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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 8/10/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. # 2 of
6. 400

Attachment

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PD-AAW-328

52169

PROGRESS REPORT NO. 2

"BIOTECHNOLOGY APPLICATION FOR CHARACTERIZATION
AND SELECTION OF BETTER-YIELDING RUBBER CLONES"

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No.936-5542-G-00-6054-00

Submitted by

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Project Profile

Country : Thailand

Grant No. : 936-5542-G-00-6054-00

Program : Program on Science and Technology Cooperation

Project Title : Biotechnology Application for Characterization and Selection of Better-Yielding Rubber Clones.

Project Leader : Dr. Rapepun Wititsuwannakul

Organization : Dept. of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai 90112, Thailand.

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Authorized Officer : Dr. Phasook Kullavanij (Rector)

Total Project Budget : US \$ 150,000

Project Duration : 3 years (27 June-1986-31 July 1989)

Reporting Period : 1 January-30 June 1987

Budget Allocation for This Period : US \$ 67,890.57

1. Background/Introductions :

Part A. Biochemical parameters for rubber yield evaluation in *Hevea brasiliensis*.

Traditional practice on yield evaluation of rubber trees is to grow the plant for at least six years until trees can be tapped and yield measured. Early attempt on yield evaluation studying relation between latex yield of *Hevea* and rubber biosynthesis *in vitro* was reported in 1965 by D'Auzac (1). The study involved latex specimens chosen from pairs of high- and low-yielding trees growing in a monoclonal plot. The efficiency of rubber biosynthesis from 2-¹⁴C acetate was shown distinctly higher in the high yielders. In 1970, C.H. Woo and E.E. Edwin performed similar incorporation experiment using trees from different clones and chosen at random (2). The results led to similar conclusion but large variations made it difficult to make reliable distinction of high and low-yielding potentials. Our studies on diurnal variation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity showed the enzyme was closely related to rubber content in the latex (3). The correlation suggested possible role of this enzyme in regulation of rubber biosynthesis. In this study we proposed to test and verify HMGR as a rate limiting enzyme in rubber biosynthesis pathway. Its possible use as an enzyme parameter in yield evaluation will be further pursued and utilized in clonal selection processes.

Part B. Disease resistance screening of *Hevea* at tissue culture stage

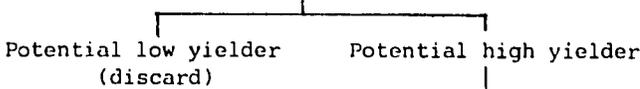
Since *Hevea brasiliensis* is a perennial tree crop and thus the duration of breeding cycle is rather long. It takes 5-6 years for *Hevea*

to grow before the first tapping can be commenced, after which it can be tapped for rubber latex for a period of 30 or more years. It is, therefore, of great importance to select for a disease resistant clones in addition to the high-yielding potential concurrently. This is an added advantage to prevent growth and rubber production failure due to disease or disturbance by the pathogens. We proposed a system to study the disease resistance at tissue culture level in this study as an early selection in conjunction with high-yielding potentials.

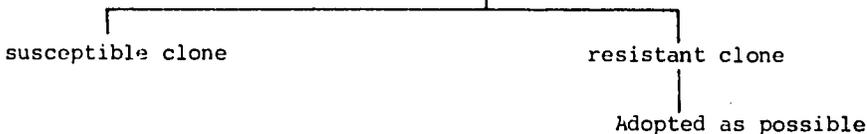
2. Objectives :

- A. To develop a reliable method for identification and selection of higher yielding rubber clones at an early stage, while the plants are still at seedling stage. This would help circumventing the time and energy needed in conventional approaches.
- B. To initiate the study of disease resistant rubber clones at the level of tissue culture stage in conjunction to the high-yielding potential screening as outlined below.

Yield screening at seedling stage (Part A)



Disease resistance screening at tissue culture stage (Part B)



high yielding disease resistant clones.

3. Materials and methods :

Part A. Biochemical parameter for rubber yield evaluation in *Hevea brasiliensis*.

Chemicals:

All chemicals and reagents used were of analytical grade. DTT NADPH, HMG-CoA, polyoxyethylene ether W-1 (Brij W-1), mevalonolactone, PEP, NADH, pyruvate kinase, lactate dehydrogenase, acetyl-CoA, acetoacetyl-CoA, polyvinyl pyrrolidone were from Sigma. 3-¹⁴C HMG-CoA was from Amersham England and Affi-Gel-Blue was from Bio-Rad. Agarose-hexane-HMG-CoA was from P-L Biochemicals.

Collection and fractionation of latex :

Latex was obtained from several clones of *Hevea brasiliensis* as indicated in the text. The trees are regularly tapped, half spirally on every other day (S.2/D.2). All clones are grown categorized at the Rubber Research Center, Hat-Yai. Rubber trees were normally tapped at 0600 h or otherwise as indicated. After tapping the fresh latex was collected into an ice-chilled beaker. The latex from each clone was pooled and fractionated into 3 major fractions by centrifugation in a microfuge at 15,000 rpm for 60 min. They are the rubber fraction, the C-serum (latex cytoplasm) and the bottom fractions, respectively. The bottom fractions, where 95% of HMGR was located (4), were separated by cutting the bottom of the microtube and used for assay of HMGR activity. The C-serum was used for assays of mevalonate kinase and HMG-CoA synthetase activities.

Assay of HMG-CoA reductase (HMGR) activity :

The HMGR activity was assayed in buffer X (0.1 M KCL, 10 mM DTT 30 mM EDTA, 0.1 M K_xPO_4 , pH 7.0) containing bottom fraction or active protein fraction or fresh latex with protein ranged from 0.1-1.8 mg, 0.4 μ moles NADPH, 120 nmoles DL-3 ¹⁴C HMG-CoA (1000 dpm/nmole) in a final volume of 50-100 μ l. For solubilized enzyme, 1% W/V of BSA was added to the

assay system to stabilize the HMGR activity. Incubation was at 37°C for 20 or 30 min as indicated. The reaction was stopped by addition of 25 µl of 10 N HCl. Unlabeled mevalonolactone was then added, and the mixture was allowed to incubate for another 30 min for complete lactonization of the incubation product (mevalonic acid). The precipitated protein was removed by centrifugation and the product of the enzyme was purified by TLC using benzene acetone (1:1 v/v) as solvent system and developed under iodine vapor according to the method of Iijima *et al.* (5). The zone of mevalonolactone was scraped into a scintillation vial containing 10 ml of dioxane fluor. Samples were allowed to stabilize overnight before counting in Packard liquid scintillation counter.

Enzyme solubilization and fractionation : Frozen sample of bottom fraction was thawed at room temperature and homogenized in equal volume of solubilization buffer A (0.1 M K_xPO_4 , pH 7.0, 10 mM DTT 0.1 M KCl, 5 mM EDTA) which containing 1% Brij W-1 and 20% glycerol. The detergent Brij W-1 was used according to method described by Bach *et al.* (6). A glass Potter Elevehjem homogenizer with a tight-fitting glass pestle was used in homogenization. After 10 downward passes of hand-driven glass homogenizer pestle, the suspension was incubated at 37°C for 30 min. The mixture was then rehomogenized and centrifuged at 190,000 g for 60 min at 4°C. The resulting supernatant was isolated and fractionated by slow addition of saturated ammonium sulfate solution to final concentration of 40%. After incubation at 4°C temperature for 3-6 hours the mixture was centrifuge at 25,000 g for 30 min. The yellowish uppermost protein layer was dissolved in buffer B containing 6% Brij W-1 and dialyzed against buffer B (50 mM K_xPO_4 5 mM EDTA, 0.1% Brij W-1, 10 mM 2-Mecaptoethanoi) before subjected to further purification by chromatography.

Chromatographic procedures :

The dialyzed sample in buffer B was slowly loaded onto a column (40 x 2.5 cm) of DEAE Sephadex A-50, previously equilibrated. The column was washed with buffer B with flow rate 0.5 ml/min. The enzyme activity was eluted by adding KCl in buffer B at concentration 50, 500 and 1000 mM respectively. The enzyme fractions were pooled and concentrated by ammonium sulfate precipitation (90% saturation). The pellet was collected by centrifugation at 25,000 xg for 30 min and dialyzed against buffer B. This active fraction was then loaded on Affi-Gel-Blue (100-200 mesh; 75-150 μ) column (1.5 x 5 cm), previously equilibrated with buffer B. The column was washed with buffer B before eluted with the same buffer by the addition of 500 mM and 1000 mM KCl respectively. Active fractions were pooled and loaded onto 1 ml HMG-CoA-hexane agarose column, previously equilibrated with buffer B. The enzyme activity was eluted by adding 100 mM KCl, 100 mM KCl + 5 mM NADPH mixture, 750 mM KCl and 1000 mM KCl respectively.

