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FINAL REPORT

PROGRAM IN SCIENCE AND TECHNOLOGY COOPERATION (PSTC)

PROPOSAL 6.400

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

GRANT NO: 936-5542-G-00-6054-00

Submitted by

Dr. Rapepun Wititsuwannakul

Principal Investigator

Department of Biochemistry

Faculty of Science

Prince of Songkla University

Hat-Yai, 90112, Thailand.

October 15, 1990

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Project Profile

Country:	Thailand
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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:	Department of Biochemistry Faculty of Science Prince of Songkla University Hat-Yai 90112, Thailand
Co-Investigators:	Dr.Wallie Suvachitanont, Dr.Rapiporn Sotthibandhu, Dr.Dhirayos Wititsuwannakul, Mr.Apichai Shuprisha, Mr.Wisut Sukonrat, Dr.Nunta Churngchow
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ABSTRACT

Biochemical parameters for rubber yield evaluation in *Hevea brasiliensis* were investigated. Diurnal variations of rubber biosynthetic enzymes and rubber contents indicated the important role of HMG CoA reductase (HMGR) as regulatory enzyme parameter. Correlation between HMGR and rubber contents was investigated in both latex and leaf studies and found to be highly positive. Intraclonal and interclonal correlation studies also indicated highly significant positive correlation. These results pointed out important role and practical use of HMGR as a biochemical parameter/marker for rubber yield evaluation.

The positive correlation between HMGR level and rubber yield was studied and also found at seedling stage. The influence of rootstock on the enzyme level and rubber yield was observed and characterized in the young seedlings. The findings indicated the potential use and application of HMGR as enzyme marker for early selection of potentially high-yielding clone. The potential high yielders could be screened and selected from either a single clone seedlings or grafted seedlings population. Mass screening of 536 Brazilian clones using HMGR parameter resulted in the identification of ten high-yielding clones which were more superior to the current high yielder (RRIM 600).

The enzyme HMGR was purified to homogeneity and characterized as in the published report (17). Basic study on biochemical regulation of HMGR was also carried out and the results was published (21). Development of ELISA for HMGR was attempted but the antibody titer was not high enough to be of practical use at the moment. Calmodulin in the latex C-serum was also found to be positively correlated to rubber yield (21) and could prove to be of practical use in the near future.

Preliminary study on disease resistance screening of *Hevea* at tissue culture stage was investigated. Callus could be initiated from male flowers as well as inner seed integument of several *Hevea* clonal types. Bioassay of leaf and cullus infected with live pathogen (*Phytophthora botryosa*) indicated GT-1 to be of the resistant clone. RRIM 600 was found susceptible to Phytophthora while GT-1 was much more resistant to the pathogen. Further study is being carried out in the subsequent investigation.

IMPACT

The HMGR marker has high potential application to selection of high yielding grafted seedlings and mass screening for the high yielders at early (seedling) stage. It is, however, needed to be proved at a plantation level. Recently, the Bangkok Rubber Land & Engineering Co. Ltd. agreed to grant us financial support and land facility for the long-term follow up at a plantation scale. It is hoped that in the near future we can produce certified high-yielding grafted seedlings for distribution among 800,000 small rubber farmers in Thailand and the nearby rubber growing countries.

KEYWORDS

Rubber (*Hevea brasiliensis*), Rubber yield, Enzyme marker, HMG CoA Reductase, Calmodulin, *Phytophthora botryosa*

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1. BACKGROUND AND INTRODUCTION

Part A. Biochemical Parameters for Rubber Yield Evaluation in *Hevea brasiliensis*.

Good rubber clones grown in most plantations are the results of a combination of rootstock and bud grafting materials. A wide range and broad variations in yield on productivity (per tapping per tree) are persistently observed (1,2). Traditional practice on yield evaluation of rubber clone is to grow the plant for at least six years until the mature trees can be tapped and yield subsequently evaluated. Various factors being proposed as related to rubber biosynthesis capacity (and hence rubber yield) were postulated and studied in various aspects (2-8).

Early attempt on yield evaluation by 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-^{14}C 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.

When enzymic activities involved in rubber synthesis from acetate were measured by F. Lynen in 1969 (5), it was found that the relative activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) was lowest when compared to other enzymes in the pathway. It is commonly observed that the rate-limiting enzyme in a biosynthesis pathway is always low and exists in an inactive form when it is not actively involved in the synthetic processes. These enzymes will be turned on or activated by certain mechanisms to become an active form when the need for synthesis arises. Furthermore, F. Lynen experiment on rubber synthesis by using HMG-CoA as starting precursor and suggested that the rate is controlled by (the rate of) mevalonate formation (6).

