8]. Unlike the Type IV reaction which is of cell-mediated delayed hyper sensitivity, the Type I allergy is of immediate hypersensitivity and is IgE-mediated[9]. Absorption of the allergenic proteins is mainly *via* cutaneous and mucosal routes. Symptoms involve urticaria, rhinitis, conjunctivitis, asthma and, only very rarely, anaphylaxis. The onset of this type of allergy is believed to be due to a myriad of factors, one of which is the sudden demand in the late 1980s for latex products such as gloves and condoms, which are very good protective barriers against viral diseases, particularly AIDS. It is thought that the increased exposure to latex products has resulted in sensitisation of, especially, the atopic individuals.

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For diagnosis of the Type I hypersensitivity, various methods[10-14] have been used by different people. The most preferred method, by far, is the skin prick test (SPT), despite the lack of a standardised latex allergen-containing reagent. This test is very sensitive, and when performed with proper reagent preparation and the prick technique, it provides a very simple, convenient and safe diagnostic method.

To-date, the prevalence of Type I latex protein allergy in the general population is still unknown, although the risk appears to be higher among the atopic than the non-atopic individuals. The high risk groups identified in the West among the predominantly latex product users, are the healthcare workers and children with *spina bifida* and urogenital abnormalities. To a lesser extent, the rubber industry workers are also included. Using mainly the SPT and the radio-allergosorbent test (RAST), a number of prevalence studies have been carried out mostly among healthcare workers[15-18] and the *spina bifida* children[19-21], and to a lesser extent, the rubber factory workers[22]. In Malaysia, (the world's largest producer of latex products) the group of people who are constantly exposed to latex or rubber are the rubber tappers, rubber factory workers and the latex product manufacturing plant workers. Prevalence of Type I latex protein allergy among these groups of people has, however, not been studied yet. Work was thus undertaken to do this. This paper concerns the study of latex factory workers.

MATERIALS AND METHODS

149 workers from a number of latex glove manufacturing plants in the states of Selangor, Negeri Sembilan and Malacca were examined. All workers were interviewed with the guide of a prepared questionnaire for information regarding age, sex, duration of time working in an occupationally exposed area, history of various allergies and family history of allergy, if any. Each subject was then skin prick tested in a hospital.

Glove Extracts (Test Reagents)

Six different brands of latex medical gloves were obtained commercially. Glove pieces from each brand, weighing 1 gram, were cut into 1 cm^2 and extracted in 5 ml of phosphate buffered saline (PBS) at pH 7.2 at room temperature for 1 h. The resulting extracts were centrifuged at $1600 \times g$ for 10 min to remove any particulate contamination, to give clear test extracts.

Protein Concentration of Glove Extracts

Protein concentration was determined essentially by the RRIM modified Lowry method[23]. Soluble proteins were first extracted from glove pieces of each brand of glove in PBS (pH 7.2) at 23°C for 3 h. After removal of the glove powder by centrifugation, protein in each clear extract was precipitated prior to measurements using trichloroacetic acid (resulting concentration of 5%) and phosphotungstic acid (resulting concentration of 0.22%). The resulting suspensions were centrifuged, and the sedimented protein from each sample was redissolved in minimum quantity of 0.2 *M* sodium hydroxide. Protein concentration was then measured by the Lowry colorimetric microassay. Absorbance values at 750 mm were read against a curve calibrated using bovine serum albumin (BSA) standard.

Skin Prick Test

A drop of test extract was introduced onto the volar surface of the foreman. A flap of the skin was gently lifted with a sterile lancet (2.4 mm, Beckton Dickinson, New Jersey) through the drop, allowing the allergens to penetrate into the skin. Reaction was observed after 15 min. Positive reactions appearing as wheals were recorded, and the wheal size measured. Histamine (1 mg/ml) and PBS (pH 7.2) were used as positive and negative controls, respectively. Wheal size was graded according to the Bencard Skin Test Reaction Chart (Bencard Allergy Diagnosis, UK) as indicated below:

–	:	no wheal and absent erythema or erythema less than 1mm in diameter
+	:	wheal absent or very slight erythema present and not more than 3 mm
++	:	wheal size not more than 3 mm diameter with associated erythema
+++	:	wheal size between 3 mm to 5 mm diameter with associated erythema
++++	:	any larger reaction possibly with pseudopodia.

Reaction showing wheal size equal or larger than ++ was considered to be positive. All glove extracts used were freshly prepared.

RESULTS

The 149 workers examined consisted of 108 females and 41 males. Their mean age was 30.6 years, ranging from 17 to 54 years. All have been working in the glove manufacturing plants for a mean duration of 4.5 years. Twenty-two (14.8%) had worked for more than 5 years in the plants. Fifty-eight (39%) had history of various allergies and thirty-two (21.5%) had strong family history of atopy. Nine complained of hand dermatitis since working in the factories (*Table 1*).

The six different brands of glove used for skin testing were shown to have extractable protein content ranging from 0.02 mg/g to 0.75 mg/g of glove (or 20 μg/g to 750 μg/g). Of the 149 subjects tested, only three (2 females and 1 male) showed positive skin test reaction. Their wheal size varied from undetectable to as large as 4 mm when tested with the extracts from gloves with increasing content of extractable proteins (*Table 2*). All three were non-atopic, with no history of hand dermatitis and have worked in the glove plants for 1-5 years. In addition, they have not experienced any Type I allergic reactions to latex products.

DISCUSSIONS

Although skin prick testing has been found to be the most sensitive diagnostic tool for detecting latex protein allergy, both the allergen reagent and the wheal size evaluation for positive reaction have not yet been universally standardised. In view of this, our study was conducted using extracts from latex gloves which were expected to contain most allergens commonly encountered by affected users. For evaluation of wheal size, the method of Bencard was adopted. According to the Bencard grading, which made no reference to the wheal size shown by the

TABLE 1. PARTICULARS OF LATEX GLOVE WORKERS SUBJECTED TO SKIN PRICK TEST

Item	Number
Number of workers tested	149
Age range (Mean)	17 – 54 yrs. (30.6 yrs.)
No. of female workers	108 (72.5%)
No. of male workers	41 (27.5%)
Mean duration of work in latex glove factories	4.5 yrs.
No. of workers with > 5 yrs. of duration	22 (14.8%)
No. of workers with history of allergies	58 (38.9%)
No. of workers with strong family history of allergies	32 (21.5%)
No. of workers with hand dermatitis	9 (6.0%)

TABLE 2. DEMOGRAPHIC DATA OF THE 3 SUBJECTS (OF A TOTAL OF 149) WITH POSITIVE SKIN TEST REACTIONS TO EXTRACTS FROM LATEX GLOVES WITH VARYING CONTENT OF EXTRACTABLE PROTEINS

Sex/Age	Atopy	Skin prick test: Allergic response (wheal size in mm)						
		Histamine (1 mg/ml)	Extractable protein content of gloves (mg/g glove)					
			0.75	0.69	0.64	0.11	0.07	0.02
F/29	N	4 mm	NR	NR	++ 3 mm	NR	NR	NR
F/20	N	3 mm	+++ 4 mm	+++ 4 mm	++ 2 mm	+ 1 mm	NR	+ 1 mm
M/20	N	4 mm	++ 3 mm	NR	++ 2 mm	NR	NR	NR

Allergic response :

NR : no reaction, no wheal or erythema < 1 mm
+ : wheal absent, or very slight erythema < 3 mm
++ : wheal < 3 mm with associated erythema
+++ : wheal between 3–5 mm with associated erythema
++++ : larger reaction possibly with pseudopodia
Reaction showing wheal size equal or larger than ++ was considered to be positive.

F : female worker
M : male worker
N : no history of atopy

histamine (1 mg/ml) control, the three cases with wheal size of 2-4 mm encountered in the present investigation, were considered positive. This gives a prevalence of 2%. It may be mentioned that the preferred assessment according to Turjanmaa[13] was not used due to the unavailability of histamine (10 mg/ml) required, at the time of the study.

Although atopy and pre-existing hand dermatitis have been reported to be factors underlying an individual's risk in developing latex protein allergy, the three positive cases found did not experience any immediate allergic reaction to latex products. They were neither associated with any form of atopy (allergic rhinitis, asthma, eczema or urticaria), nor had prior hand eczema or dermatitis. Furthermore, the positive responses were not related to the length of time they spent in the manufacturing plants. One may therefore speculate that such positive reactions could well be associated with IgE cross-reactivity involving latex proteins and certain foods[24-27], which were, however, not investigated in this study.

Compared to the 11% prevalence reported by Tarlo *et al.*[22], who skin tested 81 workers in a surgical glove factory in Canada, a prevalence of 2% shown in this study is comparatively low. Although not included, similarly low incidence has also been observed among other high risk groups in Malaysia, such as the healthcare workers, and the rubber tappers[28]. This is indeed in contrast to the comparatively high prevalence reported for the high risk groups in the West, which showed a variation of 2.8% to 16.9% among the healthcare workers[29,30], and 32% to 51% among the *spina bifida* children[31-33].

It is noteworthy that wheal size and hence allergic response shown by the positive subjects (*Table 1*) increased with increasing concentration of extractable proteins tested. Generally, very little or no response was demonstrated by these allergic persons at extractable protein content of 0.11 mg/g (or 110 μg/g) or lower. This is highly consistent with the findings by Yip *et al.*[34] which showed a well correlated relationship between the residual extractable proteins in latex gloves and the allergic responses elicited by them in latex hypersensitive persons. More importantly, extractable protein content of 0.1 mg/g or 100 μg/g and lower,(by the same RRIM modified Lowry method) were shown to be associated with very little or no allergic responses as assessed by the skin prick test. It is often wondered if sensitisation were solely due to frequent exposure to latex products, how is it that high risk groups in Malaysia, who are exposed frequently to latex and latex products, demonstrated such low prevalence, if at all? Various explanations have been proposed, one of which referred to the genetical differences between people in the West and in Malaysia. In view of the recent awareness in cross-reactivity shown by allergens from foods and latex, differences in diets of people concerned have also been suggested to be yet another possible cause. While much work is needed to further understand the allergy reactions, it may be worth noting that the Rubber Research Institute of Malaysia has developed various methods[35] for effective removal of the undesirable residual proteins from latex products, to prevent further sensitisation among users.

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Construction of a Microsatellite-enriched Library from Hevea Brasiliensis

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A microsatellite library was constructed from Hevea brasiliensis *by cloning DNA fragments of between 200 bp – 800 bp in length. These were ligated to pBluescript KS + phagemid as the vector and transformed into* Escherichia coli *DH5α. The library appeared to be highly enriched with simple sequence repeats;* $(GACA)_n$ *– 10%,* $(GATA)_n$ *– 9%,* $(GA)_n$ *– 34% and* $(GC)_n$ *– 9%.*

The oldest and most commonly used DNA marker technique is restriction fragment length polymorphisms (RFLPs). RFLP is based on the ability of an endonuclease to recognise a specific DNA sequence (recognition site) and to cleave at this recognition site, thereby producing DNA fragments of various lengths. However, changes in DNA sequence, such as base addition, deletion or substitution, will result in alteration in these recognition sites, resulting in length variation after digestion of the DNA by that same endonuclease. In the RFLP technique, DNA polymorphisms can be detected by the use of an appropriate hybridisation probe consisting of a cloned DNA fragment. Positive hybridisation signals will be obtained when a DNA probe anneals to a DNA sequence because it is either wholly or partly homologous to that DNA sequence[1, 2]. Genetic maps consisting of RFLP markers have been constructed for a number of plants in order to assist in breeding programmes[3–5]. The usefulness of the map is enhanced when it is used in conjunction with other conventional markers, e.g., morphological and biochemical markers[6]. In recent years, several new classes of molecular markers have gained popularity. One of these is microsatellites or simple sequence repeats (SSRs). Microsatellites consist of stretches of short tandem repeat elements (1–5 bp) which are scattered throughout the genome[7]. As molecular markers, microsatellites have all the characteristics of being very useful because they behave according to Mendelian laws[8], are able to distinguish between two or more individuals[9] and are abundant throughout the genome of the organism studied, *viz.* humans, animals and plants[10–12].

In this study, we attempted to construct a microsatellite-enriched library for *Hevea*

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brasiliensis so that it will serve as a source of informative probes for DNA fingerprinting and genetic mapping. At the same time, the nature and frequency of occurrence of microsatellites in the *Hevea* genome might be learned in the course of the construction of this library.

MATERIALS AND METHODS

Plant Materials

The microsatellite library was constructed according to a protocol adapted from Ostander *et al.*[13] Soft, young, light green *H. brasiliensis* clone GL1 leaves were used for DNA extraction. Harvested leaves were frozen immediately in liquid nitrogen and stored at -70°C until ready for use.

DNA Extraction and Digestion

Total genomic DNA was extracted by the method of Low *et al.*[14] A sample of genomic DNA (300 ng) was digested sequentially with *Eco*RV, *Hae*III, *Hin*fI, *Hpa*II, *Msp*I and *Taq*I with appropriate buffers at 37°C for 16 h according to the manufacturers' instructions. Digestion was terminated by the addition of 1X loading buffer containing 0.006% (w/v) bromophenol blue, 0.6% (w/v) Ficoll 400, 16 mM EDTA. DNA fragments were separated through 1.2% agarose gel in 1X TAE and a 100-bp ladder was used as size markers. That portion of the agarose gel containing DNA which corresponded to 200–800 bp (as indicated by the size markers) was cut out with a sterile scalpel. The DNA was subsequently electroeluted from the gel according to Sambrook *et al.*[15] The concentration of the resultant fragments was estimated by comparison with known concentrations of λ DNA in a gel.

The ends of the DNA fragments were repaired by adding *Escherichia coli* DNA polymerase (Klenow fragment) in a reaction mix containing 200 µl of digested DNA, 30 µl of 10X nick translation buffer [0.5 M Tris-HCl, pH 7.5, 0.1 M magnesium sulphate, 1 mM dithiothreitol (DTT)], 500 µg/ml bovine serum albumin (BSA) and 10 µl of Klenow fragment (4 U/µl). The mixture was incubated for 10 min at 16°C. Distilled water and 24 µl of 2'-deoxynucleoside 5'-triphosphates (dNTPs) containing 2.5 mM of each dNTP namely dATP, dCTP, dGTP and dTTP were added to a final volume of 300 µl. The mixture was incubated at 16°C for a further 30 min. Purification of the DNA was performed by phenol:chlofoform (1:1, v/v) extraction. DNA fragments were precipitated with two volumes of ice-cold ethanol in the presence of 0.3 M sodium acetate, pH 5.2, and stored at -20°C overnight. DNA precipitates were collected after centrifugation and washed twice with 70% ethanol. The DNA pellet was redissolved in sterile distilled water.

Preparation of pBluescript KS+

Phagemid vector pBluescript KS+ (Stratagene, USA) was used for cloning. pBluescript KS+ (10 µg) was digested with 50 U of *Sma*I in the appropriate buffer. The cleaved phagemid was dephosphorylated with 0.5 U of shrimp alkaline phosphatase (United States Biochemical, USA) at 37°C for 1 h. The reaction was terminated by heating the reaction mixture at 65°C for 15 min. After purification with phenol:chloroform followed by

ethanol precipitation, the phagemid DNA was dissolved in a minimal volume of sterile distilled water.

Ligation

DNA fragments (for cloning) and pBluescript KS+ at 50 and 10 ng/µl, respectively, were used at two weight ratios of vector DNA to insert DNA of (1:5) and (1:10). In order to prevent the formation of hairpin loops and to maintain DNA strand separation, these vector and insert DNA samples were initially incubated together at 55°C for 5 min and then plunged into ice. Ligation was then carried out at 15°C overnight. The 35 µl of ligation mix contained 25 mM Tris-HCl, pH 7.4, 5.0 mM $MgCl_2$, 5.0 mM DTT, 0.25 mM spermidine, 1.0 mM ATP, 1.25 mM hexamine cobalt chloride, 10 µg/ml BSA and 2µl of T4 DNA ligase (1 U/µl) (Boehringer Mannheim, Germany). The control reaction differed from the test reaction in that it contained all the reactants except insert DNA fragments.

Success of the ligation was confirmed by electrophoresis of an aliquot of the ligation reaction through agarose gel (1%) against control ligation reaction. A successful ligation would appear as a DNA smear with a higher molecular weight compared to the control reaction.

Transformation

Transformation was conducted according to the protocol provided by Stratagene (USA). *E. coli* DH5α was used as the bacterial host. Competent cells (DH5α) were prepared according to the method of Sambrook *et al.*[16]

Plating of Transformants

The transformed cells were pelleted and resuspended in 50 µl of LB medium. They were then plated over selective LB-agar plates. The selective plates were prepared by spreading 40 µl of X-gal (20 µg/µl) and 4 µl of IPTG (23.8 µg/µl) over LB containing ampicillin (50 µg/ml) on each plate. Plates containing transformed cells were incubated at 37°C for 16 h. White colonies (putative recombinant transformants) were picked with sterile toothpicks. These were inoculated in 3 ml of LB medium and grown overnight for subsequent amplification of DNA inserts.