Assay of mevalonate kinase (MVA) activity :

MVA kinase activity in c-serum was assayed by measuring the amount of ADP released from the reaction. 0.1 ml of C-serum was incubated with 5 μ moles ATP and 3 μ moles potassium mevalonate for 10 min at 30°C. The reaction was carried out in 50 mM phosphate buffer, pH 7.0, 5 mM MgCl₂, and 40 mM KCl in a final volume of 0.5 ml as modified from the method of Gray et al. (7). Mevalonate was omitted in the controls. The reaction was stopped by adding 0.2 ml of 1 M trichloroacetic acid and the precipitate was separated by centrifugation at 10,000 rpm for 3 min. The clear supernatant was neutralized with 0.1 ml of 2 M KOH. The aliquots (0.1-0.4 ml) were then used for ADP determination using coupled enzyme catalyzed oxidation of NADH. The ADP assay mixture contained 4 μ moles phosphoenol (0.3 mole) pyruvate (PEP), 0.3 moles NADH, pyruvate kinase (7 μ g protein, sp.act. 477 units/mg) and lactate dehydrogenase (39 μ g protein, sp.act. 490 units/mg).

The assay was carried out in 50 mM potassium phosphate buffer pH 7.0 containing 5 mM $MgCl_2$ and 40 mM KCl, in a total volume of 1.0 ml. MVA kinase activities in the C-serum were determined either immediately after collecting or kept in the cold, assayed in the morning for samples collected during the night. All determinations were done within 15 hours after latex collection.

Assay of HMG-CoA synthetase activity :

HMG-CoA synthetase activity was determined using dialyzed C-serum. The assay was carried out by following the incorporation of $1-^{14}C$ acetyl-CoA in HMG-CoA or its dehydration product, 3 methylglutaryl-CoA. The reaction mixture contained 1 M Tris HCl pH 8.2, 0.1 mM EDTA, 0.2 mM $1-^{14}C$ acetyl-CoA (4×10^5 cpm/ μ mole), 0.05 mM acetoacetyl-CoA, and dialyzed C-serum (0.5-2 mg protein/ml) in a final volume of 0.2 ml. (8,9). The reaction was initiated by adding ^{14}C acetyl-CoA to the preincubated reaction mixture (2 min at $30^\circ C$). At 2, 4, 6 and 8 minutes after ^{14}C acetyl-CoA addition, 40 μ l aliquots are transferred into glass vials containing 0.1 ml 6 M HCl. The acidified aliquots were taken to dryness at $95^\circ C$ using heating plate. Water (0.5 ml) is added to dissolve the dried nonvolatile material. Liquid scintillation cocktail, compatible with 10% final H_2O content, was then added. The labelled nonvolatile material, HMG-CoA or its dehydration product, was determined in liquid scintillation counter. Correction for and nonvolatile ^{14}C acetyl-CoA was determined using controls in which C-serum was omitted.

Gel electrophoresis :

Polyacrylamide gel electrophoresis (PAGE) was run either in the absence or presence of sodium dodecyl sulfate (SDS) using the method of Weber *et al.* (10). For silver staining of the proteins, the procedure of Wray *et al.* (11) was employed.

Protein determination :

The protein concentration was determined by the method of Lowry *et al.* (12).

Part B. Disease resistance screening of *Hevea* at tissue culture stage

Explant materials :

Young leaves, stem, and shoot were cut from young plantlet which have been raised by embryo culturing technique were used as source of explants. During the first (major flowering) season of *Hevea* (Aug-Oct), pistillate flowers located at the top of central axis and main lateral branches were collected. Male flower buds (3-3.5 mm) in which majority of the anthers are at uninucleate stage were excised and used for callusing in a differentiation media.

Medium preparation for *Hevea* tissue culture :

Modified MS medium with additions of 2,4-D, kinetin, and NAA (each at 1 mg/l), 4% coconut water, 7% sucrose and 0.6% agar was prepared as media. This was used as differentiation media for callusing the explant materials according to method described by Chen *et al.* (13).

Phytophthora botryosa culturing :

Phytophthora botryosa was obtained from Plant Disease Division, Songkla Rubber Research Center. The fungus was subcultured once every two weeks in medium containing (per liter) 0.5 gm KH_2PO_4 , 0.5 gm K_2HPO_4 , 0.5 gm $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.02 gm $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 gm thiamine, 20 gm glucose, 1.5 gm NaNO_3 and 15 gm agar.

Bioassay for possible antifungal growth compound in C-serum :

Seeded agar discs (5 mm diameter) were prepared by cutting with cork borer from seeded agar plate of two weeks old *phytophthora* culture. The disc was placed at the center of P-4 medium petri-dish. Fresh C-serum of clones RRIM 600, KRS 21 and GT 1 were filtered sterile. They were loaded on sterile filter paper disc (5 mm diameter) to study antifungal activity. Filter paper discs containing either sterile distilled water, or C-serum

(50 and 100 μ l) were placed in the center of each quarter with a distance of 2 cm away from the seeded agar disc. Three replications were made for each assay. After incubation at room temperature for 3 days, the discs were inspected and assessed for antifungal effect.

4. Results/Discussion/Tables :

Part A. Biochemical parameter for rubber yield evaluation in *Hevea brasiliensis*

A.1 Solubilization and purification of latex HMG-CoA reductase

Preparation of soluble HMG-CoA reductase : Various attempts to solubilize HMGR from the bottom fraction of fresh latex were carried out using different detergent compounds. Efforts to solubilize the enzyme with Triton x-100, Brij 35 and Tween 80 were unsatisfactory. However, we found that maximum activity can be obtained in soluble form by using polyethylene ether W-1, similar to those reported for etiolated radish seedlings by Bach et al. (6). After the solubilization process, all enzyme activity was present in the soluble or supernatant fraction. The enzyme activity remained stable in this solubilization medium.

Purification of solubilized reductase enzyme : The concentration and purification of HMGR was achieved by ammonium sulfate fractionation and affinity chromatography. Affi-Gel-Blue, a biospecific affinity for nucleotide requiring enzyme and agarose-hexane-HMG-CoA were used in the final affinity chromatography steps. Data for a typical preparation and purification are presented in Table I. The specific activity of the reductase enzyme solubilized from a freshly prepared bottom fraction ranges between 0.4 to 0.7 $\text{nmole min}^{-1} \text{mg}^{-1}$. Solubilization leads to a 1.78 fold purification so that the specific activity of the enzyme in the soluble supernatant after centrifugation is around 0.7 $\text{nmol min}^{-1} \text{mg}^{-1}$. Further purification was achieved by fractionating the supernatant with addition of saturated ammonium sulfate solution to final concentration of 40%. The uppermost yellowish enzyme protein layer was collected by centrifugation

at 25,000 g for 20 min. The ammonium sulfate fractionated enzyme resulted in about 5 fold purification. This was further purified by chromatography on DEAE-Sephadex A-50 Column. The elution profile obtained is shown in Fig.1. The reductase activity was eluted from the column with buffer B containing 0.5 M KCl. The pooled fraction contained approximately 85% of the added activity and gave 22 fold increase in specific activity. Further purification on Affi-Gel Blue (Fig.2.) and HMG-CoA-hexane agarose columns (Fig.3.) led to 77 and 257 fold of purification respectively. Further experiments for purified HMGR molecular weight determination and enzyme kinetics are in progress.

A.2 Variation of rubber productivity within individual *Hevea* clonal types

Good rubber clones grown in most plantation are resulted from a combination of root stock and bud grafting material. Generally, young buds of good yielding clones i.e. RRIM 600, GT 1, KRS 21 are grafted on young rootstock seedlings. The rootstock seedling are collected in random from rubber plantation or propagated through any available seeds. Therefore, rubber clonal type used in planting is referred by scion clonal type generated from bud grafting material (Fig.4). Very little studies were performed on compatibility of rootstock and scion. It was shown that scion of RRIM 600 will give better rubber yield if grafted on PB 5/51 rootstock.