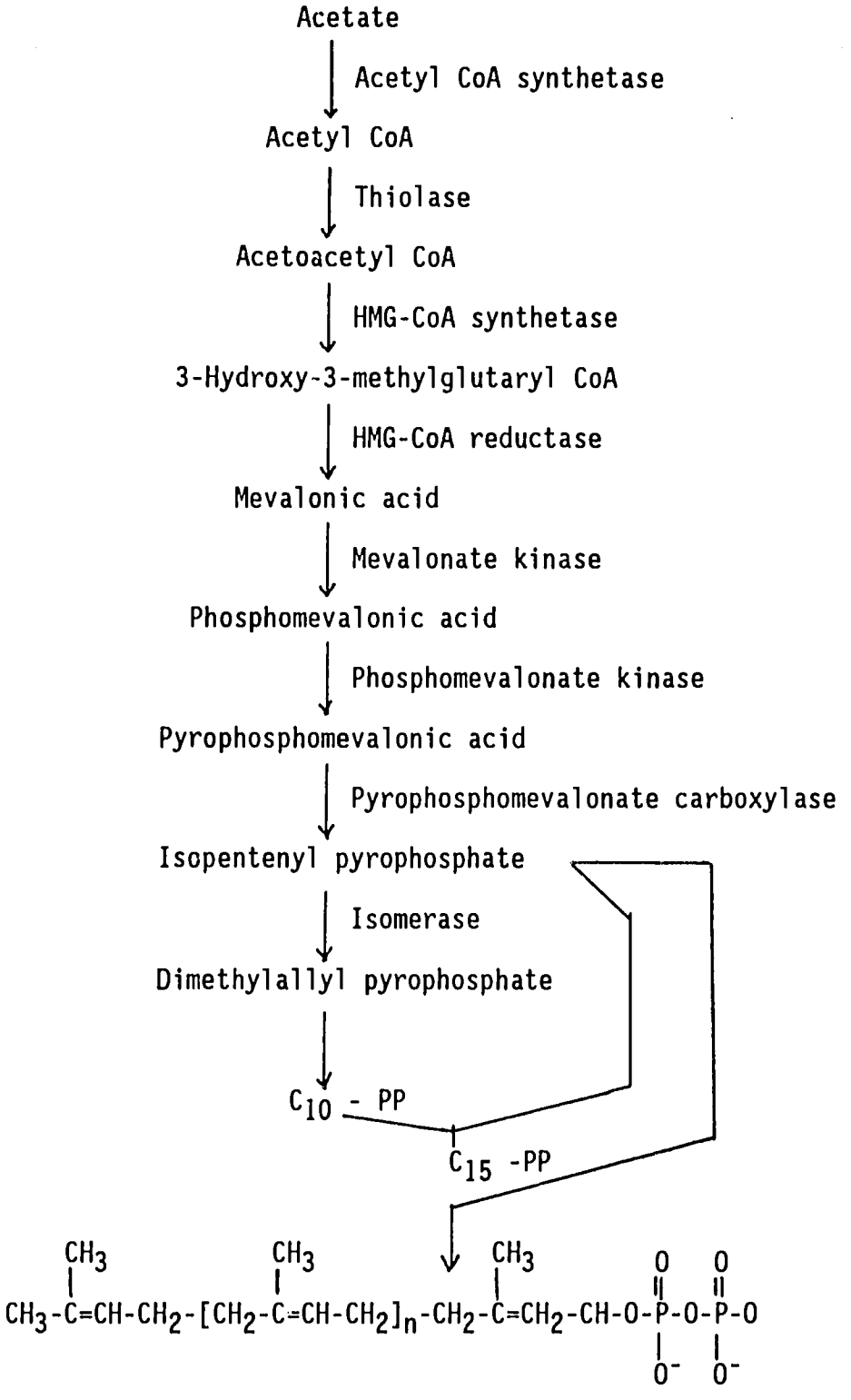
The diurnal variation study carried out by us showed a strong positive correlation between the activity of HMGR in latex and the dry rubber content (3). Several other biochemical and physicochemical parameters were also analyzed and shown to express correlation with rubber yield (i.e. total solid content, reduced thiols and C-serum pH in latex. These parameters were suggested for possible use in the early selection of new rubber clones (8).