Insert Amplification by PCR

Insert DNA was amplified by the polymerase chain reaction (PCR). Amplification reaction was carried out in 1X *Taq* DNA polymerase buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl), 100 µM dNTP, 200 nM SK/KS primers (Stratagene, USA), 1 U *Taq* DNA polymerase and 3 µl bacterial culture solution, in a final volume of 25 µl. The reaction was topped with one drop of mineral oil. DNA amplification was carried out through a programme of one cycle of 5 min at 95°C, 5 min at 48°C; 35 cycles of 90 sec at 72°C, 45 sec at 94°C, 45 sec at 48°C and a final 10 min extension step at 72°C. Amplification success was demonstrated by visualisation after agarose gel electrophoresis and staining in ethidium bromide. The amplified products were transferred to nylon filters by Southern blotting. The nylons were subsequently hybridised with oligonucleotide probes.

Generation of Oligonucleotide Probes

Synthetic concatenated oligonucleotides containing repeated sequences such as $(GACA)_n$, $(GATA)_n$, $(GA)_n$ and $(GC)_n$ of a few hundred bps in length were generated by amplification of their respective basic tandem repeat elements. These were $(GACA)_4$, $(GATA)_4$, $(GA)_8$ and $(GC)_8$. Success in amplification of these oligonucleotides was confirmed by visualisation of the PCR products after gel electrophoresis. Since the number of times these SSRs were repeated in each probe was not determined, these concatenated oligonucleotide probes were probably heterogeneous in length.

Screening of Positive Clones

Concatenated oligonucleotides were labelled with γ-P^{32}-ATP by a 5'-end labelling kit from United States Biochemical (USB, USA) and used as hybridisation probes. They were hybridised with filters containing amplified inserts as described above. Positive hybridisation signals were located and respective clones which harboured these inserts were identified.

RESULTS AND DISCUSSION

Library Construction

Digestion of genomic DNA by the six selected REs appeared to result in complete digestion, since an abundance of low molecular weight fragments were obtained.

Success of ligation was confirmed by agarose gel electrophoresis. Successful ligation resulted in an increase in molecular weight of the vector. In contrast, the control, which was devoid of insert DNA, was unchanged in its molecular weight. Comparison of the resultant ligated vector suggested that the weight ratio of vector DNA to insert DNA of (1:5) was better than (1:10), since a higher proportion of high molecular weight ligated vector was obtained at that ratio (*Figure 1*).

A transformation efficiency of 5×10^6 transformants/μg vector DNA was obtained. Though this value appeared low in comparison to commercially available competent cells which are reported to transform at an efficiency of 1×10^8 transformants/μg vector DNA, the value obtained was nonetheless considered to be satisfactory, since the competent cells used in the above experiments were prepared in-house and has been stored for some time.

Screening of the Library

The success of cloning was confirmed by PCR amplification. Nearly 400 putative transformants were obtained, but only 281 (70%) were screened and 121 were found to harbour DNA inserts (*Figure 2A*). This indicated that the generated library contained approximately 43% of positive recombinants harbouring insert DNAs of 200 to 800 bp in length.

A few clones, *e.g.* clones #76 and #83, were shown to contain two bands after PCR-amplification (*Figure 2A*). Sequence homology between one of the primers used for amplification and the insert DNA might have resulted in amplification of two DNA fragments (bands); a major DNA fragment from amplification of

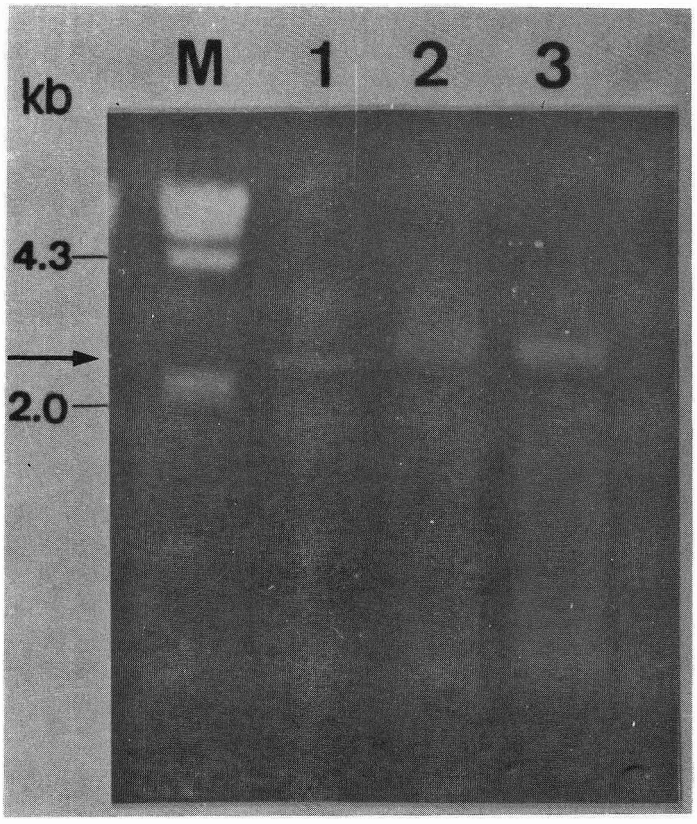

Figure 1. Analysis of success of ligation reaction.

*M: Mol. wt. marker, λ/Hind*III
1: Control ligation reaction
2: Ligation reaction at a weight ratio of (1:5) vector DNA to insert DNA
3: Ligation reaction at a weight ratio of (1:10) vector DNA to insert DNA

sequences flanking the cloning site and another band from between one of the flanking sequences and an internal region of the insert DNA. However, the disparity between the intensity of these two DNA bands and their relative sizes suggested that it was improbable. Since the higher molecular weight fragment was much brighter than the lower molecular weight fragment, the band of lower intensity was probably a product of contamination from a neighbouring clone, which appeared to be of similar size as the insert DNA. Although the reason for the above is uncertain at present, analysis of nucleotide sequence of these inserts would definitely shed some light on the question.

Enrichment of Microsatellites in the Library

Hybridisation of these recombinant clones with various SSR sequences suggested that the library was enriched with microsatellite sequences (*Figures 2B* and *2C*).

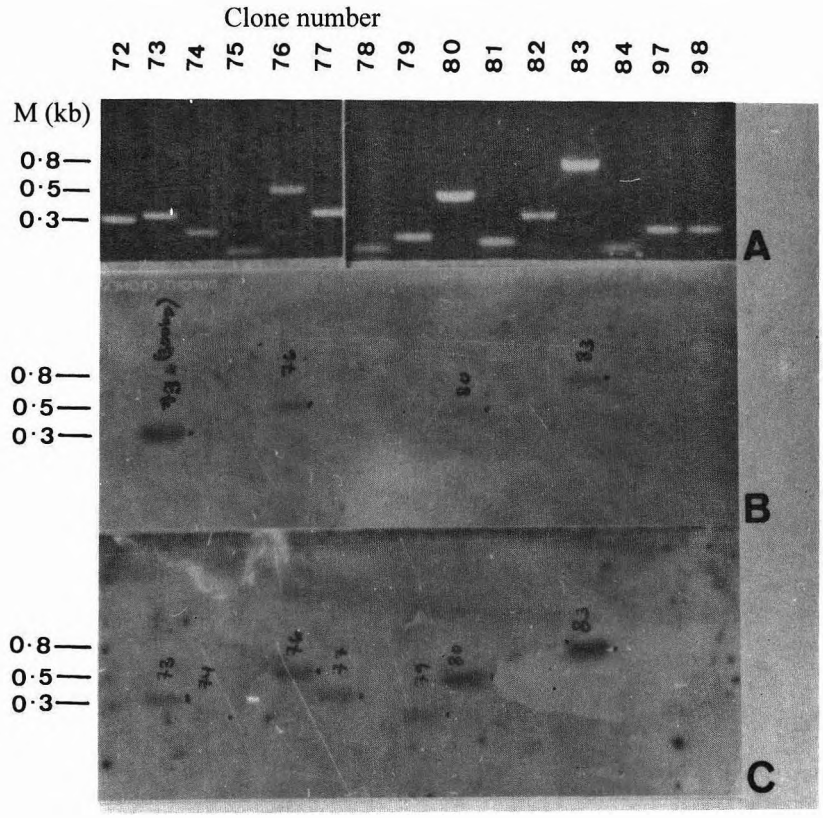

Figure 2. Screening of clones in microsatellite-enriched library.

A: Positive clones after PCR amplification with SK/KS primers
B: After hybridisation with concatenated oligonucleotide probe $(GACA)_n$ of heterogeneous lengths
C: After hybridisation with concatenated oligonucleotide probe $(GA)_n$ of heterogeneous lengths
M: Mol. wt. marker, 100 bp ladder

Four concatenated SSR sequences of heterogeneous lengths (a few hundred bp) were used as probes to test the enrichment of the library with micro-satellites. These were tetranucleotide repeats $(GACA)_n$, $(GATA)_n$ and dinucleotide repeats $(GA)_n$ and $(GC)_n$. The library appeared to be particularly enriched with $(GA)_n$ dinucleotide repeats (*Table 1*).

The high incidence (34%) of clones which were enriched with $(GA)_n$ dinucleotide repeats in this *Hevea* microsatellite library may reflect the enrichment of this particular dinucleotide microsatellite in the *Hevea* genome. This high proportion of GA repeats in *Hevea* is not uncommon in trees. The enrichment appeared to be similar to that in *Pinus radiata*[18] which was found to have a high content of GA and CA microsatellites. Similarly, Condit and Hubbell[19] reported that AG repeats were 20–40% more abundant than AC repeats in all

TABLE 1: PERCENTAGE OF CLONES POSITIVE TO SSR PROBES OF HETEROGENEOUS LENGTHS

Concatenated SSR probes	% Positive clones
$(GACA)_n$	10.00
$(GATA)_n$	9.00
$(GA)_n$	34.00
$(GC)_n$	9.00

TABLE 2: PERCENTAGE OF CLONES POSITIVE TO MULTIPLE SSR PROBES OF HETEROGENEOUS LENGTHS

Multiple Concatenated SSR probes	% Positive clone
$(GATA)_n$, $(GA)_n$, $(GC)_n$	1.40
$(GATA)_n$, $(GA)_n$	6.40
$(GACA)_n$, $(GA)_n$	5.60
$(GATA)_n$, $(GC)_n$	0.70
$(GA)_n$, $(GC)_n$	6.40

six tropical forest plants which they had examined.

Two separate surveys on plant microsatellite sequences covering 34 and 28 species by Morgante and Olivieri[20] and Wang *et al.*[21], respectively, revealed that $(AT)_n$ was the most abundant, with $(AG)_n$ as the next most abundant dinucleotide repeat sequence in plants[21].The status of dinucleotide microsatellites in *Hevea* is unclear at present, since only two dinucleotide sequences out of six were used in this study. However, between the two microsatellite $(GA)_n$ and $(GC)_n$ which were examined, $(GA)_n$ appear to predominate. The abundance of microsatellite $(GC)_n$ is probably inaccurate. When used as hybridisation probes, GC repeats will self-hybridise. This will reduce the availability of single-stranded sequences as hybridisation probes thus resulting in decreased hybridisation signals. Similarly, $(AT)_n$ was not used in this study because of its tendency to self-hybridise. On the other hand, the absence of self-hybridisation with concatenated oligonucleotide GA, lends greater confidence in its hybridisation results when it is used as a probe.

Compound Microsatellites

Several recombinant clones in the library were found to hybridise with more than one SSR (*Table 2*). This would suggest that the stringency of washing of the nylons was not high enough to remove non-specific hybridisation, or intermolecular ligation of two or more DNA fragments had occurred before ligation with a dephosphorylated vector, or these clones contained more than one class of SSR otherwise known as compound microsatellites[22]. Of these three possibilities, stringency of washing is the least likely. Either or both of the remaining possibilities could have resulted in the observed putative compound microsatellites. Whether these clones were indeed true compound microsatellites would be evident if fewer such clones were obtained in a repeat experiment where ligation was carried out with dephosphorylated DNA fragments (after repair or filling-in of the cohesive ends) and *Sma*I-digested dephosphrylated vector. This repeat experiment would be carried out at a later date, to confirm the above. In the meantime, sequencing results from one of these clones

(#76) indicated that it was an imperfect compound repeat (results not shown). The occurrence of compound microsatellites has not only been documented[18, 22, 23], but also appeared to be proportionally higher in plant than in human genome[23]. Notwithstanding that some clones might be true compound microsatellites, a portion of these putative compound microsatellites could have arisen from intermolecular ligation of multiple DNA fragments preceding cloning. This would be confirmed later, after the repeat experiment described above had been carried out as well as when sequencing of all the putative compound microsatellite clones had been completed.

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Physiological Characteristics of Latex of the IRRDB 1981 Hevea *Germplasm*

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Five physiological parameters of latex, namely total solid content, sucrose inorganic phosphorus, contents of thiol groups and plugging index were studied on Wickham and germplasm clones. Results showed difference in profiles of physiological characteristics of latex of the germplasm and Wickham groups.

*The germplasm clones were significantly higher than the Wickham clones in sucrose content of latex and plugging index (PI), but the latter (Wickham clones) were higher in the contents of thiol groups (R-HS) and inorganic phosphorus (Pi). Significant positive correlations were found between yield and R-SH ($r=0.31$ **, $df=86$) and between yield and Pi ($r=0.40$ ***, $df=85$), while a significant negative correlation was found between yield and PI ($r=0.31$ **, $df=86$) in the germplasm.*

Principal component analysis of the five physiological parameters of latex showed that the germplasm group was well separated from the Wickham group. There was no distinct separation among the three geographical groups of the germplasm.

In 1981, the International Rubber Research and Development Board (IRRDB) carried out an expedition in three states of Brazil, namely Acre, Mato Grosso and Rondonia aimed at broadening the genetic base of *Hevea* in the East[1,2]. Between 1984 and 1987, the Rubber Research Institute of Vietnam (RRIV) received 3672 genotypes from the *Hevea* Germplasm Centre located in Malaysia. Out of these, 2972 genotypes survived[3,4]. Part of the collection has been evaluated and reported for agronomic characteristics in clonal trials[4,5].

Jacob *et al.* [6,7] postulated that the cytoplasmic nature of latex as reflected by its biochemical and biophysical parameters, could provide useful data on state of health of laticiferous system and on clonal typology. It would therefore be interesting to study some of these parameters and compare differences or similarities between the germplasm and Wickham materials.

Physiological characteristics of the latex of germplasm studied are presented in this paper.

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MATERIALS AND METHODS

Five physiological parameters of latex, namely plugging index (PI), total solid content (TSC), sucrose content (SUC), inorganic phosphorus content (Pi) and content of thiol groups (R-SH) were studied using 88 germplasm and 26 selected Wickham clones in a small-scale clone trial (SSCT) sited in Lai Khe Station, RRIV.

The SSCT was established according to a randomised complete block design with three replications and each genotype was represented by eight trees per plot per replication. Yield measurement commenced when 70% of the trees reached a tappable size of 45 cm of circumference from the stock union. The tapping system used was ½S d/3 without stimulation. Mean yield over one year of tapping was used for the present study.

The physiological parameters were studied on trees during the first year of tapping. For sampling of latex, the fraction of latex flowing between the fifth and thirty-fifth minutes of tapping was collected in a small tube packed in ice. Latices from three trees per plot were collected, pooled and homogenised thoroughly and carefully to make a sample which was chilled to block the continuity of the metabolism *in vitro*[6]. The sample of latex was then used to determine TSC and to prepare a trichloroacetic extract (TCA) for determination of sucrose, thiols and inorganic phosphorus. PI was determined according to Milford *et al.*[8] with the modification that dry rubber weight was used instead of volume in order to make possible study of the large number of trees and to overcome difficulties in taking measurement of volume due to very low latex production from the germplasm clones. Other physiological parameters of latex (TSC, SUC, Pi and R-SH) were sampled and analysed according to the procedures of micro diagnosis of latex developed by IRCA[9].

Statistical Analysis

Means and standard deviations were computed for the various physiological parameters by materials of different origins (Acre, Mato Grosso, Rondonia and Wickham). Significant groupings were carried out using Duncan's Multiple Range Tests for means of the various germplasm groups. Correlations among the characters (including latex yield) of the germplasm and the Wickham materials were also performed separately. Principal component analysis was adopted on the multivariate data sets collected to provide some ideas of genotypic groupings and their possible phylogenetic relationships.

The above statistical analyses were carried out using selected procedures of SAS package[10].

RESULTS AND DISCUSSION

Latex Physiological Profile of the New Germplasm

The physiological characteristics of latex of the germplasm and Wickham clones are summarised in *Table 1*. The germplasm clones were significantly higher than the Wickham clones in SUC and PI, but lower in R-SH and especially very low in Pi *Table 1*. In general, geographical groups of the germplasm were not different from one another, especially Mato Grosso and Rondonia in these parameters.