Our recent studies on rubber productivity per tapping per tree indicated a wide range and high variation in yield (Fig.5). These differences may result from the variation of bud grafting material (scion) and/or rootstock. Rubber trees has a long productivity period ranging from 25-30 years. It is much desirable to start with a good yielding tree in order to gain better and higher output in the long run. The data shown in Fig.5 on rubber yield per tapping per tree were collected from 40 different trees of clonal types RRIM 600 and GT 1. The trees are at the same age and planted within the same

area. A wide range of yield per tapping per tree were observed within the same clonal type, from as low as 12 gm/tapping/tree to as high as 78 gm/tapping/tree in RRIM 600 clone. It was evidenced that the development of a reliable technique for yield evaluation at seedling stage will help to prevent the planting of potential poor yielding trees. Hence expected increase of total rubber yield per planting area can be achieved with reasonable confidence. Our proposed study on HMGR as yield screening at seedling stage might be used as a possible approach to achieve the goal.

A.3 Diurnal studies on HMGR activity

Two diurnal cycles were studied on latex HMGR and dry rubber content of clones RRIM 600, GT 1 and KRS 21 during Feb-March and May-June. Five trees per clonal type were used for the study. Average result on the first two diurnal cycles (Feb-March) and the second two diurnal cycles (May-June) were shown in Fig. 6-7 and 8-9, respectively. As depicted in Fig. 6 and 8, HMGR activities varied diurnally, the difference between high and low enzymic activity was about 3 fold. High specific activity of HMGR was observed between 1800-2200 h. (Fig. 6,8) while level of dry rubber content was also considerably high (Fig. 7,9). Corresponding relationship between HMGR specific activity and dry rubber content was observed during the dark period (1800-0600). Reverse relationship was also observed around mid-day period (between 1000-1400 h.).

There was a large proportion of positive corresponding correlation between HMGR activity and dry rubber content observed in the diurnal study. However it is still necessary to test for positive correlation between HMGR activity and rubber yield in latex of several other *Hevea* clonal types before a possible use of HMGR activity as yield parameter in clonal selection can be verified and evaluated.

A.4 Studies on correlation of HMGR activity and dry rubber yield in latex collected from various clonal types

The results in progress report no. 1 showed the correlation between HMGR activities and rubber contents in many different clones tested. In this report we make daily ranking on HMGR specific activity (Table 2), total rubber yield (Table 3), and total HMGR activity (Table 4) as well as rubber yield from latex of 10 different *Hevea* clonal types.

Table 2. showed different level of specific activity in bottom fraction isolated from 10 latex samples. The samples were collected from 15th - 20th min. after tapping on three different experimental dates. High HMGR specific activities were always observed with clones KRS 209, KRS 208, and KRS 240 while low HMGR specific activities were observed in clones KRS 223, GT 1 and KRS 247.

Dry rubber yield obtained from three collection dates in table 3 indicated high yield levels in clones KRS 233, RRIM 600 and KRS 208. Low yielding capacity was observed in clones KRS 223, GT 1 and KRS 247. Positive correlation between HMGR level and dry rubber yield were shown to exist in this study. The top 60% of high yielding clones as indicated in the table are KRS 226, KRS 233, RRIM 600, KRS 209, KRS 246 and KRS 208.

Similarly, if we want to select for top 60% of high yielding clones using level of HMGR specific activity as a screening parameter, clones KRS 226, KRS 233, RRIM 600, KRS 209, KRS 208, and KRS 240 should be included (Table 2, last column). The reliability on clonal choice selected by this method was approximately 80% when compared to the actual yield results shown in Table 3. Several factors accounted for the small portion of invalidity might include latex flow, latex clotting factors, as well as sample collection time.

The correlation between HMGR specific activity (Table 2) and rubber yield (Table 3) was calculated and shown to be statistically significant

($\alpha = 0.1$ and $r = 0.49$). However, more experiments needed to be carried out including improve method for data collections in order to establish the reliability of this finding.

Finally, we should evaluate whether the finding on the correlation between HMGR specific activity and rubber yield is still valid at pretapping or seedling stage of the rubber plant. A number of young rubber seedlings should be evaluated for rubber yield potential by using HMGR specific activity as a parameter. All seedlings should be tagged to indicate their yield potential and planted within the same area to study the actual tapped rubber yield at tapping age. Results obtained from this experimental design can then be used to verify the possible use of HMGR specific activity as a yield screening parameter at seedling stage.

Table 3 indicated the statistically significant ($\alpha = 0.01$, $r = 0.78$) on the correlation between total HMGR activity and dry rubber yield. The correlation obtained from total HMGR activity was more closely correlated to total dry rubber yield than HMGR specific activity. However, in term of its application, HMGR specific activity is more suitable since it can be measured in small amount of latex obtained from young rubber seedling by puncturing. The total HMGR activity can be measured only from tappable tree size only. Average results on the correlation studies between HMGR specific activity and total HMGR activity with dry rubber yield are depicted in Fig.10 and 11, respectively.

A.5 Latex mevalonate kinase activity and its diurnal variation

A procedure has been developed for the assay of MVA kinase activity in C-serum. The assay was carried out by incubating C-serum with ATP and mevalonate. The reaction was terminated with trichloroacetic acid and precipitated proteins removed by centrifugation. After neutralization, ADP

in the supernatant was determined by coupling to enzyme catalysed oxidation of NADH as described under the methods. Trichloroacetic acid was found to be suitable deproteinizing agent as auto-hydrolysis of ATP was not detected during the course of assay. An important point should be noted that neutralization of the deproteinized supernatant must be carefully done because alkalization leads to ATP hydrolysis.

Diurnal variation in MVA kinase activity was investigated in latex collected from rubber clones, RRIM 600, GT 1 and KRS 21. Fig.12 showed the variation of MVA kinase activities in C-serum obtained at different time during the 24 hour cycle. Each point was the average of two values obtained from separate tappings, 14 days apart. The MVA kinase activity of each clone tended to vary during the 24 hour cycle, being high between 1000-1400 and low between 1800-2200 h (Fig.12). The difference between level of high and low activity is about 1.8 fold. Dry rubber contents in latex samples used for MVA kinase assay also varied diurnally (Fig 7). High and low dry rubber content was observed between 14.00 - 18.00 h and 02.00 - 06.00 h, respectively. The high and low level of MVA kinase did not correspond with those of dry rubber contents in this study. There seemed to be no positive correlation as compared to the HMGR studies.

A.6 Latex HMG-CoA synthetase

As stated in the first report that the attempt to employ spectrophotometric method for HMG-CoA synthetase assay was not suitable for the *Hevea* enzyme. An effective radiochemical assay was found suitable for *Hevea* HMG-CoA synthetase by modifying the method of Clinkenbead et al. (9).

The HMG-CoA synthetase activity in C-serum from clone RRIM 600 rubber latex was assayed as described under materials and methods. Specific activity of the enzyme ranged from 4.3-5.3 nmole/min. mg protein. The enzyme was quite

stable upon deep-frozen. Almost 95% of HMG-CoA synthetase activity was remained after one week of storage at -70°C .

Levels of HMG-CoA synthetase activity in C-serum were studied on 3 *Hevea* clonal types. The specific activities of RRIM 600, KRS 21 and GT 1 were 5.3, 3.9 and 4.5 nmole/min.mg protein respectively. The small difference among there 3 clonal types seemed to indicate that there was little variation on this enzyme among the rubber clones as compared to the study on HMGR.

Part B. Disease resistance screening of *Hevea* at tissue culture stage.

B.1 Callus intiation from *Hevea* male flowers

In last report we were not able to generate callus from male flower of clone RRIM 600. Our attempt on pre-cold treatment at 15°C for 24 hour on the male flower of RRIM 600 resulted in sucessful initiation of callus. Fig 13,14 and 15 were calli derived from anther of RRIM 600 at the age of 2, 2½ and 3 months old respectivity. It was clearly evidenced that the explants selected for culturing did not response to the culture media in the same manner. More than 50% of the explants, showed good callus growth. It was interesting to note that callus in fig 13 is yellowish in color while yellowish-green and brownish calli are observed in Fig 14 and 15. This might result from callus age diferences and/or source of explants used for culturing.

Figure 16 showed calli derived from male flower of PR 255 at 4 months old. The callus surface look wet and shinier than those obtained from RRIM 600 clonal type.