This report suggests a possible use of HMGR activity level in latex as a simple and reliable biochemical parameter for rubber yield potential evaluation.

Part B. Disease Resistance Screening of *Hevea* at Tissue Culture Stage

Since *Hevea brasiliensis* is a perennial tree 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 .po6 rubber production failure due to disease or disturbance by the pathogens. We

proposed a model system to study the disease resistance at tissue cultur level in this study as an early selection in conjunction with high-yieldin potentials.



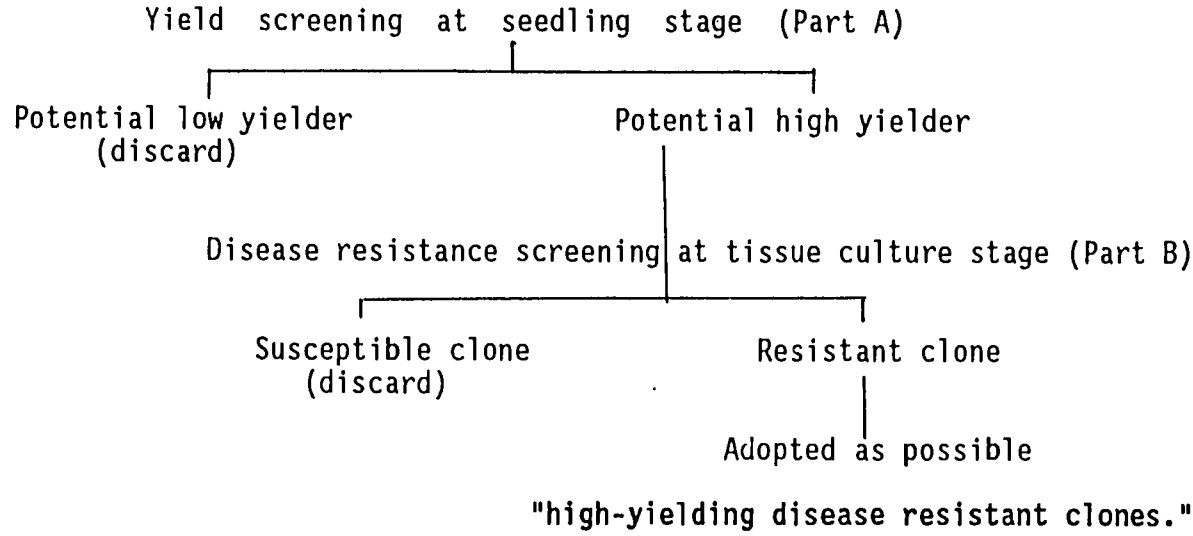
[cis 1,4 polyisoprene $(C_5H_8)_n$]

Rubber Biosynthesis Pathway Starting from Acetate (5)

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. This is in conjunction to the high-yielding potential screening as outlined below.



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, mevalonolactone, acetyl-CoA, acetoacetyl-CoA, ATP, polyoxyethylene ether W-1 (Brij W-1), mevalonolactone, chicken egg albumin, carbonic anhydrase, urease (trimer, hexamer) and bovine serum albumin (monomer, dimer) were from Sigma. DL-2-^{3}H mevalonate, 1-^{14}C acetyl-CoA, DL-3^{14}C HMG-CoA were from Amersham, England. Affi-Gel-Blue was from Bio-Rad. Agarose-hexane-HMG-CoA was from P-L Biochemicals.

Collection and Fractionation of Latex from Mature Rubber Tree

Latex was obtained from several clones of *Hevea brasiliensis* as indicated in the text. The trees are regularly tapped (half spirally) and latex collected every other day (S.2/D.2). All clones are grown and 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 directly into ice-chilled beakers. The latex from each clone was pooled and fractionated into 3 major fractions by centrifugation. Fraction was either in

a microfuge at 15,000 rpm for 60 min or in ultracentrifuge at 49,000 g for 45 min. They are fractionated into rubber fraction, the C-serum (latex cytoplasm), and the bottom fractions, respectively (Fig.1). The C-serum was separated and used for assays of HMG-CoA synthetase, MVA kinase, and HMGR activator. The bottom fractions, where 95% of HMGR was located (9,10), were pooled and used for assay of HMGR activity as well as for further purification.