TABLE 1. MEAN VALUES FOR PHYSIOLOGICAL PARAMETERS OF LATEX OF THE GERMPLASM

Germplasm	No of. clones	TSC (%)	SUC (mM)	R-SH (mM)	Pi (mM)	PI
Acre	8	34.37 b (1.11)	11.58 a (1.42)	0.48 a (0.03)	4.51 b (0.71)	4.58 a (0.77)
Mato Grosso	44	37.69 a (0.42)	9.55 a (0.70)	0.39 b (0.01)	4.32 b (0.29)	4.98 a (0.19)
Rondonia	36	37.49 a (0.43)	11.90 a (0.71)	0.41 b (0.01)	4.30 b (0.30)	4.28 a (0.17)
Wickham	26	36.79 a (0.56)	8.26 b (0.61)	0.53 a (0.02)	17.63 a (0.95)	2.99 b (0.14)

TSC: Total solid Content; SUC: sucrose; R-SH: Thiol; Pi: Inorganic phosphorus; PI: Plugging index.
Mean values followed by the same letter in the same column are not significantly different at $P \leq 0.05$
Figures in brackets denote standard errors.

TABLE 2. SIMPLE CORRELATION COEFFICIENTS BETWEEN PHYSIOLOGICAL PARAMETERS OF LATEX OF THE GERMPLASM

Parameters	TSC	SUC	R-SH	Pi	PI
SUC	-0.115^{NS}				
RSH	-0.407^{***}	0.448^{***}			
Pi	-0.371^{***}	0.250^{*}	0.574^{***}		
PI	0.319^{**}	-0.088^{NS}	-0.198 a	-0.139^{NS}	
Yield	-0.192 a	-0.136	0.306^{**}	0.403^{***}	-0.309^{**}

a $P \leq 0.10$; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; Df = 86
NS : Not significant at $P \leq 0.05$

For the germplasm clones significant positive correlations were found between yield and R-SH, and Pi; while a significant negative correlation was also found between yield and PI (*Table 2*). Significant correlations were observed among these physiological parameters of latex in the germplasm. However, for the Wickham clones no significant correlation was detected between yield and the physiological parameters studied. There was also no significant correlation detected among those physiological parameters except for relationship between sucrose and R-SH or Pi and R-SH (*Table 3*).

It is clear that in a given environment, latex production on tapping in rubber trees depends on the duration of the latex flow and on the regeneration of latex between the two consecutive tappings. The parameters investigated are either related to latex flow or latex regeneration or both, except for PI, which is an end phenomenon of latex flow[7].

Regarding the activity of the latici ferous system of the germplasm in the regeneration of rubber, the lower content of R-SH and extremely low content of Pi may reflect the inadequate synthesis or supply of those substances in the laticifers. In other words, the germplasm probably has a less active laticiferous system for latex productivity. Jacob *et al.*[6,11] reported that a laticiferous system operating weakly has a low Pi latex content. Limitation to latex regeneration by the inadequate supply of Pi and R-SH is supported by highly significant and positive correlations between yield and Pi and R-SH in the germplasm (*Table 2*), and the less effective utilisation of sucrose as rubber precursor leading to higher sucrose

TABLE 3. SIMPLE CORRELATION COEFFICIENTS BETWEEN PHYSIOLOGICAL PARAMETERS OF LATEX OF THE WICKHAM CLONES

Parameters	TSC	SUC	R-SH	Pi	PI
SUC	-0.218^{NS}				
R-SH	0.107^{NS}	0.492^{**}			
Pi	0.375^{*}	0.168^{NS}	0.111^{NS}		
PI	-0.212^{NS}	0.178^{NS}	0.195^{NS}	-0.067^{NS}	
Yield	0.139^{NS}	-0.317^{NS}	-0.018^{NS}	0.199^{NS}	-0.296^{NS}

* $P \leq 0.10$; ** $P \leq 0.01$; Df = 24 ; Others are not significantly different at $P \leq 0.05$
NS : Not significant at $P \leq 0.05$

content of the laticifers (*Table 1*). The germplasm genotypes were brought directly from the jungle where they might not have been subjected to selection for yield through latex extraction, therefore probably have less active laticiferous systems for latex biosynthesis.

Regarding the flow of latex, PI was significantly higher in the germplasm compared to the Wickham clones; and there was a significant and negative correlation between PI and production (*Table 1* and *2*). High plugging indices in the germplasm may reflect difficulties in latex flow and may be of genetic nature of the wild genotypes evolving in the absence of latex extraction and towards effective mechanism of preventing the species from excessive loss of latex on wounding. It was well established that PI is a clonal characteristic, negatively correlated to yield [8,12-14] and genetically controlled[15]. Meanwhile, the Wickham clones have been subjected to selection for high productivity which has been known associated with low plugging indices, or, in other words, easing in the latex flow. Therefore, there might have been an unconscious selection favouring lower PI over generations of utilisation of the Wickham clones. Besides this, relatively higher values of TSC in the germplasm (MT and RO) and its significant correlation with PI suggest that high values of TSC may become a limiting factor to the latex flow because of the resulting high viscosity[7].

In general, the new *Hevea* germplasm appeared to be poor in the activity of latex regeneration and showed difficulty in the flow of latex. The limitation in the latex flow seems to be more important because highly positive responses of up to 100% or above to latex stimulation were reported in wild genotypes [16,17].

Clonal Typology of Physiological Characteristics of Latex.

Because functioning roles of various physiological parameters are complex, their interactions are not well understood and the interpretation of their effects, as single factors on productivity is not easy. However, it is possible to utilise all these parameters to study the general feature of the new *Hevea* germplasm leading to clonal typology of physiological characteristics of latex[11]. It could give better understanding of physiological profile of wild genotypes and help in classification of them based on parameters studied. The study was carried out using the method of principal component analysis (PCA) of all the parameters measured.

As shown in the PCA diagram (*Figure 1*), there was a clear distinction between two groups, the germplasm's and the Wickham's, indicating their different profiles of physiological parameters of latex. However, there were also some germplasm clones which seemed to be closer to the Wickham group in terms of physiological profile. The PCA can, therefore, provide a useful tool to describe the physiological state of the new germplasm as well as a possible guide to plant breeders in evaluation and choice of wild genotypes.

Considering the geographical origin of the germplasm, there was no clear distinction among genotypes derived from different geographical origins (*Figure 2*). This implies that these germplasm genotypes may have

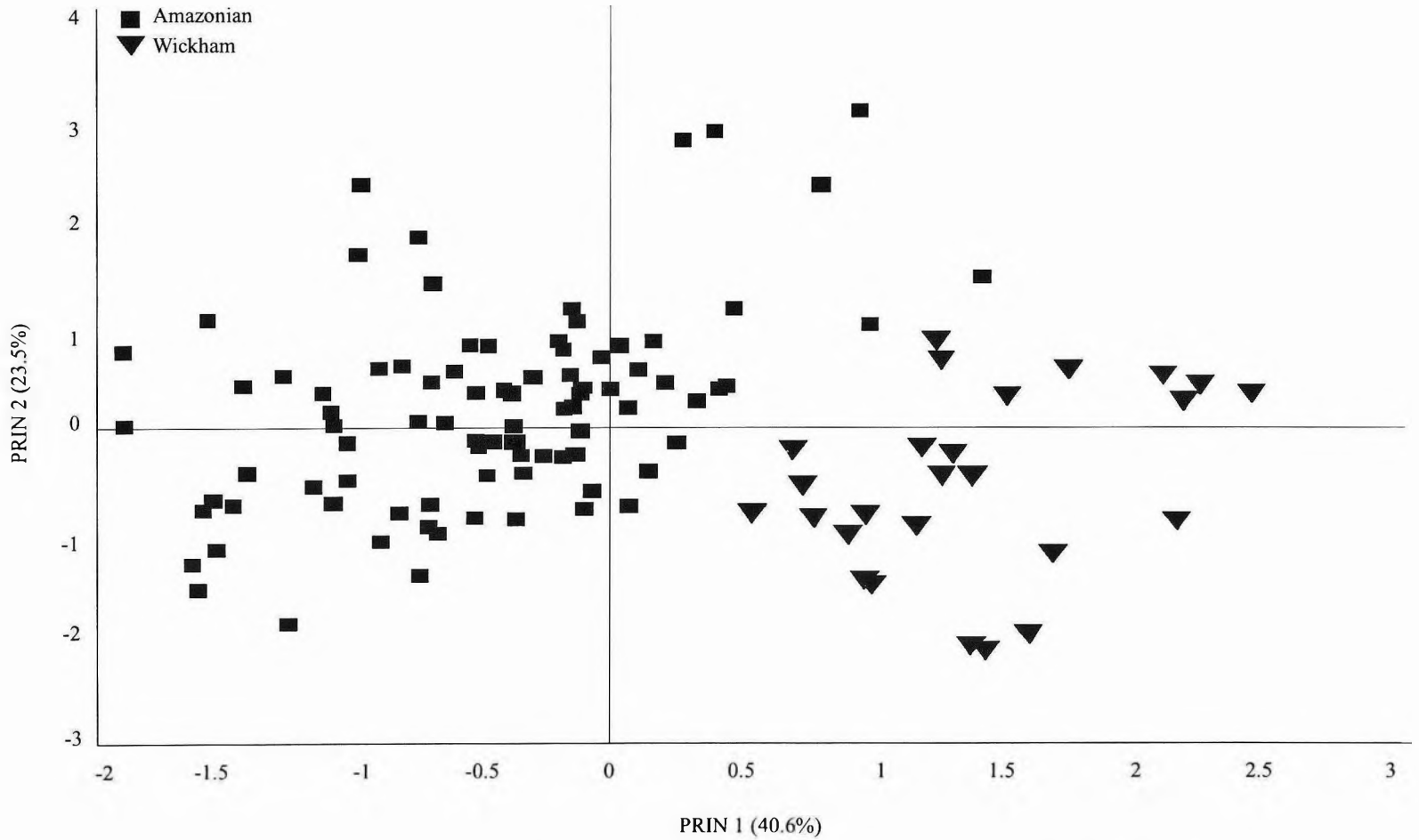

Figure 1. Principal component analysis of five physiological characteristics of latex for the germplasm and Wickham clones.

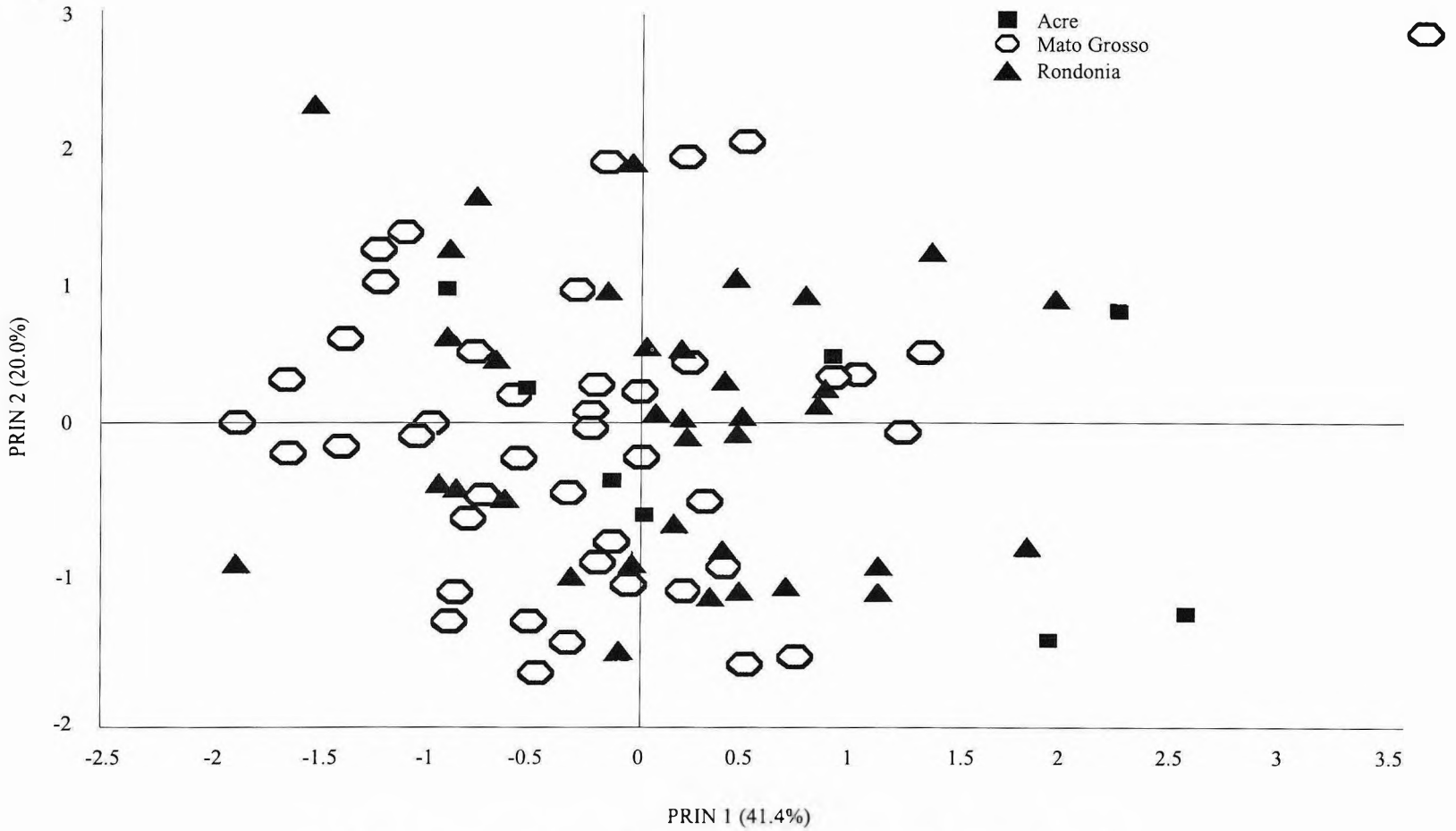

Figure 2. Principal component analysis of five physiological characteristics of latex for the germplasm clones.

the same feature of latex physiology. However, if more materials from these different geographical origins and more characteristics were included in the study, they may show out some important differences among them.

CONCLUSION

The new *Hevea* germplasm is different from the Wickham clones in physiological characteristics of latex. They appear to be poor in the activity of latex regeneration and show difficulty in the flow of latex. It would be necessary to increase clonal differences between germplasm clones to observe their maximal metabolic activity (production potential) by an appropriate stimulation treatment. Classification of the germplasm according to their physiological profiles can help in effective utilisation of germplasm.

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Effect of Interstock on Dry Matter Production and Growth Analysis of Hevea brasiliensis (*Muell. Arg.*)

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The influence of five interstock clones of contrasting vigour on dry matter production and distribution of three Hevea brasiliensis *clones grown in the ground nursery over a two-year period was studied. Results obtained after one year of planting indicate that the influence of interstock and scion upon various aspects of growth were generally found to be additive with some showing significant interaction between scion and interstock.*

The influence of interstock on scion growth was related to the inherent vigour characteristic of the interstock clones. Trees on vigorous interstock (TR 3702 and RRIM 613) produced more dry matter in the above-ground plant parts than those on less vigorous interstocks (H. spruceana *and RRIM 600). Leaf area, whole tree dry weight, mean relative growth rate and mean net assimilation rate followed a similar pattern. In these composite trees, it appears that there is competition for photosynthate between scion stem and roots with vigorous interstocks being able to divert more photosynthate to scion stem than to roots. The significance of these findings are discussed.*

Hevea tree is propagated vegetatively by grafting suitable high yielding clones onto seedling rootstocks. Rootstock therefore forms an important component of the composite tree as it has inherent ability to improve tree growth and productivity[1]. However, currently, the availability of suitable rootstocks with known potential such as PB 5/51, RRIM 623 and GT1 remain a major concern among rubber growers because these clones are no longer planted large scale nation-wide. Interstock represents a potential method to overcome this problem; since in apples, it has been shown that certain interstock such as M9 produced similar effects as the rootstock on growth and yield of scion[2-4]. Hence, interstock may present an alternative approach to obtaining more productive trees in the absence of clonal rootstocks.

In apples, research on the growth patterns and physiology of composite trees is well documented[5-7] although in other temperate fruit trees such as cherry, citrus, pears and plums this has not been intensively studied[8-12]. In apples, interstock is used mainly to control tree size. This is possible by using dwarfing interstock which reduced many growth parameters such as scion height, girth, leaf area, dry matter production

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and relative growth rate resulting in smaller tree size than given by invigorating interstocks[4,13,14].

In *Hevea*, early experiments on the use of interstock were aimed at reducing the variability due to illegitimate seedling rootstocks[15]. Ostendorf[16] in his study on three-part-trees in which the trunk can be regarded as long 'interstock', reported that the use of vigorous *Hevea brasiliensis* clones as 'interstock' did not improve growth of scion of *Hevea* species with reduced growth potential such as *H. spruceana*, *H. guianensis* and *H. collins*. In more recent years, Leong and Yoon[17] have reported that scion growth was substantially reduced when interstock with reduced growth potential such as *H. brasiliensis* 'Dwarf' clone and *H. spruceana* were used. It is obvious from these reports that detailed studies of the growth patterns of these composite trees in *Hevea* are lacking.