Fig 17 is half year old callus derived from male flower of RRIC 6 clonal type after subculturing into the same media at 3 months old. Most cells in the center of the callus mass became brownish but growth were still observable, especially on the outer layer surface.

Experiments on callus growth rate upon subculturing of calli obtained from RRIM 600 and PR 255 are underway.

Our attempts to initiate callus growth from young leaves, stem or shoot were not successful. We are in the process of finding ways to improve methods for callusing of these explants.

B. 2 Studies on antifungal compound in C-serum

The C-serum used for bioassay of antifungal activity were obtained from normal healthy rubber trees during a non infectious season. Apparently, similar growth rate on mycelium proliferation toward C-serum discs of different clonal types was observed (Fig. 18). It was thus evidenced that under normal condition antifungal compound against phytophthora growth was not present, especially in the resistant clone, GT 1. Lesion studies with live pathogen infection on leaves as well as toxin and phytoalexin isolation are planned for our future work. This aspect of study will be conducted concurrently with the study on high-yielding clone selection using enzyme as parameter. The ultimate goal will be to select for the high-yielding disease resistant rubber clones.

5. Conclusion remarks :

- Purified HMGR was obtained by methods described above.
- Supportive evidence on potential use of HMGR parameter as yield index came from our following studies :
 - a) Diurnal variation of HMGR and MVA kinase activities in relation to latex dry rubber content.
 - b) Correlation of HMGR activity and dry rubber yield in latex collected from various clonal types.
- Method for radiochemical assay of HMG-CoA synthetase was established.
- Callus could be initiated from male flower of clone RRIM 600.
- Antifungal compound in latex was not detected from healthy rubber trees.

6. Work plan for next period :

- We plan to perform diurnal studies on HMG-CoA synthetase during next period.
- Immunological studies on purified HMGR will be carried out.
- Lesion studies with live pathogen infection on *Hevea* leaves as well as toxin and phytoalexin isolations are planned for next period.

7. References :

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8. Annex/Picture :

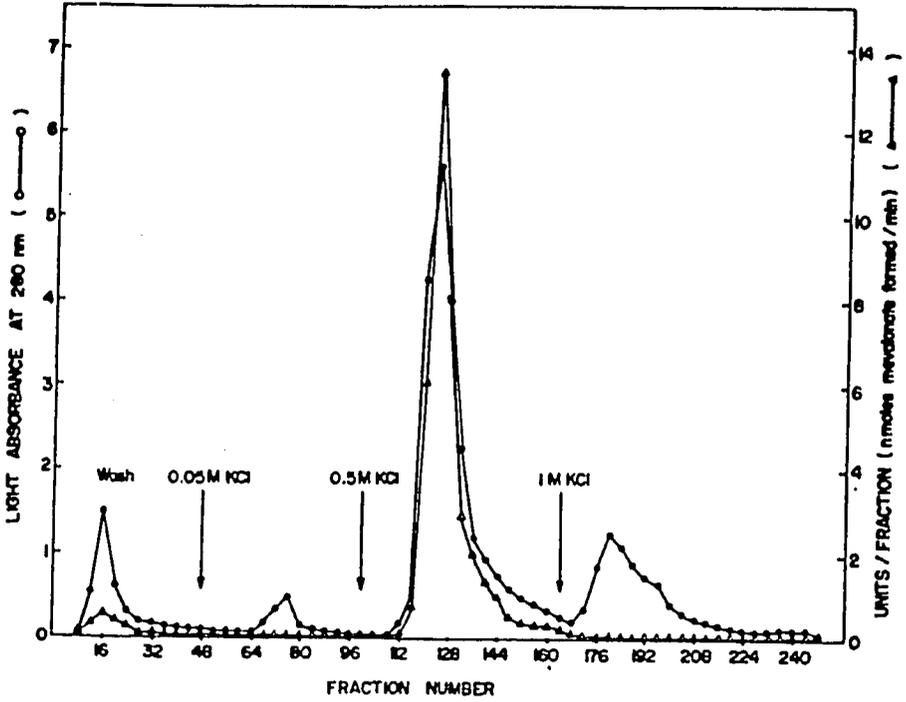


Fig.1. Purification of solubilized *Hevea* HMG-CoA reductase from DEAE-Sephadex A 50. Position of arrows indicating changes of salt concentrations.

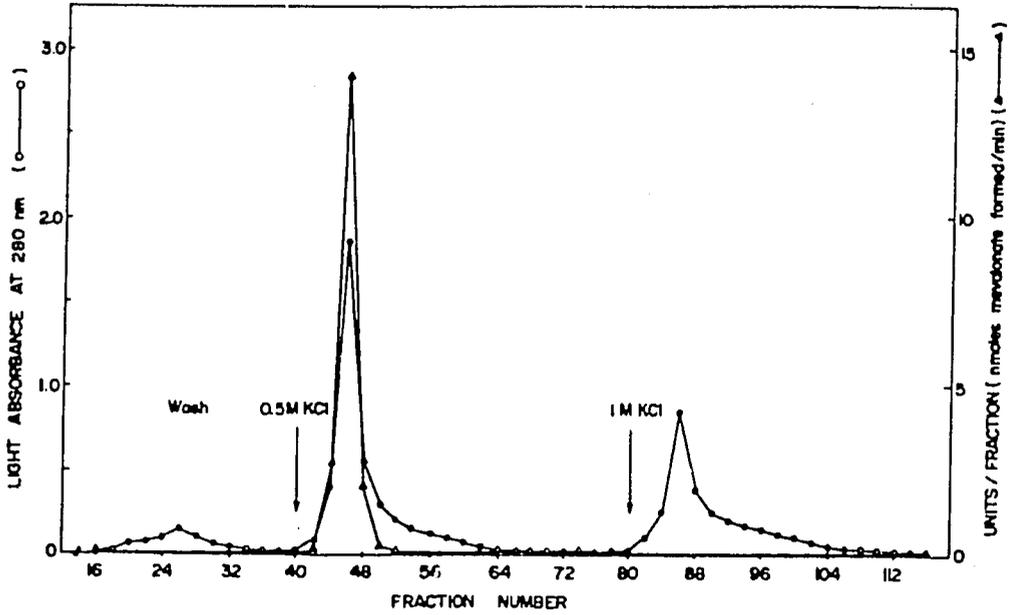


Fig.2. Purification of active fractions obtained from the DEAE-Sephadex column on Affi-Gel Blue column. Position of arrows indicating changes of salt concentrations.

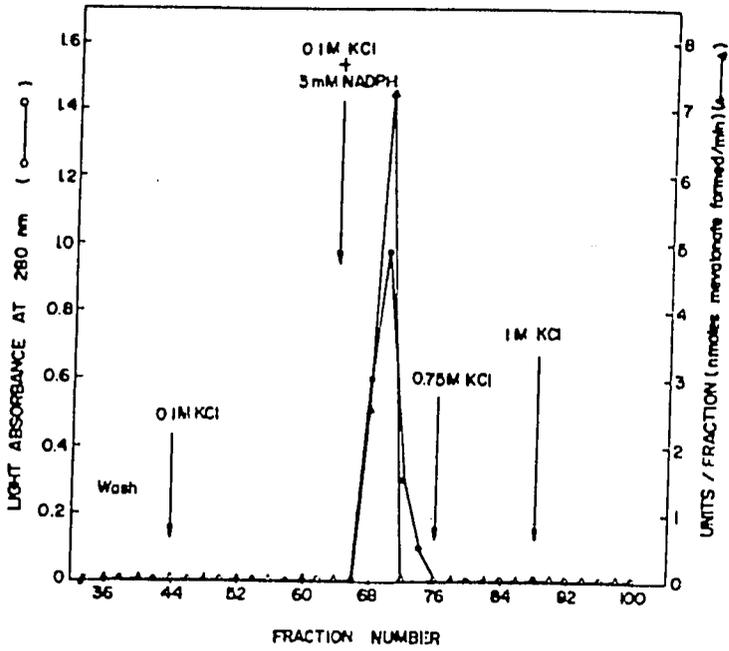


Fig.3. Purification of *Hevea* HMG-CoA reductase on affinity chromatography column using HMG-CoA-hexane-agarose. Arrows indicate changes of salt concentrations with or without NADPH addition.