Collection of Latex from Young Grafted Seedling

The sharp end of syringe was used for tapping young rubber seedling stem at a site approximately 5-6 inches above the grafted area. The young buddings of new *Hevea* germplasm were similarly tapped after 8 months of grafting on a 4 year-old stock. After tapping, the exuded latex was immediately pipetted into the assay buffer. The tapping time is around 0600 h. or otherwise indicated.

Collection of Leaf Samples for Enzymes and Rubber Yield Analysis

Young rubber seedlings approximately 6 month-old of various *Hevea* clonal types were illuminated (6,000 lux for 12 hours, 0600-1800 h) daily in an experimental control room. The plants were allowed to adapt to the new environment for at least one month before use in various experiments. After the adaptation period, newly mature leaves were detached from the young rubber plants and used for determinations of HMGR, MVA kinase, HMG-CoA synthetase, petiole rubber value, and dry rubber content.

Preparation of Leaf Cell-free Extract

The middle leaflet of *Hevea* compound leaf was detached at experimental time indicated from young rubber seedlings kept in the experimental control room. A 2 gm sample of freshly detached leaves were washed with distilled water, sliced into small pieces and quick frozen at - 70°C for 5 min. The sample was then ground in 2 ml of grinding solution (50 mM Tris-HCl buffer pH 7) containing 0.2 gm of polyvinyl porrolidone and sand. The homogenate was then squeezed through miracloth and the filtrate was centrifuged at 3,000 g for 30 min at 4°C. The supernatant was then centrifuged at 30,000 g for 20 min at 4°C. The resulting 3 K supernatant (leaf cell-free extract) was used for HMGR, HMG-CoA synthetase, and MVA kinase assays.

Determination of Leaf Dry Rubber Content

Leaf dry rubber content was determined by the method of Garrot et al. (11). The leaves, detached at time indicated, were parboiled for 15 min., sliced into small pieces and then frozen in liquid nitrogen. The frozen material was immediately ground with a grinder, mixed to obtain a homogeneous mixture, and divided into 2 equal parts. The first part was placed in a petri dish and dried for 24 h at 80°C to obtain leaf dry weight. The other replicated wet sample was homogenized for 10 min in 100 ml acetone at 65% power. The mixture was vacuum filtered through Whatman No.1 paper and the cake rinsed with 50 ml of acetone. The filtrate was placed in a pre-weighed glass petri dish and dried to constant weight at room temperature. The dried cake was homogenized in 50 ml of hexane. The homogenate was filtered through Whatman No.1 paper and the filtrate collected into a pre-weighed petri dish. The residue obtained after first hexane extraction was reextracted twice as previously described. The resulting filtrates were pooled and dried at room temperature for complete evaporation of hexane and then oven dried at 80°C for

24 h. The yield of rubber fraction was then measured after constant weight was obtained following oven drying.

Determination of Petiole Rubber Value

The petiole latex method of Zhongyu et al. (12) was used for petiole rubber value determination. Rubber latex exuded from newly detached leaf was smeared on a preweighed weighing paper. The sample was dried at 60-70°C until constant weight obtained. The petiole rubber value was determined as followed.

$$A = \frac{R_1 - R_2}{W}$$

A = petiole rubber value
R_1 = dry latex smear + paper weight
R_2 = paper weight
W = dry leaf weight

Assay of HMGR Activity in Latex and Leaf Cell-free Extract

The HMGR activity was assayed in buffer B (0.1 M KCl, 10 mM DTT 5 mM EDTA, 0.1 M K_xPO_4, pH 7.0). The incubation mixture containing assay sample with protein ranged from 0.1-1.8 mg, 2 mM NADPH, 120 nmoles DL-3^{14}C HMG-CoA (1000 dpm/nmole) was assayed in a final volume of 50-100 ul. The incubation was run at 37°C for 20 or 30 min as indicated. The reaction was stopped by addition of 12.5-25 ul 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 separated 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. (13). 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.