This study evaluates the influence of five interstock clones of contrasting vigour on production and distribution of dry matter to various plant parts in order to provide a better understanding of the physiological basis for growth differences of the composite tree. Some of the results have been communicated in an abstract form[18].

MATERIALS AND METHODS

Interstock plants were produced by grafting three scion clones (RRIM 600, RRIM 802 and PB 235) on to five interstock clones, TR 3702, PB 5/51, RRIM 613, RRIM 600 and *H. spruceana*. Both the interstock and scion clones were selected for their contrasting vigour characteristics before tapping[19-22]. *H. spruceana* was chosen because it is known to depress scion growth when used as an interstock and rootstock[17, 23]. All interstocks were 20 cm in length. The rootstock was RRIM 600 monoclonal seedling. Controls were plants with the same scion and interstock clones.

The interstock plants at first and second-whorl stage were planted in 1982 in the ground nursery at the RRIM Experimental Station, Sungai Buloh, Selangor in a triangular pattern spaced out at 90 cm x 90 cm. The experiment, consisting of 18 treatments, were laid out in a completely randomised design within each harvest block. The plants were harvested for the determination of dry matter production at the time of planting and thereafter at half-yearly intervals over a period of two years to give a total of five harvests. The total number of plants per treatment at each harvest ranged from five to ten. At each harvest, dry weights of various plant components (laminae, petiole, scion and interstocks stem and roots) were determined after drying for 48 h at 85°C. For leaf area (LA) determination, leaf discs (2.7 cm^2 area) were sampled from a total of 4-15 leaflets per plant. The total LA of a tree was estimated based on the formulae given by Watson[24]. Standard growth analysis parameters such as leaf area ratio (LAR), specific leaf area (SLA), mean net assimilation rate (NAR) and mean relative growth rate (RGR) of whole plants were calculated from data of LA and dry weights of leaf and whole plant according to the formulae and assumptions given by Briggs *et al.*[25], Fisher[26], Williams[27] and Redford[28].

Data Analysis

All data were subjected to a two-way analysis of variance to test for the scion and interstock main effects and their

interaction. F statistics at P≤0.05 was used for test of significance; least significant difference (LSD) at the same probability level was used for comparison of individual means.

RESULTS

In the present study, many parameters of plant growth were affected by interstock only at one year after planting (*Appendix 1*). Interactions between scion and interstock clone were detected at or after the first year for some of these variables.

Leaf Area and Dry Matter Production

Leaf area and biomass of various plant parts are shown in *Table 1* and *Figures 1–5*. At one year after planting, trees on TR 3702, RRIM 613 and PB 5/51 interstocks had comparable LA and dry weights of laminae, petiole and scion stem; these were significantly higher than those produced by *H. spruceana* interstocks (*Table 1*). Leaf area of trees on vigorous interstocks were about 21% to 23% larger than the control while trees on *H. spruceana* interstock had comparable LA to the control. TR 3702, RRIM 613 and PB 5/51 interstocks also produced 6% to 22% larger dry weight of scion stem than the control. In contrast, trees on *H. spruceana* interstock had the poorest growth as their scion stem dry weights were only 77% of the control.

After 1.5 years of planting, the effect of interstock on LA and dry weights of petiole, scion stem and whole tree depended on scion clone since there was a significant interaction between these two effects (*Appendix 1*). For LA, the (scion × interstock) interaction appears to have arisen from the lack of interstock influence on LA of RRIM 802 scion (*Figure 1*). However, PB 235 scion clone had significantly higher LA on *H. spruceana* and RRIM 613 interstocks than on TR 3702 and RRIM 600 interstocks. For

TABLE 1. EFFECT OF INTERSTOCK ON LEAF AREA, LEAF AREA RATIO AND DRY WEIGHT OF VARIOUS PLANT PARTS AT ONE YEAR AFTER PLANTING [a]

Interstock clone	Leaf area	Dry weight (g)				
	$(cm)^2$	Laminae	Petiole	Scion stem	Whole tree	Leaf area ratio
TR 3702	194	174	41	555	1363	14.6
RRIM 613	194	173	39	529	1345	15.2
PB 5/51	190	175	39	470	1208	16.4
Control [#]	157	137	34	421	1113	14.9
H. spruceana	152	132	30	342	960	16.5
RRIM 600	151	134	32	421	1132	13.6
Mean	173	154	36	460	1193	15.2
LSD (P<0.05)	36	31	7	96	211	1.6
Level of probability	*	*	**	***	**	**

[a] Each figure is an average of 3 scion clones

[#]: Control consists of plants in which the interstock and scion are of the same clone (RRIM 600, RRIM 802 or PB 235)

*, **, ***: F - test significant at P<0.05, 0.01 or 0.001, respectively

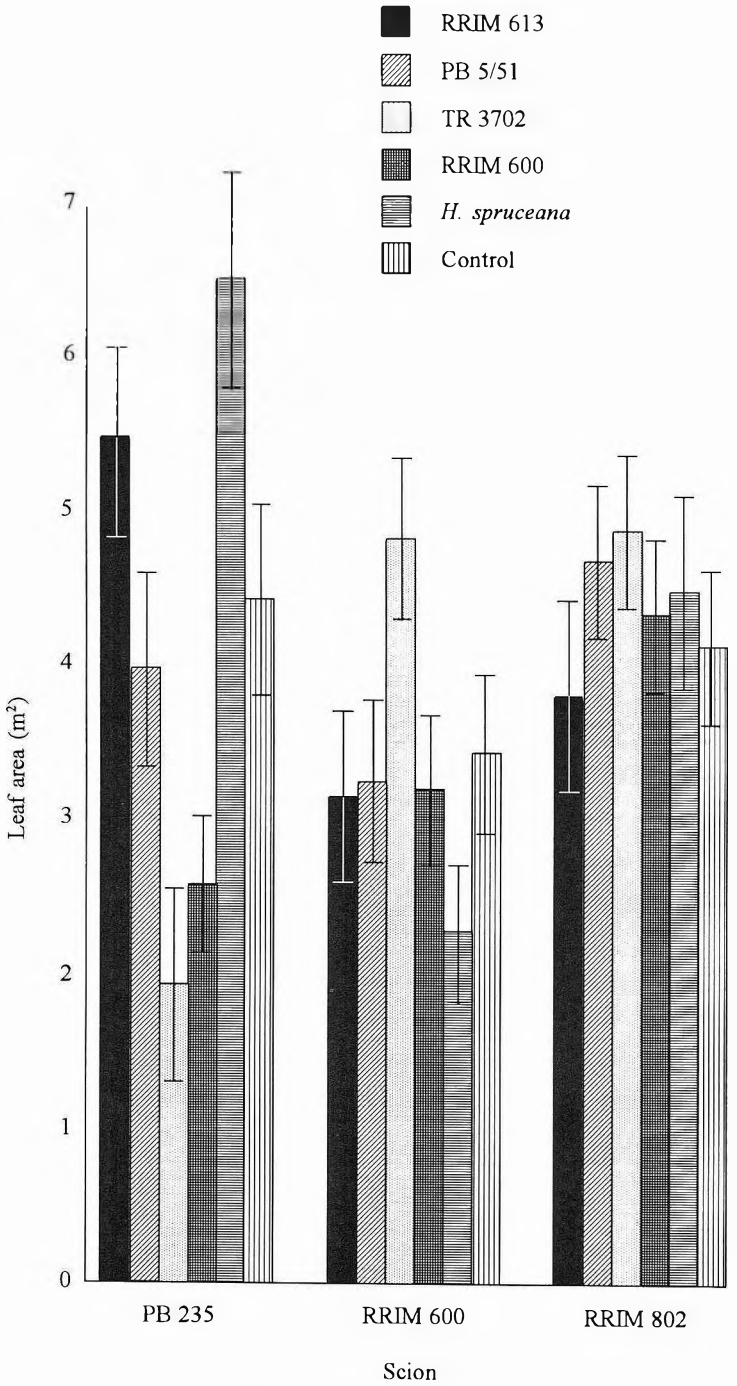

Figure 1. Effect of interstock clone on leaf area at 1.5 years after planting. (Vertical lines represent SE associated with each combination mean.)

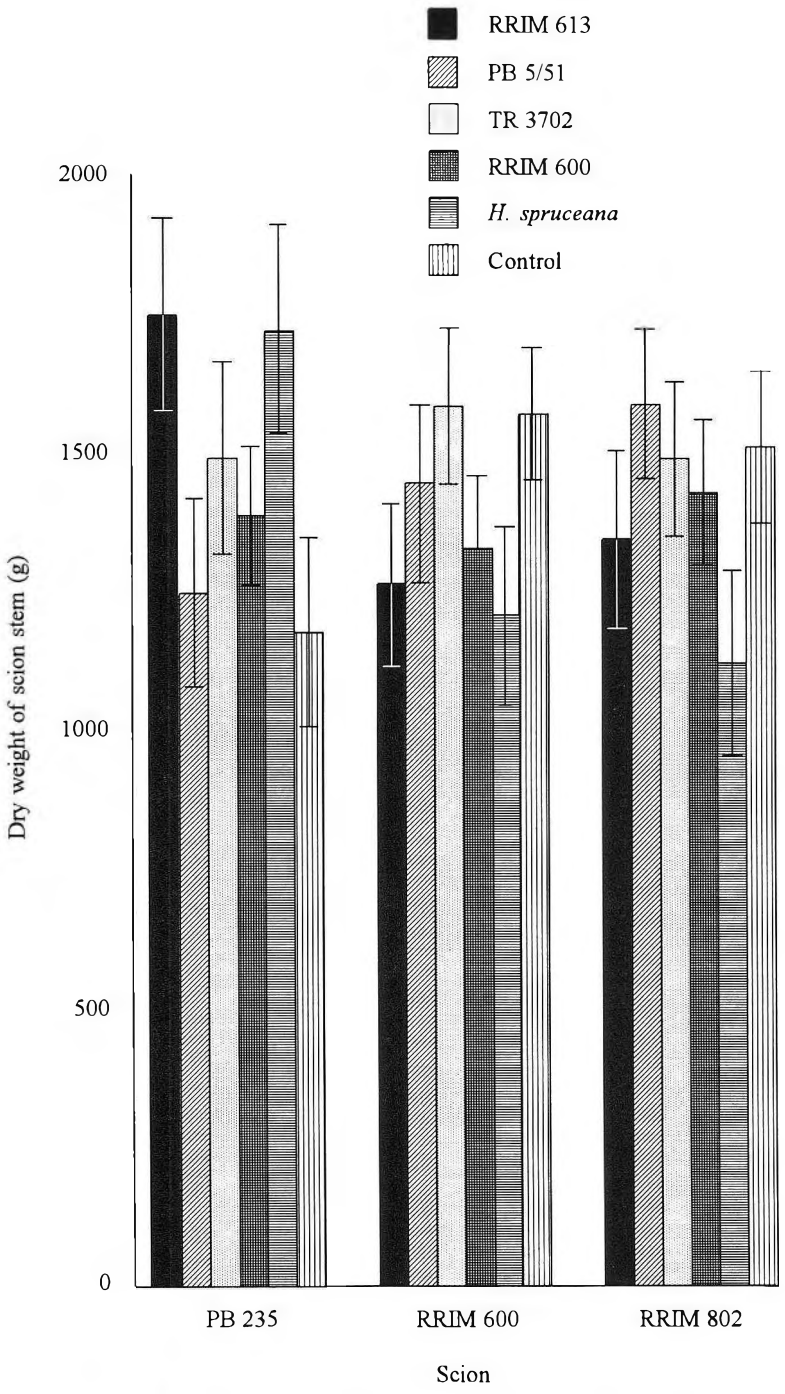

Figure 2. Effect of interstock clone on dry weight of scion at 1.5 years after planting. (Vertical lines represent SE associated with each combination mean.)

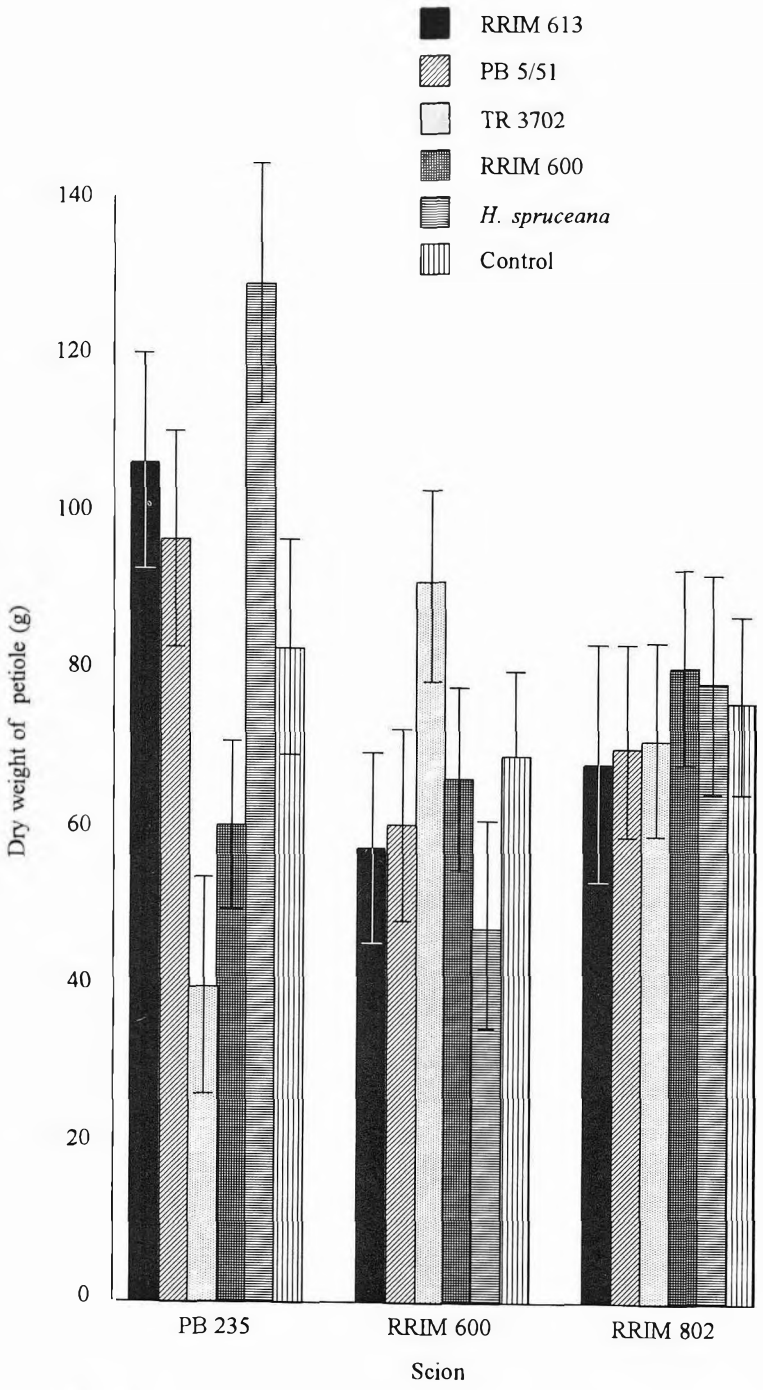

Figure 3. Effect of interstock clone on dry weight of petiole at 1.5 years after planting (Vertical lines represent SE associated with each combination mean.)