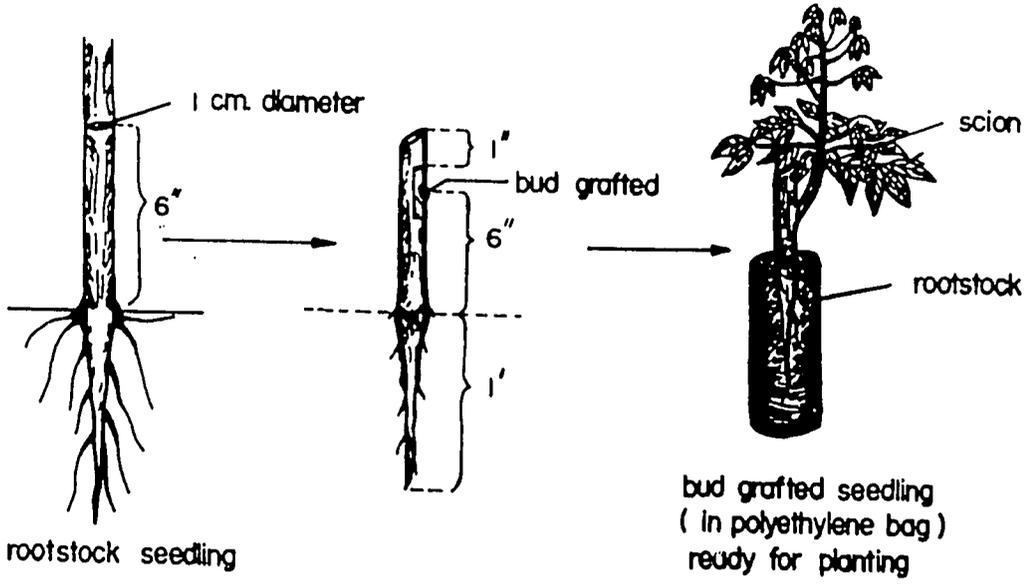


Fig.4. Bud grafting on rootstock seedling of *Hevea brasiliensis*.

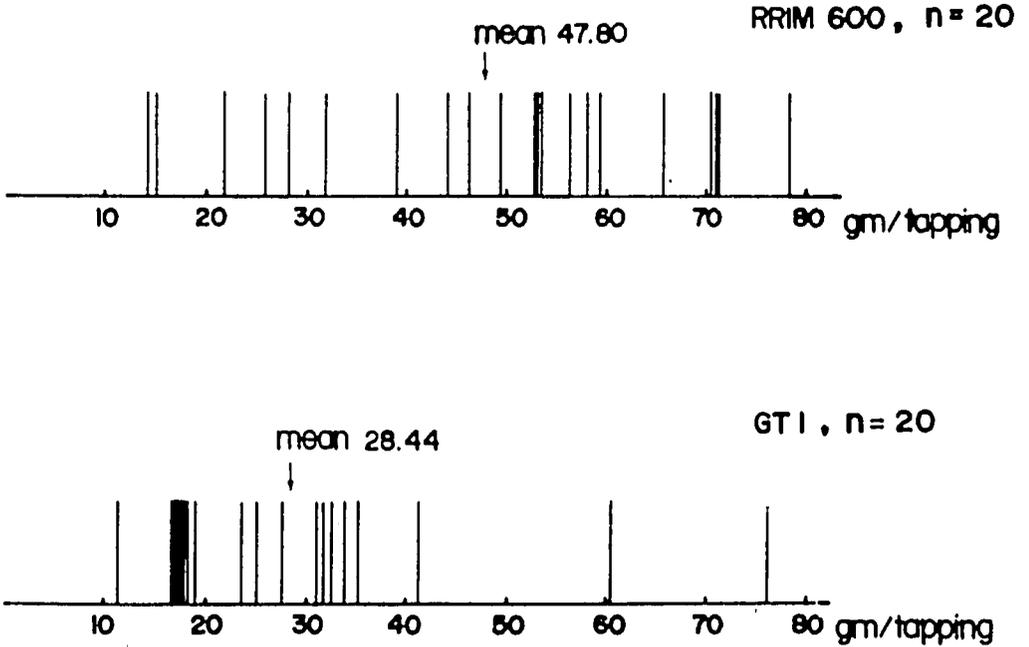


Fig.5. Variations on rubber yield per tapping. Each perpendicular line represents rubber yield per tapping of different trees of the same clones (RRIM 600 and GT 1).

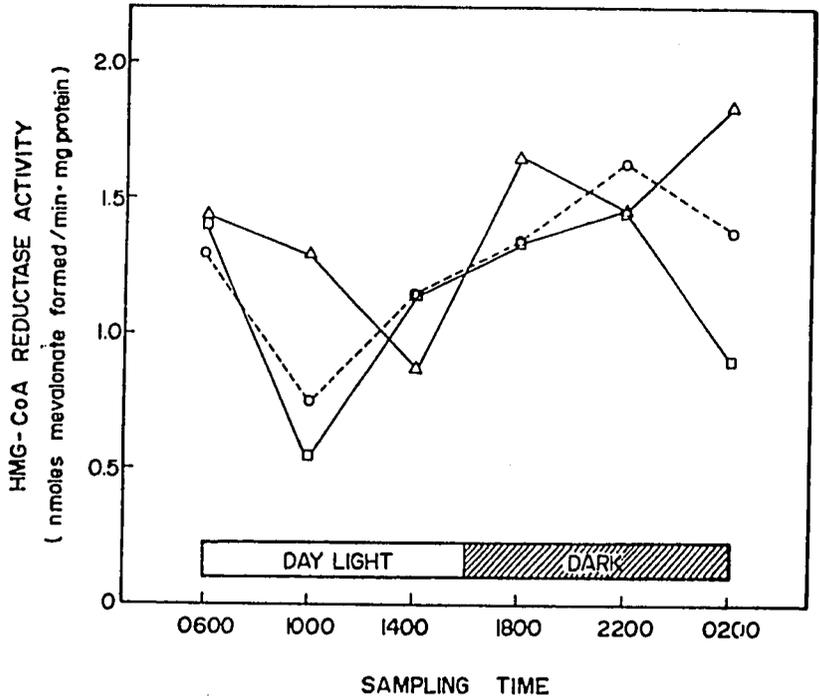


Fig.6. Diurnal variation of HMG-CoA reductase activity in *Hevea* latex clones RRIM 600 (□—□), KRS 21 (△ △) and GT 1 (○---○). Each point was the average of two values obtained from different tappings.

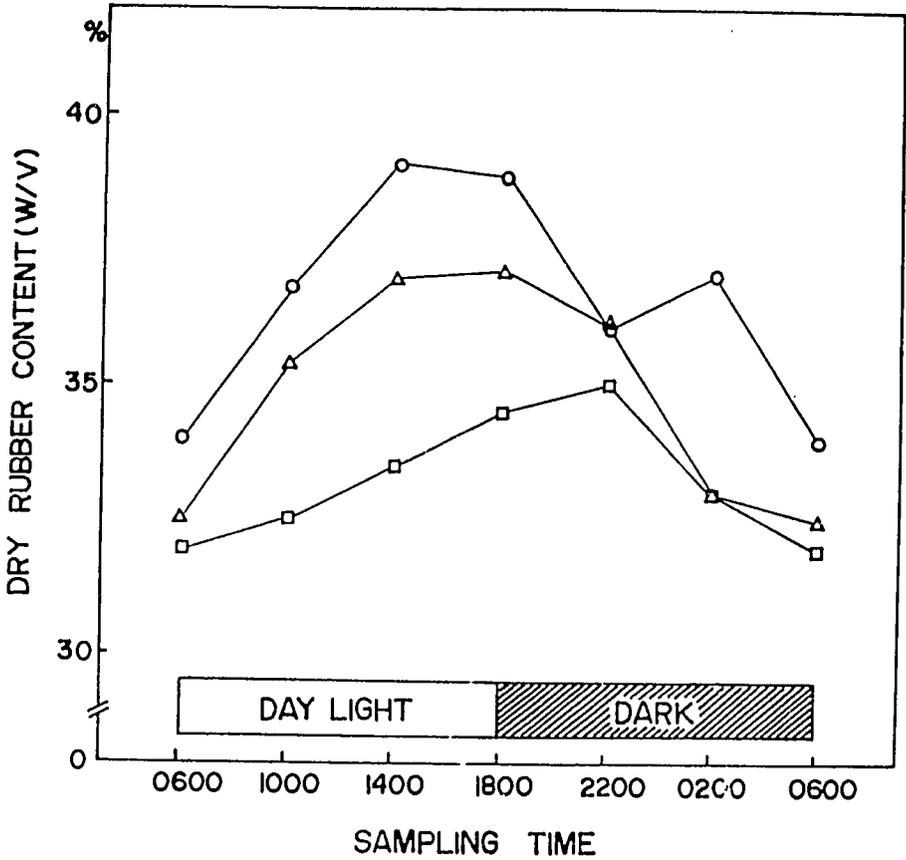


Fig.7. Diurnal variation of HMG-CoA reductase activity in *Hevea* latex clones RRIM 600 (\square — \square), KRS 21 (Δ — Δ) and GT 1 (\circ — \circ). Each point was the average of two values obtained from different tappings.