Assay of MVA Kinase in Leaf Cell-free Extract

MVA kinase activity of leaf homogenates was assayed by radiochemical procedure which was modified from that of Gray et al.(14). Incubation mixtures contained DL-[2-^{3}H]-mevalonate (1.025 umol, sp. radioactivity 0.15 uci/mol), ATP (2 umol), $MgCl_2$ (1 umol), and 0.05 ml of leaf homogenate in 50 mM potassium phosphate buffer pH 7.0, in a final volume of 0.2 ml. Reactions were started by addition of mevalonate and ATP after preincubation of the assay mixture at 30°C. The reaction was allowed to proceed for 10 minutes and stopped by boiling for 2 minutes. The proteins were removed by centrifugation. Portions of the supernatant (100-150 ul) were spotted on Whatman 3M filter paper strips (2.5 x 22 cm). Descending chromatographic separation was carried out with butan-1-ol : formic acid : water (77 : 10 : 13 by volume). The paper sections containing the radioactive product (mevalonate 5-phosphate) were cut out between R_f 0.12-0.25 (15) and counted in the liquid scintillation counter using toluene-dioxane cocktail.

Assay of MVA Kinase in C-serum

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 umoles ATP and 3 umoles potassium mevalonate for 10 min at 30°C. The reaction was carried out in 50 mM phosphate buffer, pH 7.0, 5 mM $MgCl_2$, and 40 mM KCl in a final volume of 0.5 ml as modified from the method of Gray et al. (14). 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 0.2 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 umoles phosphoenol pyruvate (PEP), 0.3 moles NADH, pyruvate kinase (7 ug protein, sp. act. 477 units/mg) and lactate dehydrogenase (39 ug protein, sp. act. 490 units/mg). The assay for ADP 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 on or kept frozen and 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 in C-serum and Leaf Cell-free Extract

Assay of HMG-CoA-synthetase in leaf cell-free extract was carried out by following the incorporation of 1-^{14}C-acetyl-CoA into HMG-CoA or its dehydration product, 3 methylglutaryl-CoA. The reaction mixture was in 1 M Tris-HCl, pH 8.2 and contained 0.1 mM EDTA, 0.2 mM 1-^{14}C-acetyl-CoA (4 x10^5 cpm/umole), 0.05 mM acetoacetyl-CoA, and leaf homogenate (50 ul) in a final volume of 0.2 ml (16). The reaction was initiated by adding ^{14}C-acetyl-CoA to the preincubated reaction mixture (2 min at 30°C). At 2, 4, 6 and 8 minutes after ^{14}C-acetyl-CoA addition, 40 ul aliquots were transferred into glass vials containing 0.1 ml 6 N HCl. The acidified aliquots were taken to dryness at 95°C using heating plate. Water (0.5 ml) was added to dissolve the dried nonvolatile material. Liquid scintillation cocktail, compatible with 10% final H_2O content, was then added. The labeled nonvolatile product material, HMG-CoA or its dehydration product, was determined in liquid scintillation counter. Correction for nonvolatile ^{14}C-acetyl-CoA was determined by using control in which leaf cell-free extract was omitted.

Purification of HMGR

Enzyme Solubilization and Fractionation (see also reference # 17)

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) containing 1% Brij w-1 and 20% glycerol. The detergent Brij w-1 was used according to the method described by Bach et al. (18). A glass Potter Elvehjem 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 standing at 4°C temperature for 3-6 hours the mixture was centrifuged 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-mercaptoethanol) 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 with buffer B, and washed with buffer B with flow rate 0.5 ml/min. The enzyme protein was eluted by adding KCl in buffer B at concentrations of 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 u) column (1.5 x 5 cm), previously equilibrated with buffer B. The column was washed with buffer B before eluted with the same buffer containing 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.

Molecular Weight Determination of HMGR

Active HMGR molecular weight determination was performed by the methods described in Sigma Tech. Bulletin No. MRK-137 (10-86). This was for MW of the active conformation HMGR in nondenaturing condition.

Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was run either in the presence or absence of sodium dodecyl sulfate (SDS) using method of Weber et al. (19). The method of Wray et al. (20) was used for silver staining of the proteins.

Purification of HMGR Activator (see also reference # 21)

The procedure used for calmodulin purification was modified and applied for *Hevea* HMGR activator (calmodulin) purification(22,23). Pooled C-serum was heat-treated at 85°C for 15 min and centrifuged at 2,000 x g for 60 min. The supernatant was subjected to batch binding by stirring gently for 30 min at 4°C with DEAE cellulose in 20 mM Tris-HCl buffer, pH 7.4 containing 1 mM $MgCl_2$. The mixture was then washed with 0.2 M KCl and eluted with 0.7 M KCl. The eluate was fractionated by ammonium sulfate precipitation between 30-90%. The pellet was dialyzed in 50 mM Tris-HCl buffer, pH 7.4 containing 0.3 M NaCl, 1 mM $CaCl_2$ and 1 mM mercaptoethanol. The dialysate was applied to Affi-Gel Phenothiazine affinity column, washed with 3 M NaCl and eluted with 10 mM EGTA, respectively.