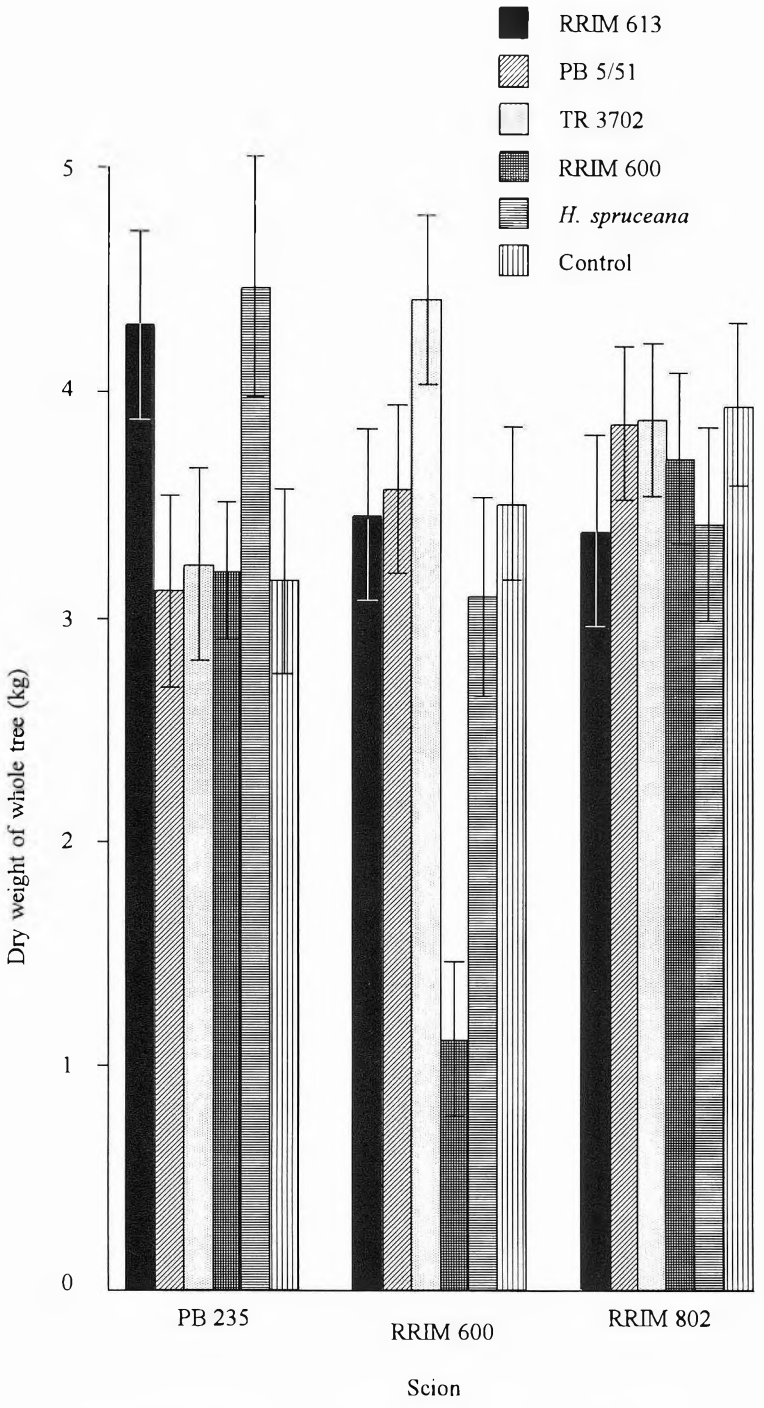

Figure 4. Effect of interstock clone on dry weight of whole tree at 1.5 years after planting. (Vertical lines represent SE associated with each combination mean.)

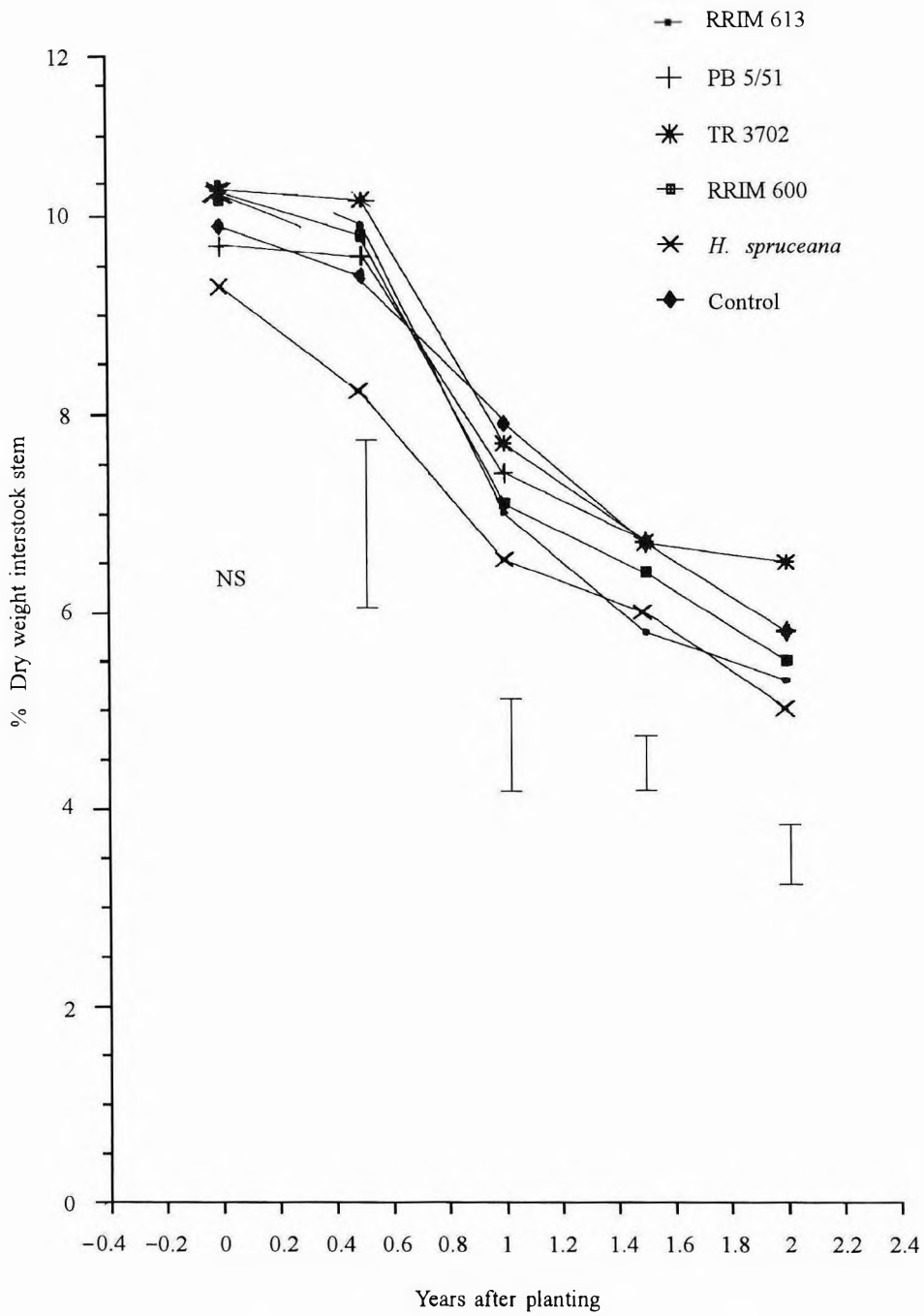

Figure 5. Effect of interstock clone on percentage dry weight of interstock stem. (Verticle bar represents least significant difference at P<0.05)

plants with RRIM 600 scion, TR 3702 interstock produced significantly higher LA than given by *H. spruceana* interstock.

At this interval, dry weights of RRIM 600 scion was not much affected by various interstocks (*Figure 2*). However, for combination with PB 235 scion, RRIM 613 and *H. spruceana* interstocks resulted in significantly higher dry weight of scion stem than given by control and PB 5/51 interstocks. For combination with RRIM 802 scion clone, the highest and lowest scion stem dry weight were given by PB 5/51 and *H. spruceana* interstocks, respectively. Petiole dry weight of RRIM 802 scion after 1.5 years of planting was not much affected by interstock clone (*Figure 3*). For PB 235 scion clone, *H. spruceana* and RRIM 613 interstocks produced substantially higher dry weight of petiole than those given by the control, RRIM 600 and TR 3702 interstocks. This pattern was reversed in RRIM 600 scion with TR 3702 and *H. spruceana* interstocks producing the highest and the lowest petiole dry weight, respectively.

The clonal differences in dry weight of interstock stem were also very highly significant at one and two years after planting (*Appendix 1* and *Table 2*) Additionally, there was a significant interaction between scion and interstock clone at these intervals. For all scion clones, differences in dry weight of interstock stem varied over the two harvesting intervals, although with the exception of the first-year harvest for RRIM 802 scion clone, there was a tendency for *H. spruceana* to have the lowest and TR 3702 interstock the highest stem dry weight.

At one year after planting, the influence of interstock on mean dry weight of

TABEL 2. DRY WEIGHT OF INTERSTOCK STEM AT ONE AND TOW YEARS AFTER PLANTING

Interstock clone	One year after planting Scion clone			Mean	Two years after planting Scion clone			Mean
	PB 235	RRIM 600	RRIM 802		PB 235	RRIM 600	RRIM 802	
RRIM 613	118.6	93.4	66.8	92.9	245.8	301.8	245.4	264.3
PB 5/51	90.3	79.2	93.9	87.8	270.7	317.5	399.6	329.2
TR 3702	136.7	109.2	64.2	103.3	424.4	295.8	422.5	380.9
RRIM 600	83.0	73.3	77.6	78.0	331.5	265.1	253.9	283.5
H. spruceana	61.0	68.4	53.2	60.9	252.3	191.8	285.8	243.3
Control	116.4	88.4	62.3	89.0	414.6	339.7	312.1	355.4
Mean	101.0	85.3	69.7	85.3	323.2	285.3	319.9	309.5
LSD (P<0.05) and level of probability:								
Scion	(S)		10.621	***		NS		
Interstock (I)			15.038	***		59.806	***	
Interaction (SxI)			26.53	**		105.182	*	

NS,*,**,***: F-test indicates non-significant or significant at p<0.05, 0.01 and 0.001, respectively

roots depended on scion clone as indicated by significant interaction between scion and interstock clones (*Appendix 1*). The scion × interstock interaction was attributed to the fact that root dry weight of combinations with RRIM 600 scion clone was not much affected by interstock clones (*Table 3*). In comparison with the control, PB 235 scion in combination with RRIM 613 and TR 3702 interstocks increased root mass by 38% to 51% while a decrease by about 10% was recorded for combination with *H. spruceana* interstocks. For plants with RRIM 802 scion clone, PB 5/51 interstock increased root mass by 29% while those on *H. spruceana* interstock were reduced by about 17% compared with the control.

The effect of interstock clones on whole tree dry weight at one year after planting followed more or less the order expected from their effects on LA and dry weight of scion stem (*Table 1*). Trees on TR 3702 and RRIM 613 interstocks were similar in size as reflected by whole tree dry weight; these were 20% to 22% larger than the control. Trees on *H. spruceana* interstock were the smallest as their dry weights were only about 86% of the control.

At 1.5 years after planting, the interstock influence on whole tree dry weight differed among the scion clones because of the scion × interstock interaction. Whole tree dry weight of RRIM 802 clones was not influenced by interstock clone (*Figure 4*). For RRIM 600 scion clone, TR 3702 interstock clone resulted in the highest dry weight and *H. spruceana* interstock the lowest dry weight of whole tree. For combinations with PB 235 scion clone, *H. spruceana* and RRIM 613 interstocks produced comparable

TABLE 3. EFFECT OF INTERSTOCK ON DRY WEIGHT OF ROOTS AT ONE YEAR AFTER PLANTING

Interstock clone	Dry weight of roots (g)			
	Scion Clone			Means
	PB 235	RRIM 600	RRIM 802	
RRIM 613	712.1	480.8	363.6	518.8
TR 3702	651.1	523.5	360.6	511.7
RRIM 600	525.3	394.3	428.2	449.3
Control #	472.1	491.7	372.4	445.4
PB 5/51	454.7	378.3	480.5	437.8
H. spruceana	424.3	456.3	310.6	397.1
Means	539.9	454.2	386.0	460.0

LSD (P<0.05) and level of probability:

Scion (s)	60.906	***
Interstock (I)	NS	
Interaction (S × I)	152.138	*

#: Control consists of plants in which the interstock and scion are of the same clone

dry weight of whole tree which were significantly higher than those given by other interstocks.

Distribution of Dry Matter to Various Plant Parts

The ratios of plant parts to whole tree dry weights were calculated to estimate the relative partitioning of photosynthates to the plant parts. A close relationship has been reported in apples between the allocation of ^{14}C labelled assimilates of a particular plant part and its percentage dry weight[29]. In the present study, harvest date significantly influenced all percentage dry weight of plant parts tested while interstock clones only influenced percentage dry weight of scion stem, interstock stem and roots (*Appendix 2*). No significant interaction between harvest dates and interstock clones was detected for all plant parts indicating that the interstock effects were consistent across the harvest intervals. There was also no significant interaction between scion and interstock clones for these variables.

During the period of study and regardless of scion clones, *H. Spruceana* interstock stem consistently received the least allocation of dry matter followed by RRIM 613 interstock while the most allocation of dry matter went to TR 3702 followed by PB 5/51 and control interstock stems (*Figure 5*). The proportion of dry matter for scion stem was the lowest for *H. spruceana* interstock and highest for TR 3702 interstock following a pattern similar to its distribution in interstock stem (*Figure 6*). In contrast, *H. spruceana* and RRIM 600 interstocks gave significantly higher allocation of dry matter to roots than did TR 3702, RRIM 613 and PB 5/51 interstocks (*Figure 7*).

Growth Characteristics

Leaf area ratio. Harvest date significantly influenced LAR (*Appendix 2*). There was no significant difference in LAR due to the interstock clones when the results were analysed with harvest date as one of the variables. However, when the results were analysed separately for each harvest date, interstock influence on LAR was evident at one year after planting (*Table 1*). There was also no significant interaction between the effect of scion and interstock on LAR.

Specific leaf area. Scion clone and harvest date significantly influenced specific leaf area (SLA) ($P \leq 0.001$) (*Appendix 2* and *Figure 8*). However, SLA was not significantly influenced by interstock clones. There was also no significant interaction between the effects of interstock and harvesting date on SLA although such interaction was evident between the effects of scion clone and harvest date ($P<0.05$). The (scion × harvest date) interaction was because the scion difference in SLA was greater at time of planting when leaves were expanding than at other intervals. On the average, SLA of PB 235 and RRIM 802 scion clones was comparable and significantly higher than the value for RRIM 600 scion.

Mean Relative Growth Rate and Mean Net Assimilation Rate

Mean net assimilation rate (NAR) and mean relative growth rate (RGR) were derived from data of total LA and total above-ground dry matter accumulation, respectively. Mean NAR were significantly influenced by interstock clones and by the interaction effect between scion and

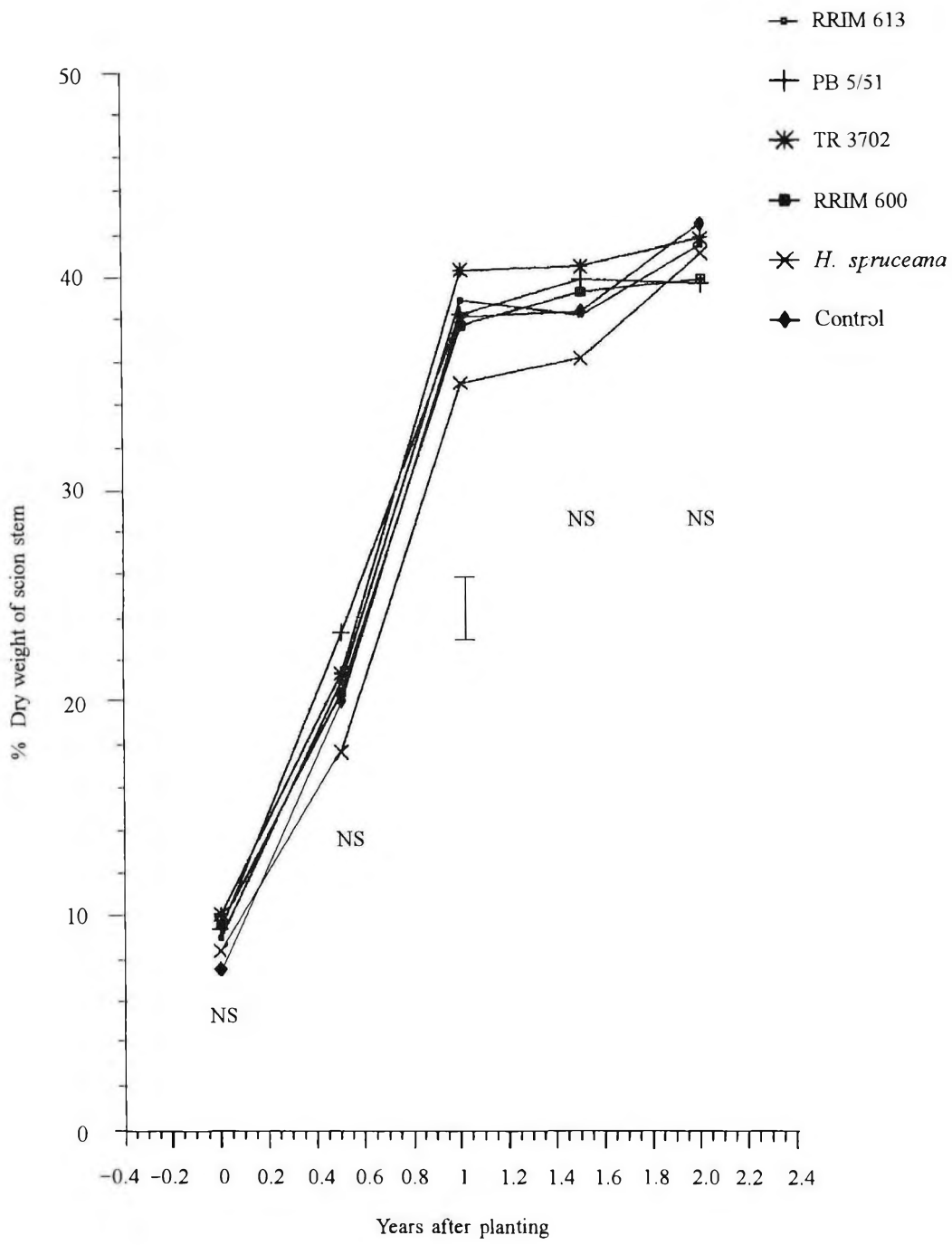

Figure 6. Effect of interstock clone on percentage of scion stem dry weight. (Verticle bar represents least significant difference at P<0.05)