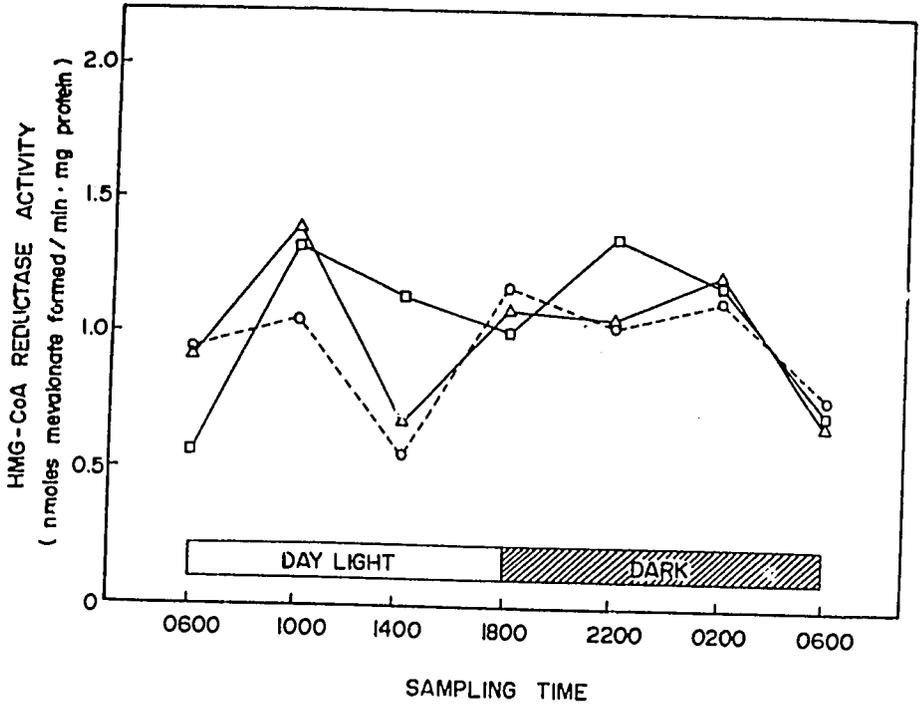


Fig.8. Diurnal variation of percentage of rubber content (w/v) in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT-1 (○---○). Each point was the average of two values obtained from different tappings.

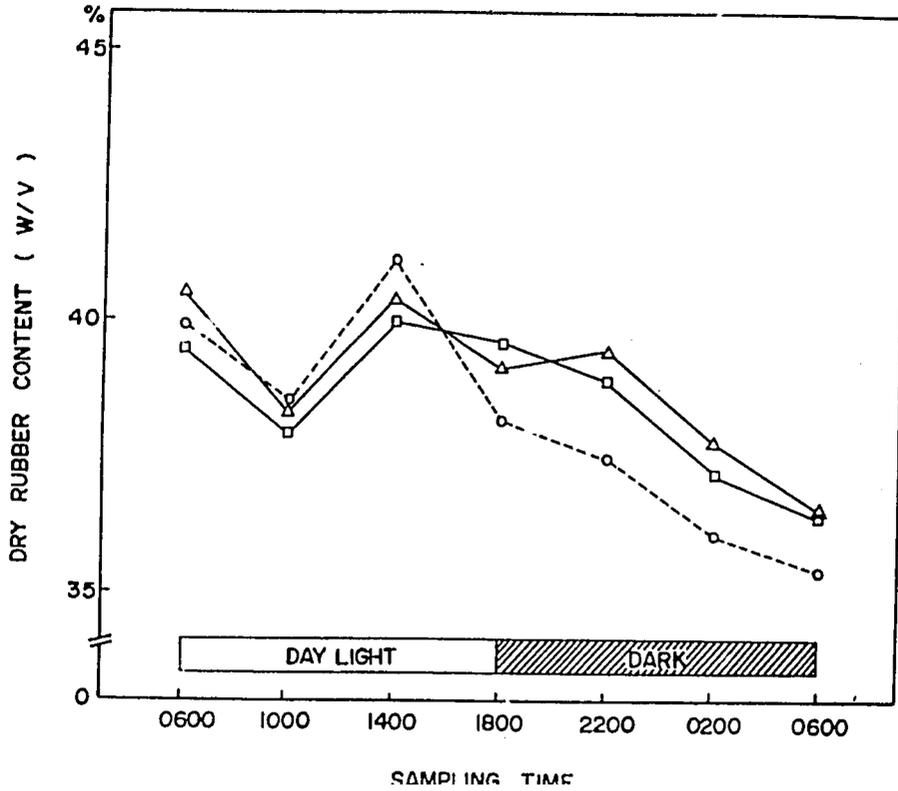


Fig.9. Diurnal variation of percentage of rubber content (w/v) in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○---○). Each point was the average of two values obtained from different tappings

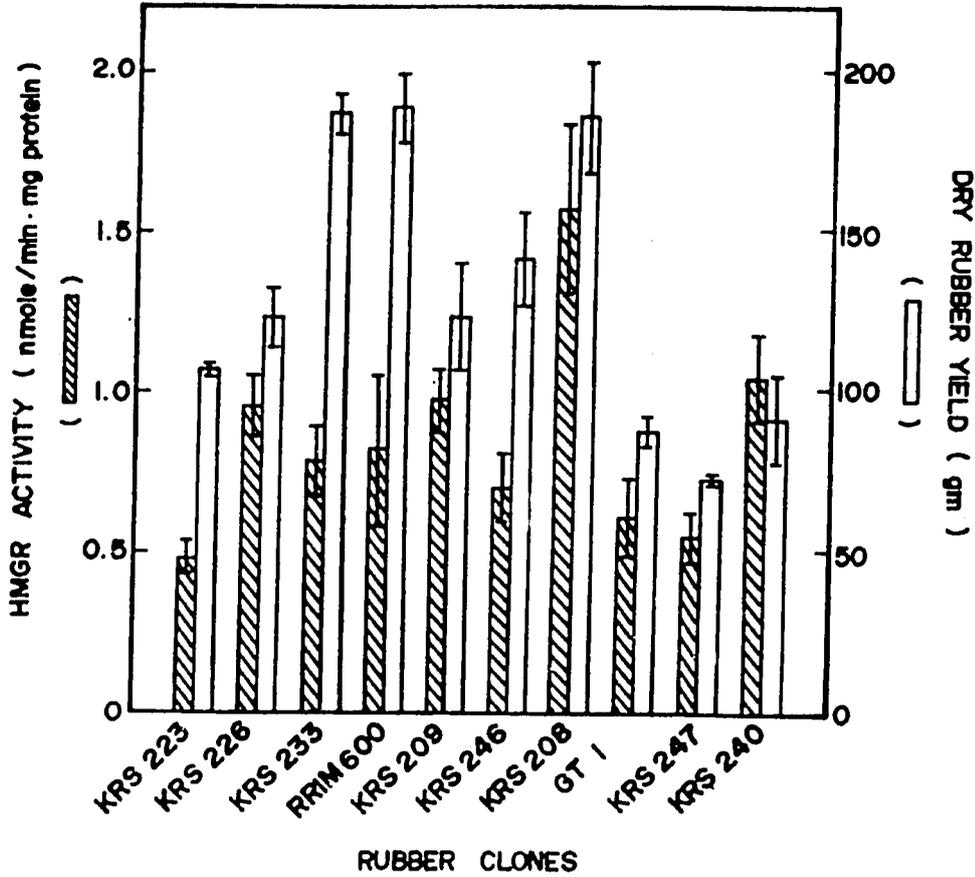


Fig.10. Correlation of latex HMG-CoA reductase specific activity () and dry rubber yield () among ten rubber clones. Each value represents the mean \pm S.E. of results obtained from 3 experiments. Three trees were used per *Hevea* clonal type.