Assay of NAD Kinase

The method for NAD kinase assay in the latex bottom fraction (24) was also adopted for assay of commercial NAD kinase (purified from liver) and NAD kinase from the latex bottom fraction. The protein (0.1-0.8 mg) was assayed in 0.1 M Tris-Hcl, pH 8.0 containing 3 mM NAD, 4 mM ATP, 0.5 mM $MgCl_2$ with final volume of 1.0 ml. The assay was initiated by the addition of NAD kinase and incubation was carried out at 37°C for 30 min. The assay was terminated by boiling for 5 min and the NADP produced was measured by method of Apps. (25).

Preparation of Antibody Against HMGR : Rabbit Immunization

Approximately 50 ug of purified HMGR in 0.5 ml of complete Freund's adjuvant was prepared for each injection dose. The protein antigen mixture was injected subcutaneously to rabbits at 1 week interval. The titer of antibody was tested at least 4 days after the third injection.

Immunotitration of Enzyme

Antibody against HMGR was tested for its specificity by Ouchterlony

immunodiffusion against purified enzyme, enzyme in fresh latex, and crude extracts of leaf and callus. Various amount of serum from immunized rabbit was incubated with purified HMGR and tested for its ability to inhibit HMGR activity. A preimmunized rabbit serum was used as a control sample.

Protein Determination

The protein concentration was determined by the method of Lowry et al. (26).

Part B. Disease Resistance Screening of *Hevea* at Tissue Culture Stage Explant Materials.

a) *Hevea* flowers obtained during the flowering season were used for callus initiation (Fig. 2). 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 callus initiation in various types of culture media as indicated.

b) Very young *Hevea* seeds with diameter size of 1.5 cm was used to prepare seed tegument. An outer greenish skin of seeds was peeled off and the inner seed tegument was cut by using sterile blade. Thin longitudinal radial slices (1 mm thick) of inner seed tegument were separated and placed in culture medium. The tissues were incubated at room temperature with 12 hrs. of dark-light cycle.

Medium Preparation of *Hevea* Tissue Culture

1. **MB Medium (27)**

<u>Macronutrients</u>

KNO_3	954	mg/l
NH_4NO_3	650	mg/l
$MgSO_4.2H_2O$	370	mg/l
$CaCl_2$	530	mg/l
KH_2PO_4	503	mg/l

<u>Micronutrients</u>

H_3BO_3	1714	mg/l
$MnSO_4.4H_2O$	1003	mg/l
$ZnSO_4.7H_2O$	8325	mg/l
$Na_2MoO_4.5H_2O$	241.9	mg/l
$CuSO_4.5H_2O$	249	mg/l

<u>Iron Salts</u>

Na_2-EDTA	40.9	mg/l
$FeSO_4.7H_2O$	30.5	mg/l

<u>Organic gr. Substance</u>

Nicotinic acid	492.4	mg/l
Glycine	2252.1	mg/l
Thiamine	505.9	mg/l
Myo-inositol	100.8	mg/l
Pyridoxine	493.2	mg/l
Sucrose	69829.2	mg/l
Water coconut	4%	

Hormone		
Kinetin	4.6	uM
2,4-D	4.5	uM
NAA	5.7	uM
pH	5.8	
Agar	1.3%	

Broad Spectrum Culture Media (28)

MZZ (ZM) and MLLH media

M	Z	Z	(Z M)	Medium conc. of sucrose
Medium conc. of minerals	Zero auxins	Zero cytokinins		Zero growth factors and amino acid
M	L	L	H	High con. of growth factors, amino acids and sucrose
Medium conc. of minerals	Low conc.of auxins	Low conc. of cytokinins		

L = Low concentration
M = Medium concentration
H = High concentration
Z = Zero concentration

Concentrations of Minerals

Macronutrients (mM)	Low	Medium	High
NH_4NO_3	2	10	20
KNO_3	2	10	20
NaH_2PO_4	0.2	1	2
$CaCl_2$	0.3	1.5	3
$MgSO_4$	0.3	1.5	3