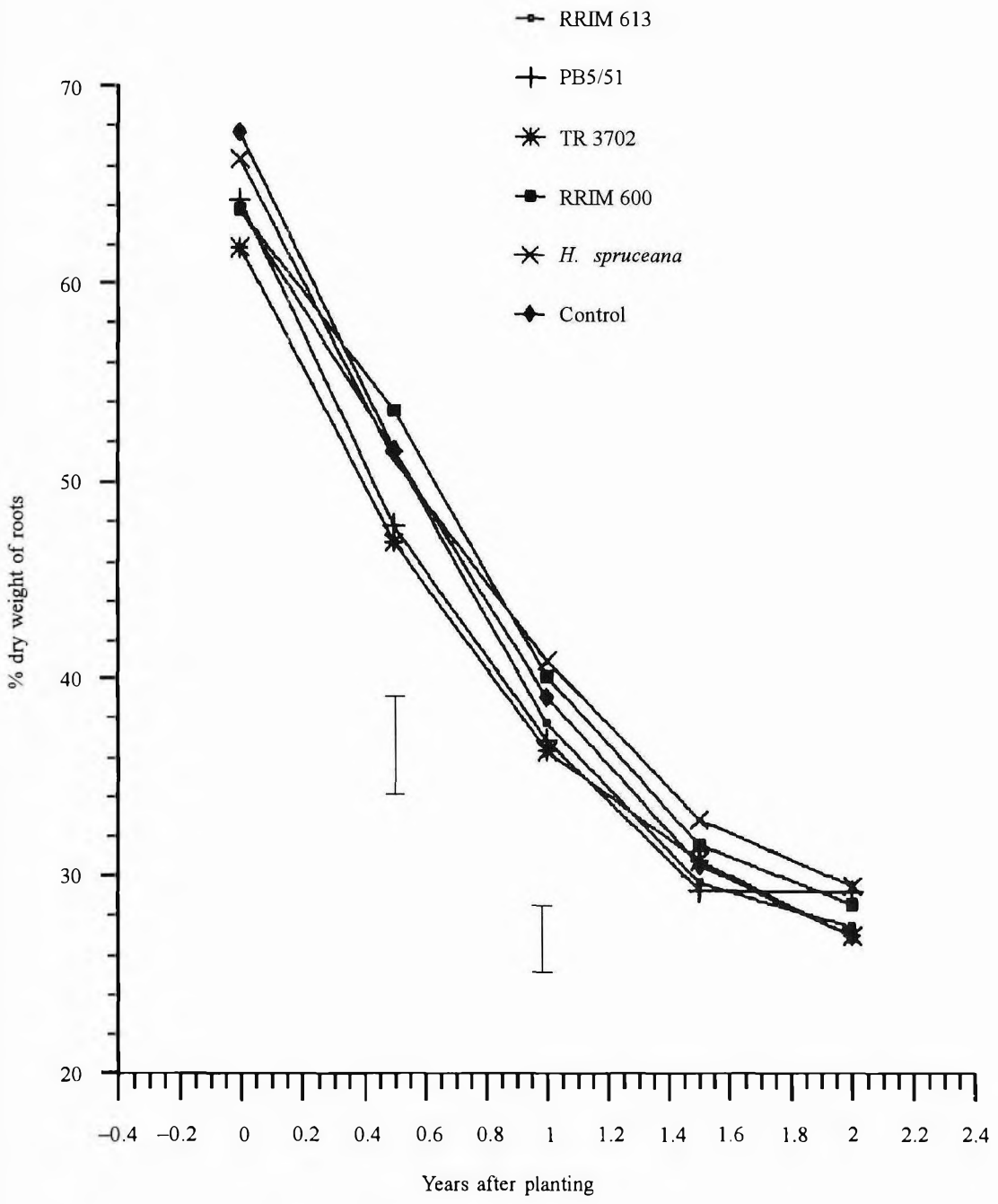

Figure 7. Effect of interstock on percentage dry weight of roots.
(Vertical bar represents least significant difference at P<0.05)

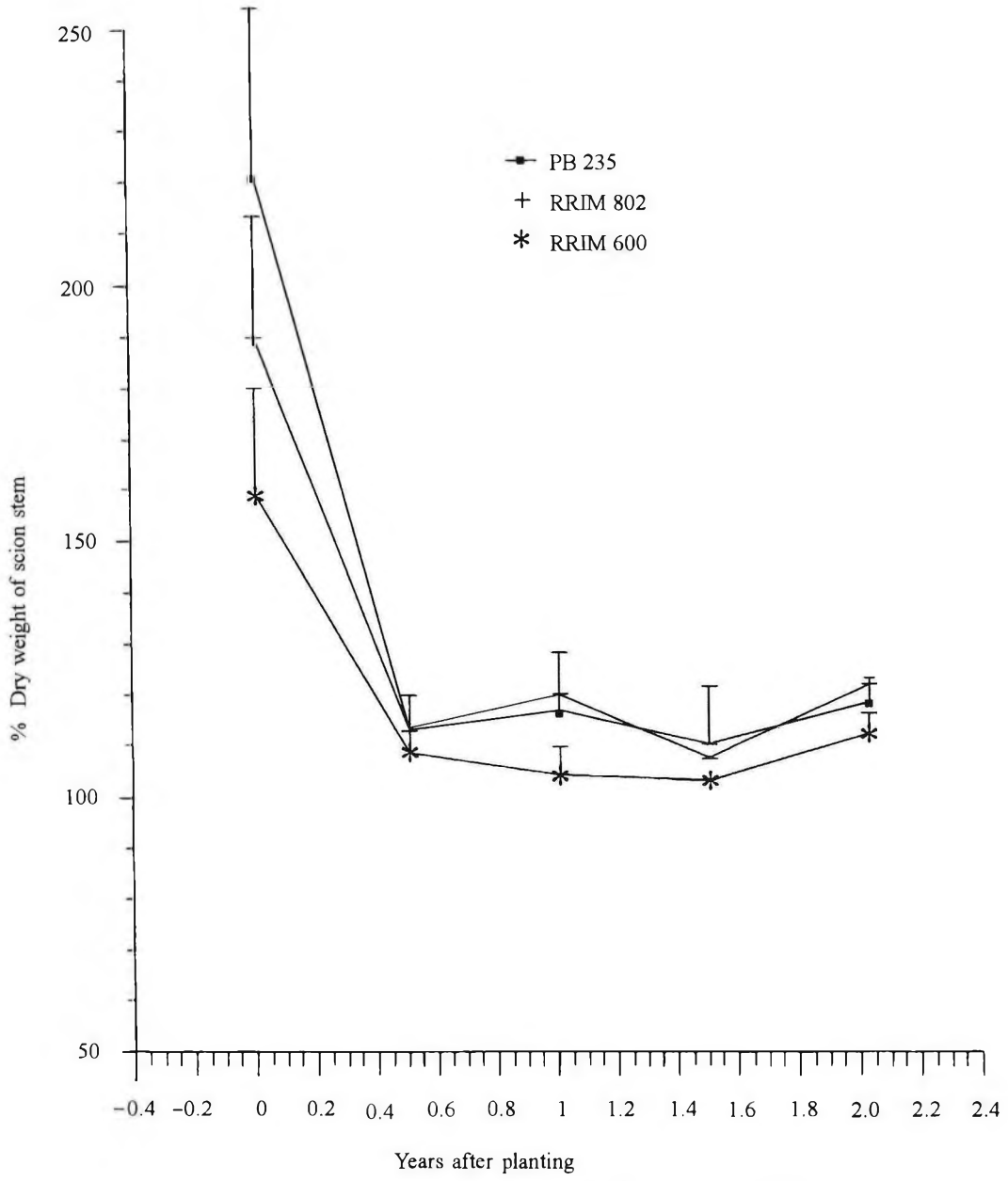

Figure 8. Specific leaf area of three scion clones from 0 to 2 years after planting. (Each point represents the mean of 6 interstocks +SD of the mean; some SDs are smaller than the symbols representing each point.)

interstock clones at all harvesting intervals (*Appendix 3*) Generally, for each scion clone, interstocks did not produce any consistent trend in mean NAR across the harvesting intervals (*Figure 9*). Mean NAR for all treatments generally increased two-fold between the 0-0.5 year and the 0.5-1.0 year intervals before declining slightly at the 1.0-1.5 year interval except for RRIM 802 scion clone which attained optimum values at the 1.0-1.5 year interval. At the final harvesting interval, mean NAR of all treatments declined two-to five-fold from the peak values with the exception of combinations with PB 235 scion grafted on TR 3702 interstock.

Reduced mean NAR observed from 1.5 year after planting reflects intense inter tree competition for light as the canopies began to overlap in the close planting stand[30, 31]. This would eventually cause a decrease in mean RGR values (*Figure 10*) since biomass is directly dependent on the daily radiation incident on the top of the canopy and on the fraction of incident radiation intercepted by the canopy[32]. Due to the presence of inter tree competition, only mean NAR and mean RGR results obtained during the first year of growth after planting merit discussion.

At 0–0.5 year interval, interstock clones did not improve mean NAR of RRIM 802 and PB 235 scion clones (*Figure 9*). However, PB 5/51 and TR 3702 interstocks resulted in higher mean NAR of RRIM 600 scion compared to the effect produced by control and *H. spruceana* interstocks. At 0.5-1 year interval, mean NAR of RRIM 600 scion clone on *H. spruceana* and control interstocks were significantly higher than those on PB 5/51 interstock. In contrast, RRIM 802 scion on PB 5/51 and RRIM 600 interstock clones and PB 235 scion in combination with RRIM 600, TR 3702 and RRIM 613 interstocks were significantly higher in mean NAR than their respective controls.

Results on mean NAR suggest that RRIM 600 scion had significantly higher photosynthetic capacity than had PB 235 and RRIM 802 scions, the values of which were not increased by the interstock clones. In contrast, photosynthetic capacity of RRIM 802 and PB 235 scion clones were further improved by RRIM 600, RRIM 613, TR 3702 and to a smaller extent by PB 5/51 interstock clones.

Mean RGR of the interstock plants more or less followed a similar trend as mean NAR across the harvesting intervals (*Figure 10*). Interstock clones significantly influenced mean RGR at all harvesting dates except at the final sampling interval. As with the mean NAR, there were also highly significant interaction between scion and interstock clones for mean RGR; thus results are presented for each scion clone. At 0–0.5 year interval, mean RGR of RRIM 802 and PB 235 scions were little improved by interstock clones compared to the control. However, PB 5/51 and TR 3702 interstocks resulted in higher mean RGR of RRIM 600 scion than produced by other interstocks. At 0.5–1 year interval, mean RGR of RRIM 600 scion was not much improved by various interstocks compared to the control. PB 5/51 interstock resulted in the highest and control interstock the lowest mean RGR of RRIM 802 scion. For combinations with PB 235 scion clone, RRIM 600, TR 3702 and

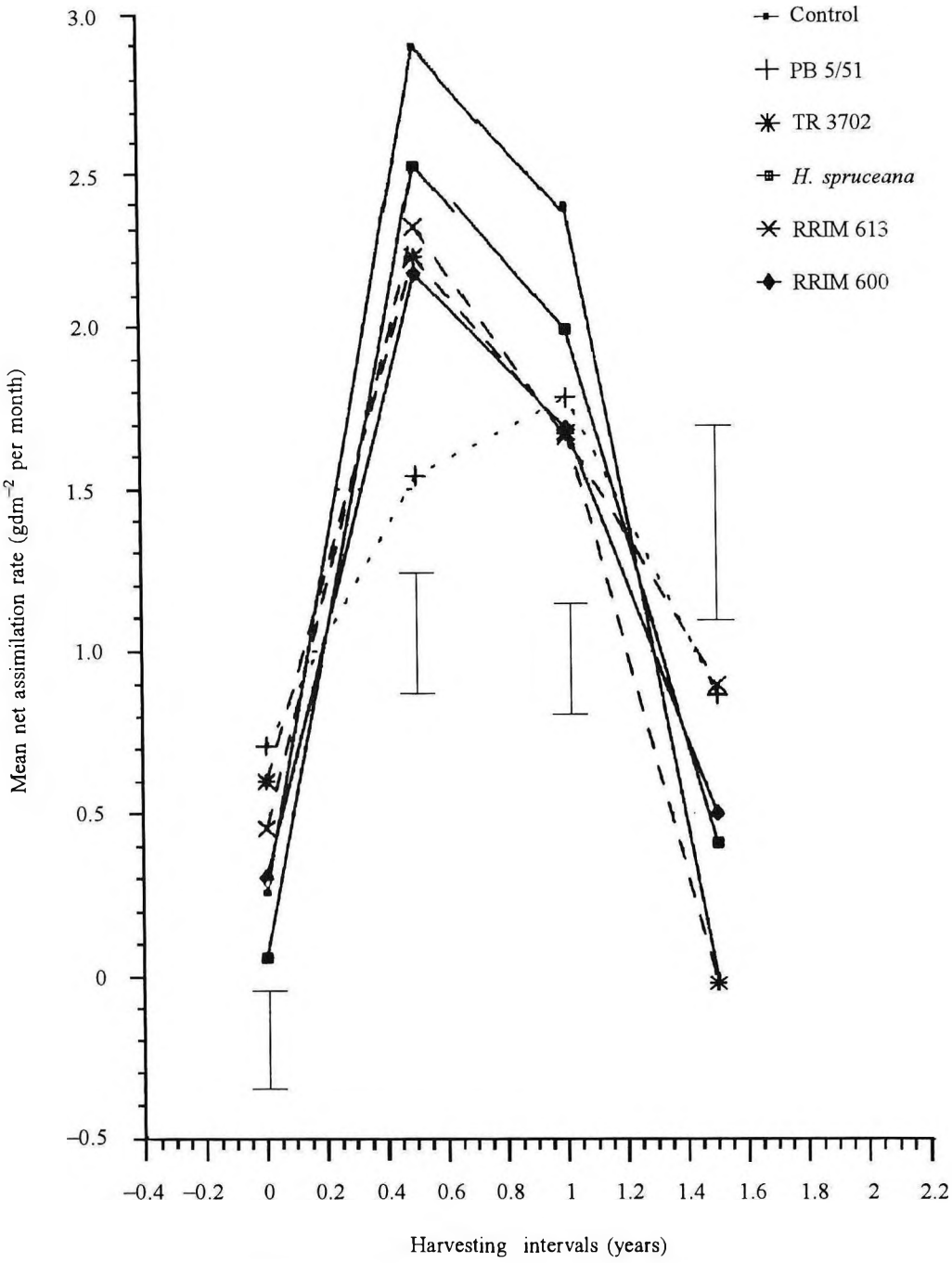

Figure 9a. Effect of interstock on net assimilation rate of RRIM 600 Scion. (Vertical bar represents least significant difference at $P<0.05$)