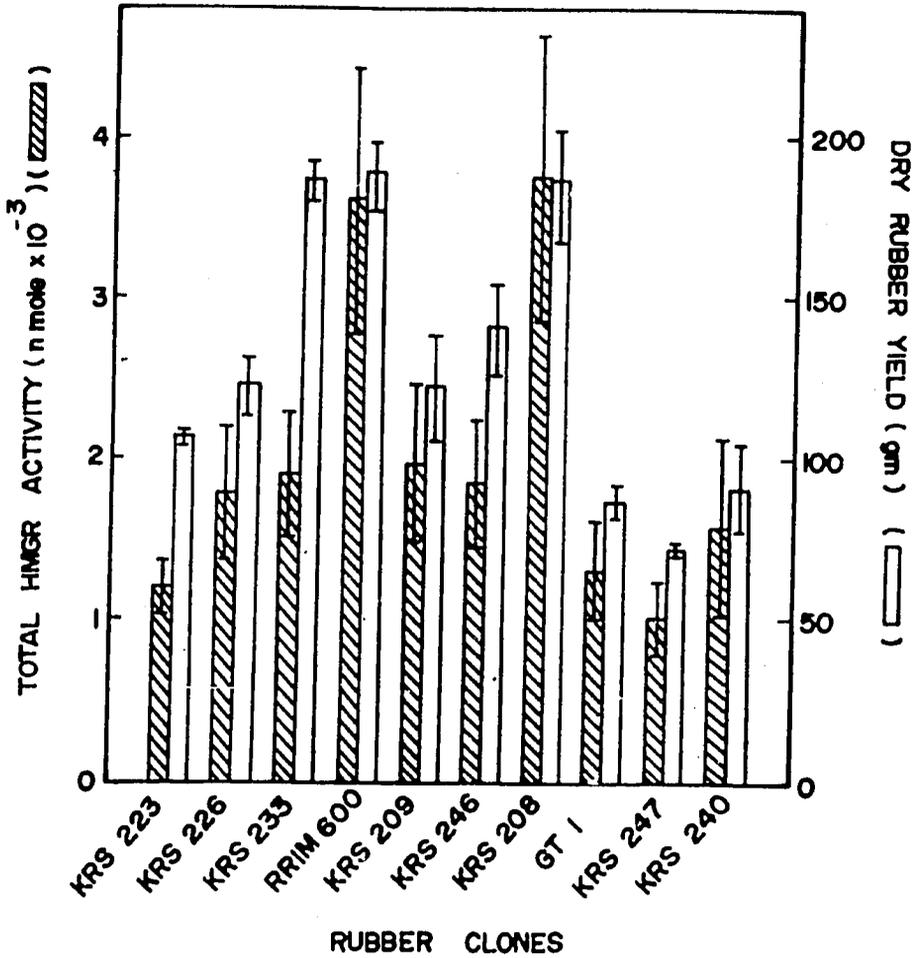


Fig.11. Correlation of latex HMG-CoA reductase activity (hatched bars) and dry rubber yield (white bars) among ten rubber clones. Each value is the mean \pm S.E. of results obtained from 3 experiments. Three trees were used per *Hevea* clonal type.

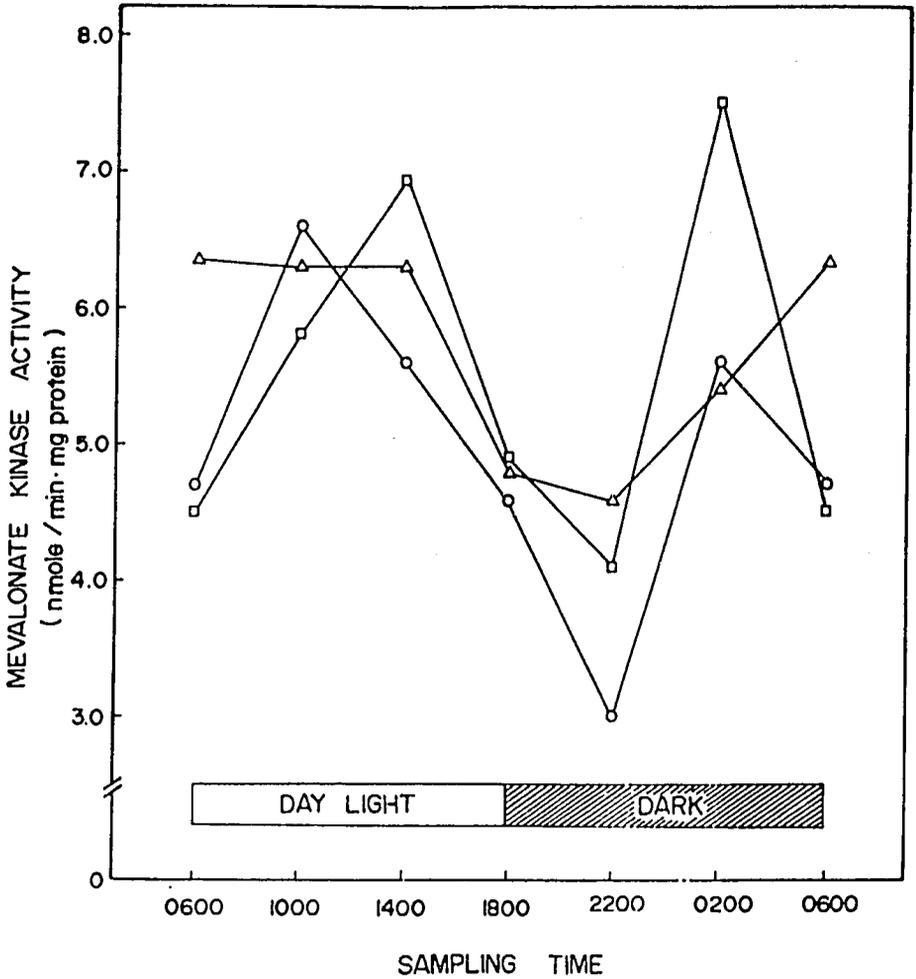


Fig.12. Diurnal variation of mevalonate kinase activity in *Hevea* latex clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○). Each point was the average of two values obtained from different tappings.

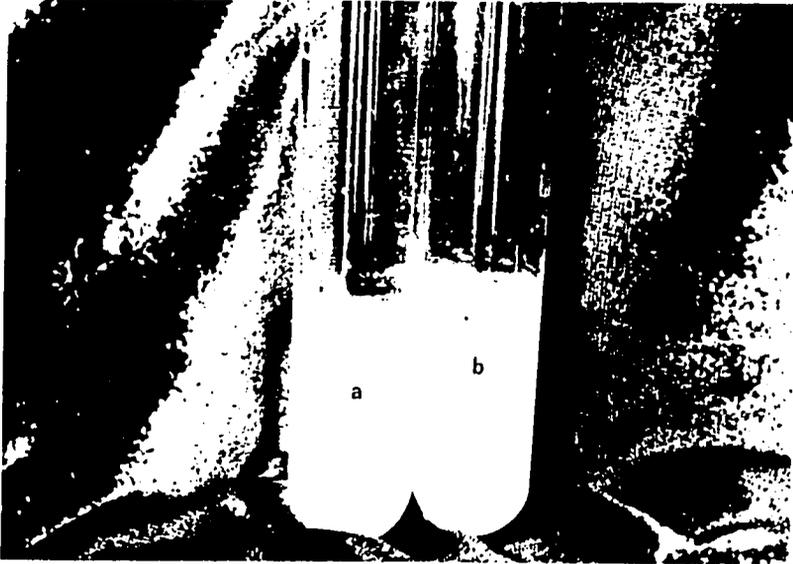


Fig.13 a,b. Callus initiation from male flower (anther) of clone RRJM 600 at the age of 2 months old.



Fig.14 a,b. Callus derived of RRIM 600 anther at 2½ months old



Fig.15 a,b. Callus mass from *Hevea* anther culture of clone RRIM 600 at 3 months old.