Micronutrients (mM)	Low	Medium	High
H_3BO_4	1.5	75	150
$MnSO_4$	10	50	100
$ZnSO_4$	4	20	40
$CuSO_4$	0.01	0.05	0.1
Na_2MoO_4	0.1	0.5	1
$CaCl_2$			

Concentrations of Minerals

Macronutrients (mM)	Low	Medium	High
NH_4NO_3	2	10	20
KNO_3	2	10	20
NaH_2PO_4	0.2	1	2
$CaCl_2$	0.3	1.5	3
$MgSO_4$	0.3	1.5	3

Micronutrients (mM)	Low	Medium	High
H_3BO_4	1.5	75	150
$MnSO_4$	10	50	100
$ZnSO_4$	4	20	40
$CuSO_4$	0.01	0.05	0.1
Na_2MoO_4	0.1	0.5	1
$CaCl_2$	0.01	0.05	0.1
KI	0.5	2.5	5
FeNaEDTA	10	50	100

Concentrations of Growth Factors

Growth Factor (uM)	Low	Medium	High
inositol	60	300	600
nicotinic acid	4	20	40
pyridoxine HCl	0.6	3	6
thiamine HCl	4	20	40
biotin	0.1	0.5	1
D-Ca-pantothenic	0.5	2.5	5
riboflavin	1	5	10
ascorbic acid	1	5	10
choline chloride	1	5	10
Amino Acid (uM)			
L-cysteine HCl	12	60	120
Glycine	5	25	50
Concentration of Auxin (uM)			
IAA	0.1	1	10
IBA	0.1	1	10
NAA	0.1	1	10
2,4-D	0.1	1	10
Concentrations of Cytokinin (uM)			
kinetin	0.1	1	10
BAP	0.1	1	10
sucrose (mM)	12	60	120
pH 5.5			
agar 1.3%			

Phytophthora botryosa Culturing

Phytophthora botryosa was obtained from Plant Disease Division, Songkla Rubber Research Center. The fungus was subcultured once every two weeks in potato dextrose agar (PDA) solid medium containing (per liter) 3.9 gm PDA and 0.02 gm lecithin. P-4 broth medium [containing (per liter) 0.5 gm KH_2PO_4, 0.5 gm K_2HPO_4, 0.5 gm $MgSO_4.7H_2O$, 0.02 gm $CaCl_2.2H_2O$, 1 gm thiamine, 20 gm glucose, 1.5 gm $NaNO_3$ and 0.2 gm lecithin] was used for suspension culture.

Extraction of *Phytophthora* Toxin

An agar disc (5 mm diameter) containing *Phytophthora* was prepared by cutting around the margin area of 7-10 day-old *Phytophthora* colony grown on PDA solid medium. It was then cultured into 500 ml of suspension culture medium and incubated for 7 days. Approximately 4500 spores/ml were obtained from a week old culture. The culture fluid was prepared by filtration through three layers of cheesecloth. The culture filtrate was concentrated at 60°C to 10% of its original volume. An equal volume of methanol was added before an overnight stored at 5°C. Precipitate resulting from methanol addition was removed by filtration through Whatman no.1 filter paper. Methanol was evaporated at 60°C and the water fraction was partitioned with three volumes of chloroform. The chloroform extract was evaporated in the hood and the remaining dry substance was weighed and dissolved in small quantities of methanol. The volume was adjusted to 2 volumes of the original amount of the culture filtrate. The concentration of extracted toxin was determined to be 3000 ug/ml. Toxin with final concentrations of 2.5, 25, 250, 275, 300 ug/ml in MB medium were prepared. The medium was used to culture 45 day-old callus initiated from RRIM 600 and GT 1 male flowers for studying the toxin effect on callus growth.

4. RESULTS/DISCUSSION/TABLES

Part A. Biochemical Parameters for Rubber Yield Evaluation in *Hevea brasiliensis*.

A.1 Variation of Rubber Productivity within Individual *Hevea* Clonal Type

High yielding rubber trees commonly grown in southern Thailand are of two-clone-trees derived from by grafting (Fig.3). The scion part is commonly derived from clones RRIM 600 or GT 1. The rootstock are taken from seed-germinated seedlings. The variation on yield or out-put of these two-clone-trees are quite broad. The yield variations were measured as dry rubber content per tapping per tree and the results shown in Fig. 4. The population of high-yielding trees in this study were accounted for less than 20 %. They gave as much as 5-6 times higher in rubber-yield than those of the low yielder ones. The results pointed out the necessity on a developing a reliable technique for early yield evaluation this will help preventing the potentially poor-yielding-trees from being planted. The rubber trees have a rather long economics life-span. Once a rubber tree is planted, it can be tapped for at least 25 years. Therefore, the planting of poor yielders should be minimized or avoid if possible.