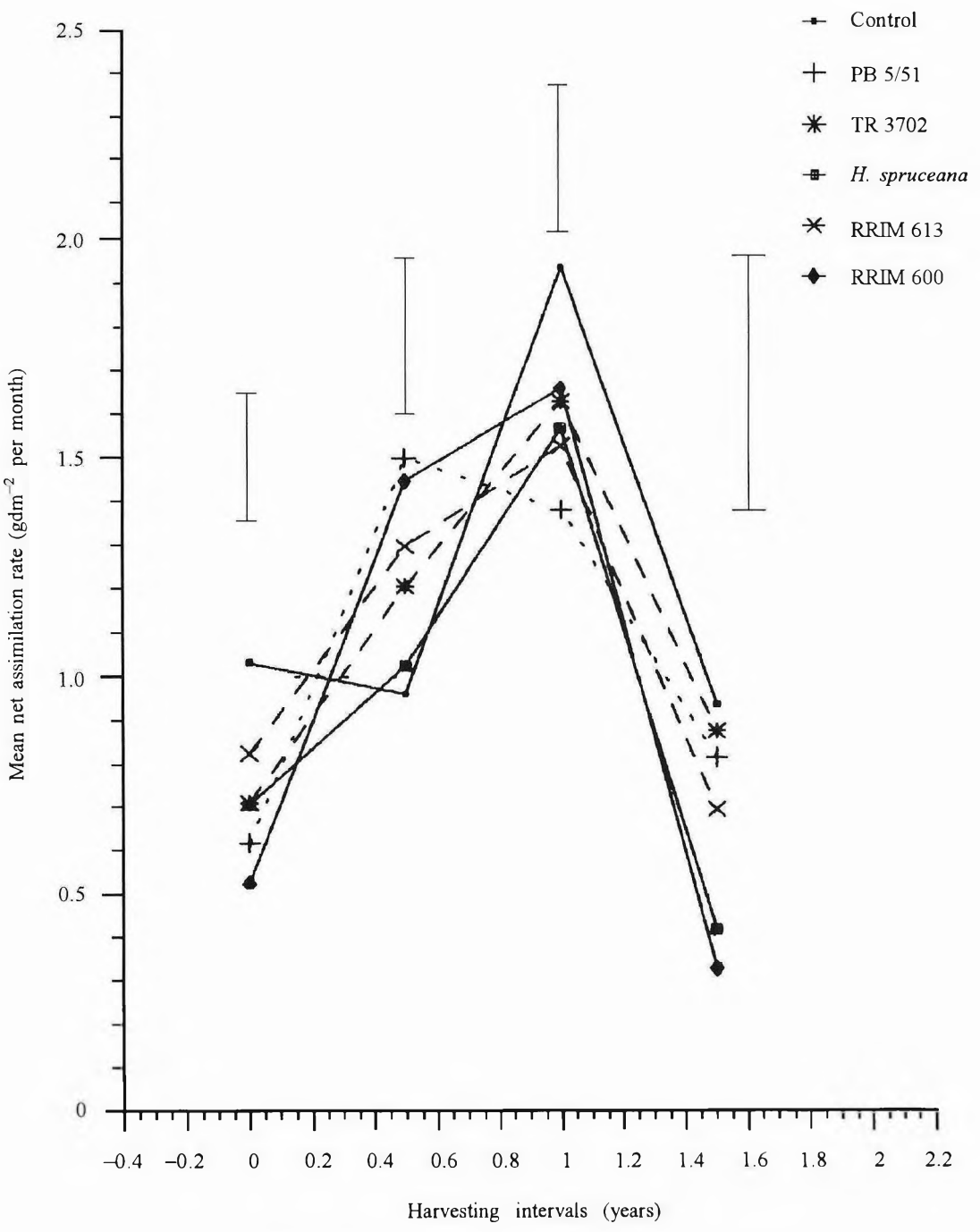

Figure 9b. Effect of interstock on net assimilation rate of RRIM 802 Scion. (Vertical bar represents least significant difference at P<0.05)

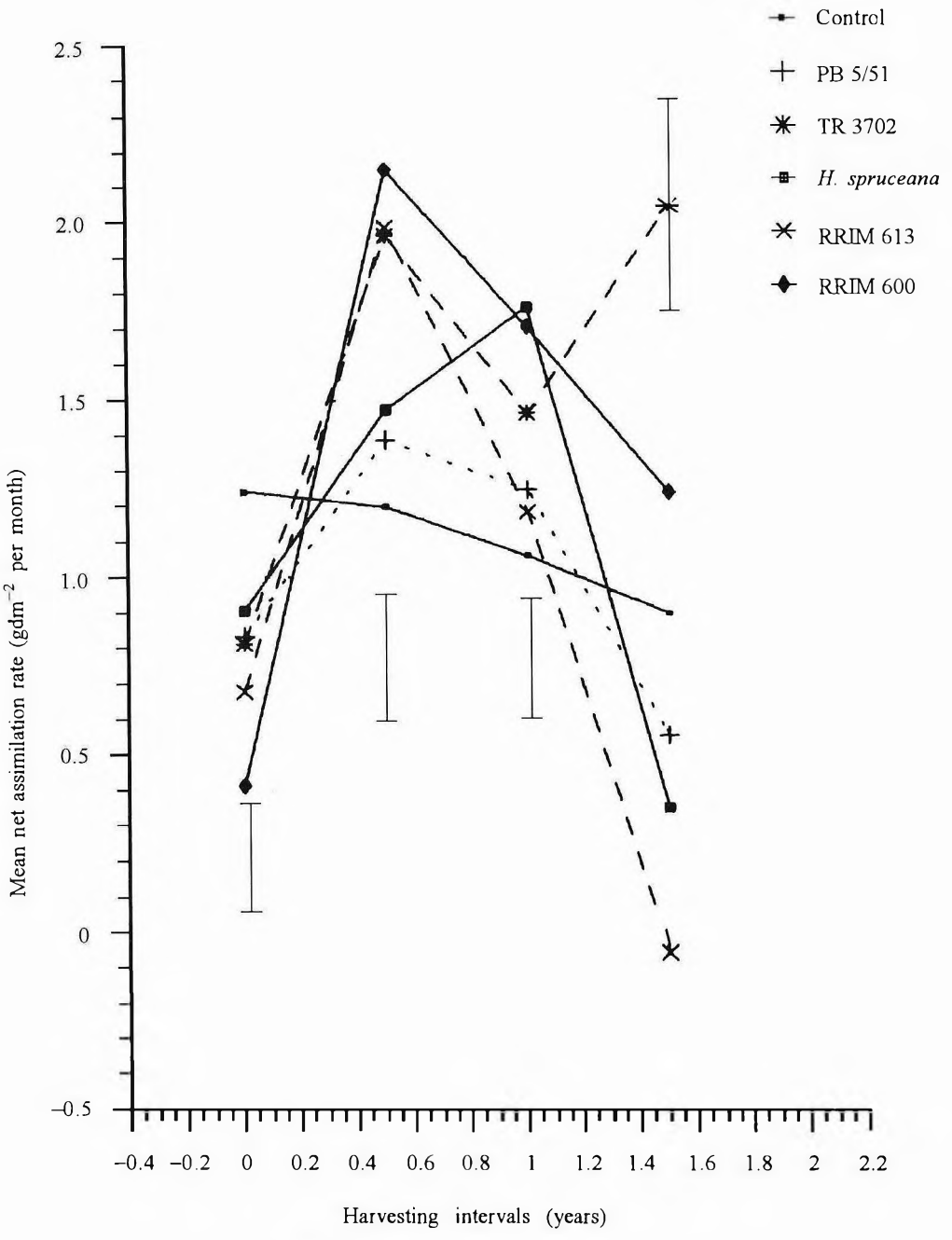

Figure 9c. Effect of interstock on net assimilation rate of PB 235 scion. (Vertical bar represents least significant difference at P<0.05)

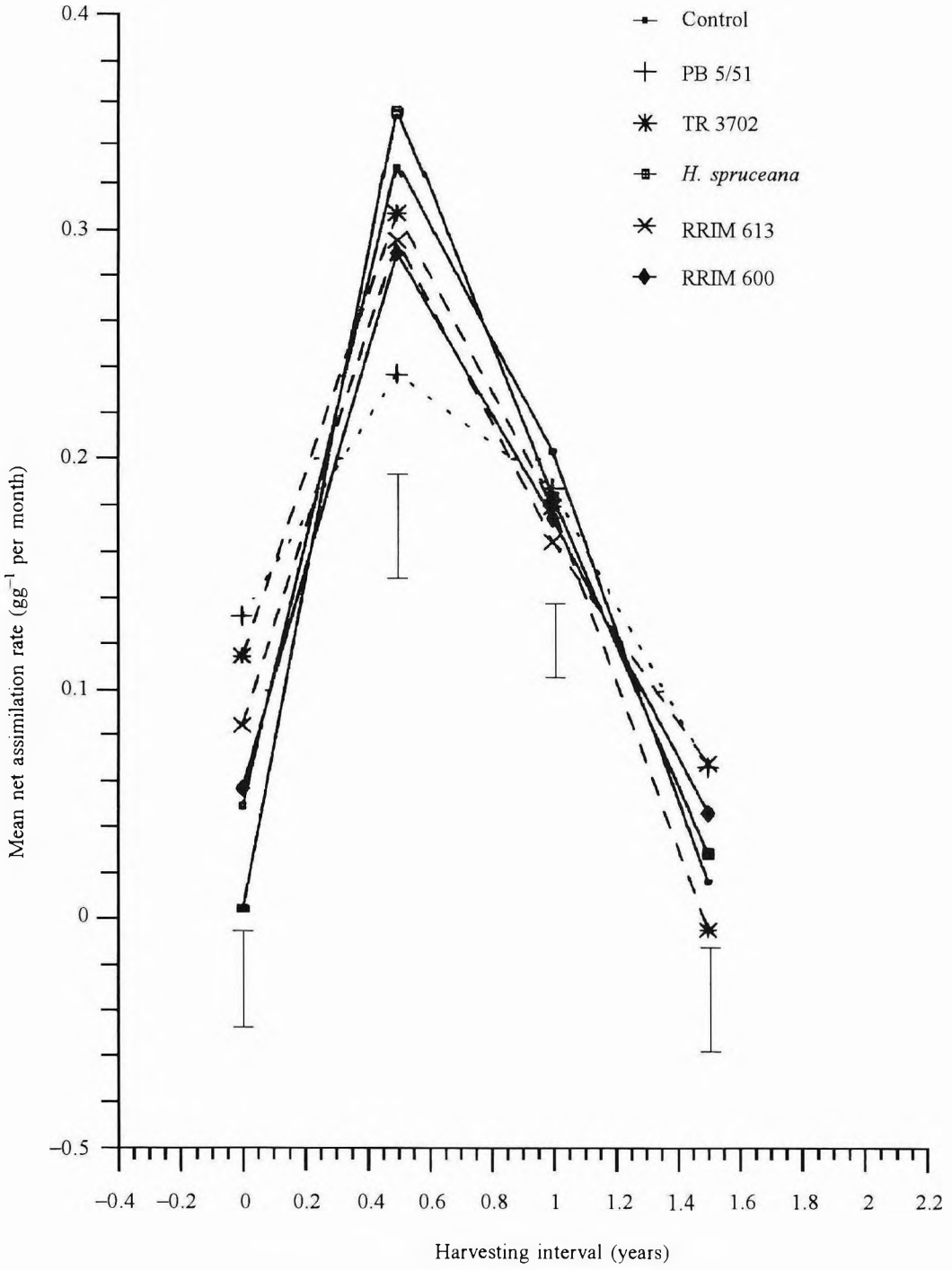

Figure10a. Effect of interstock on mean relative growth rate of RRIM 600 scion. (Vertical bar represents least significant difference at P<0.05)

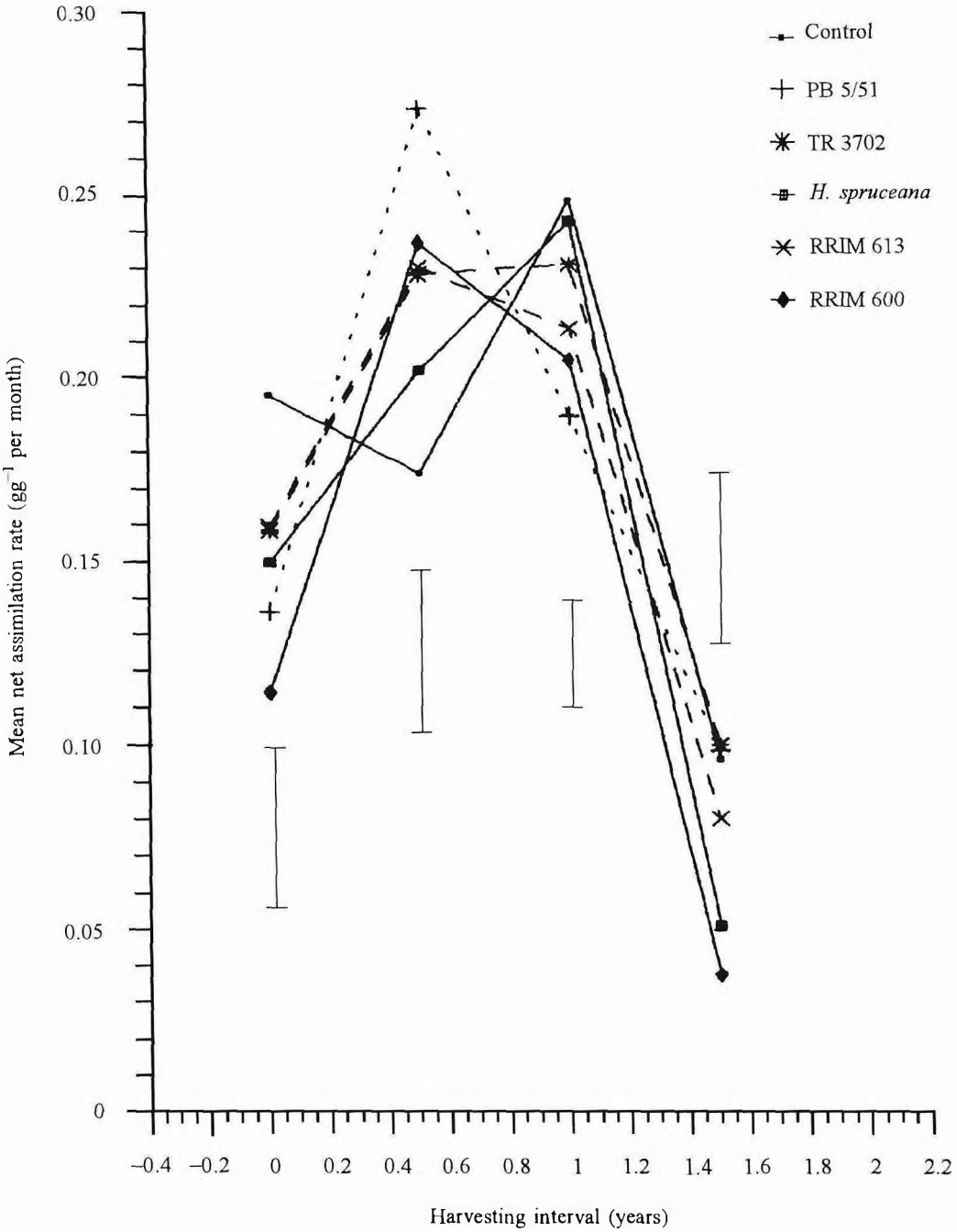

Figure10b. Effect of interstock on mean relative growth rate of RRIM 802 scion. (Vertical bar represents least significant difference at $P<0.05$)

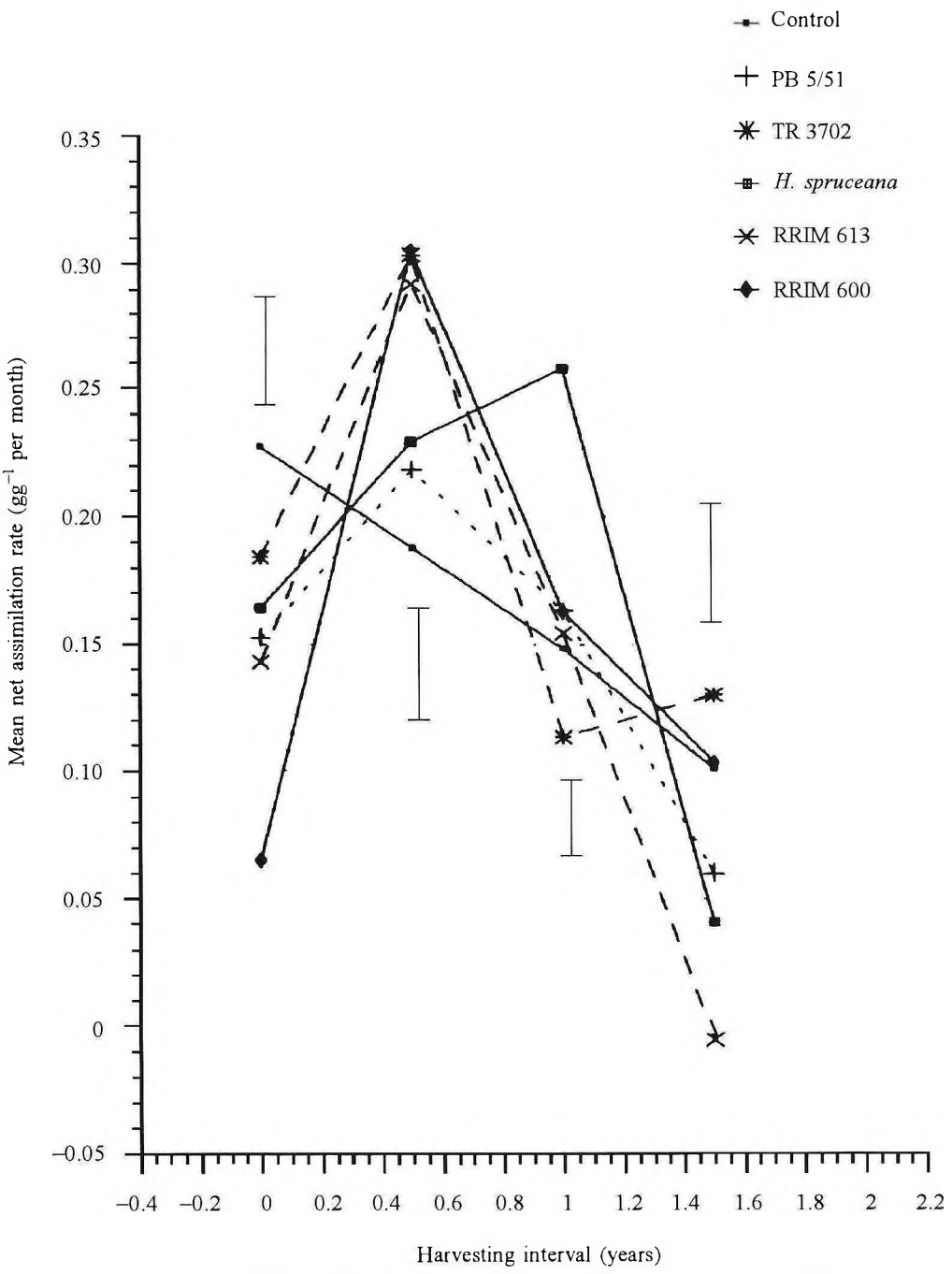

Figure10c. Effect of interstock on mean relative growth rate of PB 235 scion. (Vertical bar represents least significant difference at P<0.05)

RRIM 613 interstocks resulted in significantly higher mean RGR than those produced by *H. spruceana,* PB 5/51 and control interstocks.