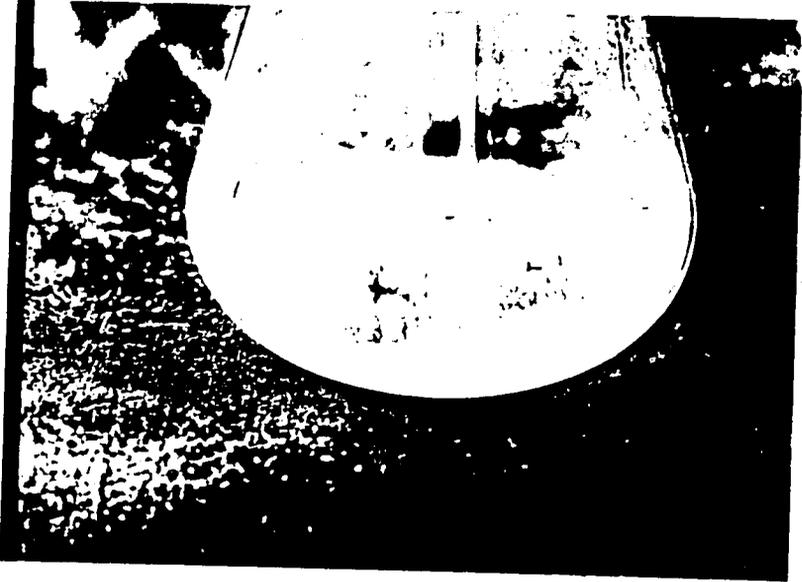


Fig.16. Callus derived from *Hevea* male flower of PR 255 at the age a 4 months old.



Fig.17. Callus mass from male flower of *Hevea* clone RR1C 6 at the age of six months old.

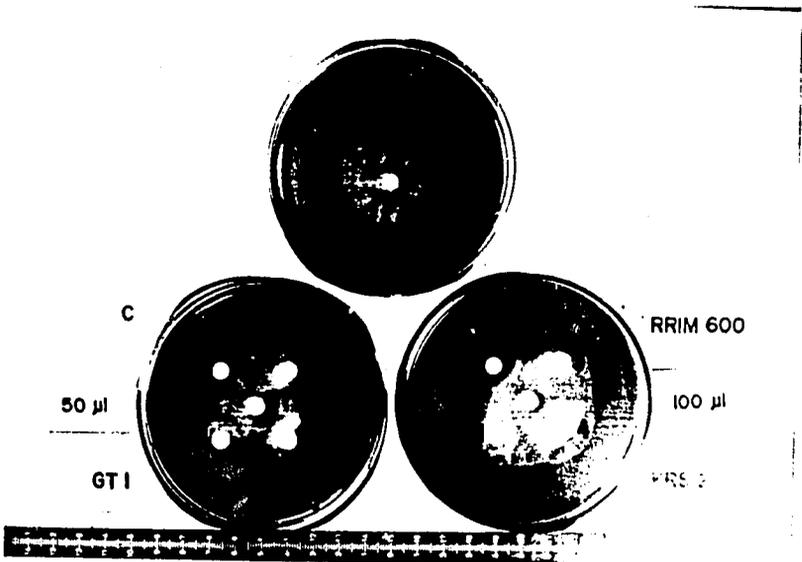


Fig.18. Bioassay of antifungal activity in latex C-serum obtained from clones RRIM 600, KRS 21 and GT 1. Top plate : phytophthora growth after 3 days of inoculation. Lower plates : four radial discs on each plate were loaded with C-serum from GT 1(1), water(2), RRIM 600(3) and KRS 21(4) respectively. Amount of samples applied on each disc was 50 μ l on the left and 100 μ l on the right disc respectively.

Table 1
Purification protocol of *Hevea* HMG-CoA reductase.

Fraction/step	Total volume (ml)	Total protein (mg)	Total activity (nmoles MVA formed /min)	Specific activity (nmoles MVA formed /min.mg)	yield (%)	purification (fold)
Bottom fraction in Buffer B						
+ Brij w-1 (1.1)	80	848	347.7	0.41	100	1
+ Glycerol (20%)	100	866.7	449.5	0.52	129.3	1.3
190,000 x g supernatant	95	570	404.7	0.71	116.4	1.78
0-40% (NH ₄) ₂ SO ₄	20.5	136.4	267.4	1.96	76.9	4.8
DEAE-Sephadex	109	24.8	227.4	9.19	65.4	22.4
0-90% (NH ₄) ₂ SO ₄	6	7.3	88.6	12.13	25.5	29.6
Affi-Gel Blue	10	1.5	47.3	31.95	13.6	77.9
HMG-CoA-hexane-agarose	4	0.37	39.1	105.65	11.3	257.7

Table 2

Specific activity of HMG-CoA reductase (HMGR) in bottom fraction of latex from 10 different *Hevea* clonal types

<i>Hevea</i> clones	Exp #1 (14 Oct. 86)		Exp #2 (17 Oct. 86)		Exp #3 (30 Oct. 86)		Ranking accumulation from Exp # 1-3			Top 60% with high HMGR activity
	⁺ HMGR activity	ranking	⁺ HMGR activity	ranking	⁺ HMGR activity	ranking				
KRS 223	0.585	10	0.344	10	0.51	7	10	10	7	
KRS 226	1.151	5	0.718	4	1.002	2	5	4	2	*
KRS 233	1.036	7	0.602	5	0.702	5	7	5	5	*
RRIM 600	1.386	3	0.579	6	0.492	8	3	6	8	*
KRS 209	1.212	4	0.830	3	0.892	3	4	3	3	*
KRS 246	1.007	6	0.525	7	0.566	6	6	7	6	
KRS 208	1.728	1	0.915	1	2.057	1	1	1	1	*
GT 1	0.896	8	0.498	9	0.413	0	8	9	10	
KRS 247	0.698	9	0.516	8	0.423	9	9	8	9	
KRS 240	1.377	2	0.892	2	0.850	4	2	2	4	*

⁺HMGR activity unit was defined as n mole/min. mg protein

Three rubber trees of each clone were used in the experiments.

Table 3

Total rubber yield obtained from latex of 10 different *Hevea* clonal types

<i>Hevea</i> clones	Exp #1 (26 Dec. 86)		Exp #2 (7 Jan. 87)		Exp #3 (24 Feb. 87)		Ranking accumulation			Top 60% with high rubber yield
	Dry rubber yield ing (gm)	Rank- ing	Dry rubber yield ing (gm)	Rank- ing	Dry rubber yield ing (gm)	Rank- ing	From Exp # 1-3			
KRS 223	104	8	113	5	104	5	8	5	5	
KRS 226	133	6	139	4	99	6	6	4	6	*
KRS 233	188	3	174	2	200	1	3	2	1	*
RRIM 600	203	2	202	1	163	3	2	1	2	*
KRS 209	164	5	113	5	92	7	5	5	7	*
KRS 246	172	4	111	7	142	4	4	7	4	*
KRS 208	228	1	167	3	163	2	1	3	2	*
GT 1	91	9	91	8	79	9	9	8	9	
KRS 247	71	10	73	9	73	10	10	9	10	
KRS 240	125	7	66	10	80	8	7	10	8	

Table 4

Studies on HMGR Activity and its relation to rubber yield in latex of 10 different *Hevea* clonal types

Hevea clones	Dry Rubber yield (gm)	Exp #1 (26 Dec. 86)			Exp #2 (7 Jan. 87)				Exp #3 (24 Feb. 87)			
		Total HMGR activity	Ranking		Dry rubber yield	Total HMGR activity	Ranking		Dry rubber yield (gm)	Total HMGR activity	Ranking	
			Dry rubber yield (gm)	HMGR activity			Dry rubber yield (gm)	HMGR activity			Dry rubber yield (gm)	HMGR activity
KRS 223	104	1199	8	9	113	1616	8	6	104	779	5	6
KRS 226	133	2020	6	7	139	2568	4	4	99	759	6	7
KRS 233	188	2090	3	6	174	2231	2	2	200	1422	1	3
RRIM 600	203	4836	2	2	202	4411	1	1	163	1589	3	2
KRS 209	164	2957	5	5	113	2080	5	5	92	873	7	5
KRS 246	172	2995	4	4	111	1646	6	7	142	900	4	4
KRS 208	228	5945	1	1	167	3197	2	3	163	2170	2	1
GT 1	91	1761	9	8	91	1627	7	8	79	482	9	10
KRS 247	71	1173	10	10	73	1096	9	9	73	745	10	8
KRS 240	125	3009	7	3	66	997	10	10	80	737	8	9