DISCUSSION

The interstock influence on many parameters of growth was evident only at one year after planting (*Appendix 1*). Any tendency towards the expression of interstock influence in terms of growth during the first six months of growth after planting may have been masked by the great variability in growth as a result of non-uniform bud sprouting after grafting and to the different flushing rate of shoots after establishment. Similarly in apples, it was reported that the interstock influence on scion growth was evident only after the composite tree had reached a certain stage of maturity or stability in its growth which occurred after the first year of growth[33, 34].

In the present experiment, the absence of any interstock influence on leaf area and biomass of plant parts after the first year is probably because the interstock influence, being weaker than the scion influence (*Appendix 1*), was more prone to be confounded by the inter tree competition than the influence of scion. The inter tree competition is expected after the first year of growth due to the close planting distances in the ground nursery[35] which resulted in a sharp decline in mean NAR and mean RGR of the interstock plants in our study (*Figures 9* and *10*).

With the exception of data on mean RGR and mean NAR, and dry weights of interstock stem, roots and whole tree, the effects of interstock and scion on other aspects of growth were found to be additive, with mean squares for scion and interstock interaction being often statistically insignificant (*Appendix 1–3*). This is in concurrence with other reports on temperate fruit trees[12]. In the present investigation, most of these significant (S×I) interaction effects occured at 1.5 years after planting when the inter tree competition had set in; thus the interaction effect was obviously attributed to external factors and not due to the effects of treatment. On the basis of these explanations and unless otherwise stated, the following discussion on LA, LAR and dry matter production will refer to the observations made at one year after planting mainly on the main effect of interstock representing a mean response for the three scion clones. Similarly, results on growth analysis will refer to data taken during the first year of growth after planting. As this experiment was mainly concerned with interstock influence, the main effects of scion will only be mentioned whenever they are relevant to the influence of interstocks. This is despite the fact than in most of the growth parameters determined, scion influence was generally greater or equal to the influence of interstocks (*Appendix 1–3*).

The present experiment indicates that even though the interstock stem is only 20 cm in length and its development took only less than 10 percent of the total dry matter accumulation (*Figure 5*), yet it is an important sink as it influenced scion vigour, dry matter production, partitioning of assimilates to other vegetative plant parts and photosynthesis.

Data presented in *Table 2* show that in the composite tree, the differences in inherent vigour of the interstock clone, as reflected by the interstock stem dry weights, were very highly significant and were as strong as the influence of scion. This is to be expected since the interstock clones were selected

based on their inherent vigour. Thus vigorous interstocks (TR 3702 and RRIM 613) generally had the largest stem dry weights while *H. spruceana* and RRIM 600 interstocks, with poor inherent vigour, had relatively lower stem dry weights.

The relationship of dry weights of interstock stem to LA and dry weights of other plant parts and whole tree were examined by correlation analysis to illustrate the importance of interstock vigour in determining scion and whole tree growth. Leaf area was used instead of laminae dry weight as preliminary studies had shown very highly significant and positive relationship between leaf area and laminae dry weight (r^2 = 0.933 or better). *Table 4* shows that there were significant and positive relationship between dry weights of interstock stem and scion stem for all three scion clones indicating that interstock vigour is important as it directly determines growth of scion. Absolute growth in terms of scion stem biomass and mean RGR calculated (*Table 1* and *Figure 10*) also showed this to be true. Thus vigorous interstock clones (TR 3702 and RRIM 613) produced better scion vigour than that of the less invigorating interstocks (RRIM 600 and *H. spruceana*) resulting in better overall growth of trees on the former interstocks. Similarly, Hewetson[33] working on apples had reported that the use of interstocks of variable vigour resulted in a range in tree size. However, for root dry weight and LA, significant relationship with interstock stem dry weights were only evident for RRIM 802

TABLE 4. THE RELATIONSHIP OF INTERSTOCK STEM DRY WEIGHT TO SOME GROWTH CHARACTERS AT ONE YEAR AFTER PLANTING

Scion clone	Growth character	Regression equation	Level of probability	r^2
PB 235	LA	0.8843 + 0.0104 x	*	0.661
	Scion stem DW	119.42 + 3.609 x	**	0.936
	Root DW	232.35 + 3.045 x	NS	0.531
	Whole tree DW	436.11 + 8.993 x	*	0.811
RRIM 802	LA	0.6774 + 0.0164 x	NS	0.581
	Scion stem DW	75.47 + 4.1021 x	*	0.755
	Root DW	87.36 + 4.305 x	***	0.974
	Whole tree DW	197.66 + 11.563 x	**	0.953
RRIM 600	LA	0.5159 + 0.0107 x	NS	0.473
	Scion stem DW	69.69 + 5.3404 x	**	0.922
	Root DW	213.94 + 2.815 x	NS	0.542
	Whole tree DW	−298.81 + 15.968 x	NS	0.241

n: 6
LA: Leaf area
DW: Dry weight
NS, *, **, ***: F-test non-significant or significant at $p<0.05$, 0.01 or 0.001, respectively

and PB 235 scion clones, respectively (*Table 4*). This indicates that interstock clones affected growth of scion stem more than they did on LA and root growth. In contrast, studies in apple trees have shown that vigour potential rather than absolute growth of the interstock clone was important in influencing scion vigour[14]. It was also shown that dwarfing interstocks exerted greater influence on root growth than on shoot growth resulting in the interstock trees to be less firmly anchored than control trees. In the present experiment, significant relationship between interstock stem and whole tree dry weights was evident only for RRIM 802 and PB 235 scion clones but not for RRIM 600 scion (*Table 4*). This confirms the result of another experiment[36] in which there was a small significant interaction between scion and interstock clone with respect to scion girth; the interaction effect was attributed to the failure of RRIM 600 scion to be invigorated by various interstock clones.

The data presented here seem to indicate that the vigour produced by PB 5/51 interstock was comparable to that of RRIM 600 interstock (*Table 1*), while PB 5/51 as a rootstock was reported to exert more superior influence on scion growth than that of RRIM 600 rootstock[1]. This seems to suggest that in *Hevea*, an interstock may not have similar effect as a rootstock; this concurs with the view that the influence of a clonal material on *Hevea* performance when present either as a scion or rootstock and probably interstock too, is not necessarily the same and may differ markedly[1]. This phenomenon has also been reported for other temperate crops such as apple, citrus, quince, cherry and plum[37,38].

Several explanations can be advanced to account for the invigorating effect of interstock clones on scion growth in the present experiment. Improved scion growth seems to involve a greater allocation of photosynthetic assimilates to scion stem (*Figure 6*). Associated with this greater allocation has been a parallel increase in the photosynthetic rate of scions as indicated by mean NAR calculated (*Figure 9*) and in parameters associated with photosynthesis such as LA and stomatal size[36].

This is consistent with the concept of interstock stem being part of a source sink system of the composite tree whereby the demand for assimilates by active sinks (interstock and scion stems) would invariably lead to an increase in photosynthetic rate of scion leaves[39]. Maggs[40] also reported increased NAR of cropping apple trees where fruits are active sink compared to either deblossomed or defruited trees. However, photosynthesis and LA may not be the limiting factors for growth of these composite plants. This is based on the observation that LAR values which reflect the relative size of the assimilatory apparatus, did not seem to be related to vigour induced in the scion (*Table 1*). Moreover, the consistently lower SLA values of RRIM 600 scion relative to the other two scion clones over the study period (*Figure 8*) may also reflect thicker leaves and/or carbohydrate accumulation in leaves of RRIM 600 clones[41]. Thicker leaves usually have higher photosynthetic capacity than thinner leaves since the resistance to CO_2 diffusion to the chloroplast are substantially reduced due to an increase in thickness of palisade parenchyma or to greater pore space in the mesophyll layer[42-44]. Since mean NAR in RRIM 600 scion was 34% to 83% greater than those of

PB 235 or RRIM 802 scion clones (*Figure 9*), this would lend support to this explanation.

It is apparent in the present experiment that vigorous interstocks (TR 3702 and RRIM 613 clones) are active sinks which improved scion vigour by increasing the sink strength of scion stem (*Figure 6*), a process probably involving plant hormones such as gibberelic acid[45]. The active sinks would then have a greater capacity to remove assimilates from the phloem, thereby giving it a competing edge over other sinks especially the roots for dry matter[39]. In other plants, it has been shown that treatments which increased gibberellin activity in a particular organ has led to a concomitant increase in its sink strength for available assimilates[46,47]. Similarly in apples, vigorous interstock also resulted in greater accumulation of dry matter in branches and stem than in roots[48]. Evidently, in the present experiment, root growth was the most seriously affected by the competition from vigorous scion stem for assimilates than growth of leaves and interstock stem. However, in the presence of weakly growing competing sinks such as *H. spruceana* and RRIM 600 interstocks, sink strength of scion stem was not increased; more assimilates would then be available for extra growth in the roots. This is consistent with the evidence found in other perennial trees that the root system becomes a major sink for photosynthates when active growth of shoot is reduced[29,49].

CONCLUSION

The use of interstock in the propagation of *Hevea* may be costly because of the additional budding process involved. However, where cost is not a limiting factor, interstock may be a practical approach to improve tree growth with no suitable monoclonal seedling rootstocks. Before interstock can be recommended as a planting material, more tests need to be carried out for the most suitable interstock and scion combinations because of the presence of scion × interstock interaction. Future research might also be directed to study the relative influence of interstock and rootstock on scion performance to ascertain whether the slight increase in scion vigour would justify the expense of making the three-part-tree. This paper shows that two outstanding interstocks were RRIM 613 and TR 3702 clones which increase the sink capacity of scion and provide an efficient dry matter partitioning towards scion growth during the early stage of plant growth. Early vigour of high yielding scion clone will ensure a higher potential for latex and timber yields during the later part of the economic life of the tree. This is particularly pertinent in the current context where there is a high demand for rubberwood by the timber industry.

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APPENDIX 1.
ANALYSIS OF VARIANCE FOR LEAF AREA AND DRY WEIGHTS OF VARIOUS PLANT PARTS MEASURED OVER THE EXPERIMENTAL PERIOD

Source of variation	df	Mean squares						
			Dry weight					
		Leaf area	Laminae	Petiole	Scion stem	Interstock stem	Root	Whole tree
At time of planting								
		NS	NS	NS	NS	NS	NS	NS
Scion clones (S)	2	0.053	55.188	3.892	63.099	13.229	2000.844	2704.045
		NS	NS	NS	NS	NS	NS	NS
Interstock clone (I)	4	0.026	49.167	3.472	49.738	59.626	2106.071	4398.548
		NS	NS	NS	NS	NS	NS	NS
Interaction (S×I)	8	0.048	66.148	4.308	35.145	12.407	490.480	1465.133
Error	94	0.038	73.683	4.058	53.416	25.577	971.287	2527.758
0.5 year after planting								
		NS	NS	**	NS	NS	NS	**
Scion clone (S)	2	0.109	582.658	107.673	482.079	247.319	9577.420	75229.916
		NS	NS	NS	NS	NS	NS	NS
Interstock clone (I)	5	0.032	203.481	17.108	1424.198	205.326	1667.657	18281.447
		NS	NS	NS	NS	NS	NS	NS
Interaction (S×I)	10	0.035	236.114	28.095	277.895	67.031	2389.259	16768.326
Error	105	0.035	273.391	19.165	998.239	104.884	2486.443	10736.480
1.0 year after planting								
		***	*	***	***	***	***	***
Scion clone (S)	2	2.721	11968.629	4012.484	286756.042	9671.352	234397.223	1180173.677
		*	*	**	*	***	NS	**
Interstock Clone (I)	5	0.923	9379.771	446.861	99431.142	4150.722	43009.159	450801.423
		NS	NS	NS	NS	***	*	NS
Interaction (S×I)	10	0.440	39166.411	205.653	22991.175	1853.636	45081.402	200625.257
Error	104	0.339	2426.293	120.329	26253.808	582.414	19153.451	118481.294

APPENDIX 1. (CONTD)
ANALYSIS OF VARIANCE FOR LEAF AREA AND DRY WEIGHTS OF VARIOUS PLANT PARTS MEASURED OVER THE EXPERIMENTAL PERIOD

Source of variation	df	Mean squares						
			Dry weight					
		Leaf area	Laminae	Petiole	Scion stem	Interstock stem	Root	Whole tree
1.5 year after planting								
		*	*	*	NS	*	NS	NS
Scion clone (S)	2	12.11	84627.88	4506.41	26696.65	12563.95	58528.91	152509.98
		NS	NS	NS	NS	NS	NS	NS
Interstock Clone (I)	5	2.51	21219.19	843.60	73525.23	6902.65	88691.41	451491.00
		***	***	***	*	NS	NS	NS
Interaction (SxI)	10	11.87	94215.69	4028.16	338036.17	5321.22	120522.54	2331459.75
Error	113	2.64	19304.61	1091.49	157720.16	3674.34	95108.89	1081512.45
2 years after planting								
		***	***	**	*	NS	***	*
Scion clone (S)	2	132.97	733736.40	16730.21	2578037.70	17958.25	1342917.28	16240624.09
		NS	NS	NS	NS	***	NS	*
Interstock clone (I)	5	3.18	225499.47	555.34	1474617.58	58961.28	252667.39	6468391.20
		NS	NS	NS	NS	*	NS	NS
Interstock (SxI)	10	10.44	75938.41	5005.43	1254718.49	20289.82	246685.02	6843455.20
Error	108	8.45	57813.07	2524.70	693536.30	9580.68	179335.62	3699823.77

NS *, **, ***: F-test indicates non-significant or significant at P<0.05, 0.01 and 0.001, respectively

APPENDIX 2.
HARVEST DATE, SCION AND INTERSTOCK EFFECTS ON LEAF AREA RATIO (LAR), SPECIFIC LEAF AREA (SLA) AND PERCENTAGE DRY WEIGHTS OF VARIOUS PLANT PARTS

Source of variation	df	Mean squares						
				Percentage dry weight				
		LAR	SLA	Laminae	Petiole	Scion stem	Interstock stem	root
Scion clone (S)	2	150.610 ***	498.611 ***	46.192 ***	3.953 ***	20.198 *	3.220 **	49.967 **
Interstock clone (I)	5	6.643 NS	15.519 NS	3.233 NS	0.091 NS	16.478 *	2.982 ***	27.174 **
Harvest date (H)	4	720.400 ***	430.101 ***	173.228 ***	12.646 ***	3563.485 ***	65.339 ***	4109.497 ***
Interaction (S × I)	10	7.700 NS	19.002 NS	1.575 NS	0.223 NS	4.124 NS	0.472 NS	4.666 NS
Interaction (S × H)	8	12.175 *	55.891 *	4.203 NS	0.278 NS	24.725 ***	0.763 NS	12.897 NS
Interaction (I × H)	20	5.902 NS	15.197	1.630 NS	0.147 NS	3.991 NS	0.375 NS	5.337 NS
Error	40	4.780	19.775	2.250	0.178	4.933	0.563	6.828

NS, *, **, ***: F-test indicates non-significant or significant at P<0.05, 0.01 and 0.001, respectively

APPENDIX 3.
ANALYSIS OF VARIANCE FOR MEAN NET ASSIMILATION RATE AND MEAN RELATIVE GROWTH RATE OVER THE EXPERIMENTAL PERIOD

Source of variation	df	Mean Squares x 10^2			
		Harvest dates (years after planting)			
		0–0.5 year	0.5–1.0 year	1.0–1.5 year	1.5–2.0 year
Mean net assimilation rate		***	***	***	***
Scion (S)	2	185.91	1021.32	198.04	151.11
		***	***	***	*
Interstock (I)	5	42.15	48.83	40.33	75.36
		***	***	***	***
Interaction (S × I)	10	30.48	102.30	41.53	207.31
Error	101	7.01	10.49	9.16	29.38
Mean relative growth rate					
		***	***	***	***
Scion (S)	2	8.26	5.97	3.05	1.83
		***	***	***	NS
Interstock (I)	5	1.72	0.77	0.82	0.42
		***	***	***	***
Interaction (S × I)	10	0.98	1.29	0.55	1.06
Error	101	0.14	0.16	0.07	0.17

NS, *, **, ***: F- test indicates non-significant or significant at P<0.05, 0.01 and 0.001, respectively

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