A.2 Comparison Studies on Specific Activities of the Enzymes involved in Rubber Biosynthesis in Fresh Latex

Our studies on diurnal variations of latex HMGR showed that the enzymic activity level was closely related to the rubber content (3). The correlation suggested a possible role of this enzyme in the regulation of rubber biosynthesis. In this study section, we proposed to test and verify HMGR as a rate-limiting enzyme in the rubber biosynthesis. This will lead to a practical way of using HMGR as an enzyme parameter for screening of potential high-yielders.

It is generally accepted that HMGR is a rate-limiting enzyme in cholesterol biosynthesis pathway by limiting the formation of isoprene units (29) Similarly, HMGR was also observed and suggested to be a rate-limiting enzyme for rubber biosynthesis (5,30). The regulation was by controlling isoprene formation through HMGR in forming the polyisoprene of rubber molecules (5). The HMGR enzyme measured in the stored latex has been shown to be lowest among the enzymes involved in rubber biosynthesis in earlier study (5). The use of stored latex (5) was doubtful as to validity of the studied results. In order to eliminate uncertainty due to instability of the latex enzymes. We therefore performed a similar study using freshly collected latex. The results showed the HMGR activity of fresh latex was much lower than that of HMG-CoA synthetase and mevalonate kinase. The two enzymes are required before and after the HMGR catalyzing step in the isoprene and hence rubber biosynthesis pathway (Table 1). These results supported and confirmed the earlier findings suggesting that HMGR was likely to be a limiting and controlling factor in the rubber biosynthesis.

A.3 Comparison Studies on Diurnal Correlations of Certain Enzymes in Rubber Biosynthetic Pathway and Rubber Yield from the Leaves of Various *Hevea* Clonal Types

Our results in part A.1 on specific activity levels of various enzymes in rubber biosynthesis pathway were in good agreement with F. Lynen's suggestion

that HMGR might be a rate-limiting enzyme in rubber biosynthesis (5).

In this study section, we carried out experiments to compare the degrees of diurnal correlations between HMGR and rubber yield with enzymes before (HMG-CoA synthetase) and after (MVA kinase) the HMGR catalyzing step. The aims was to point out whether the highest degree of positive correlation existed between HMGR and rubber yield. The comparison results would ascertain and verify HMGR to be the rate-limiting enzyme in rubber synthesis and hence the yield. To eliminate variable factors and errors due to environmental changes (i.e. moisture, rain and sunlight intensity etc.), in the open field condition, the controlled system and specimens were used in this study. The plant materials from leaves of young grafted seedlings grown in experimental control room with 12 hrs of daily illumination at 6000 lux was employed in the study. The specimens and plant materials under specified control conditions were used to carry out the experiments that followed.

A.3.1 Diurnal Variation of Leaf HMG-CoA Synthetase Activity and Its Relation to Petiole Rubber Value

This study was carried out to see if any correlation existed between the enzyme HMG-CoA synthetase and the rubber yield. Three *Hevea* clonal types were used in the study and the results of the enzyme levels and rubber values were shown in Fig. 5 and Fig. 6. There was clearly no positive correlation between HMG-CoA synthetase activity (Fig.5) and petiole rubber value (Fig.6) from three *Hevea* clonal types (RRIM 600, PB 235 and PBM 24) used in the study. The maximum change on HMG-CoA synthetase activity level was observed in clone BPM 24 at 1800 h with 6.3-fold increase from its basal level while there was only a slight increase in petiole rubber value. The result pointed out that relationship between HMG-CoA synthetase and petiole rubber value might not play any significant role in controlling the rubber biosynthesis. This was in contrast to the correlation observed for HMGR and the rubber content.

A.3.2 Diurnal Variation of Leaf MVA Kinase Activity and Its Relation to Petiole Rubber Value

Three *Hevea* clonal types RRIM 600, PB 235 and PBM 24 were used in this study. Diurnal variation and correlation experiments were carried out similar to the other two enzymes. A certain pattern of correlation was observed between MVA kinase activity and petiole rubber value in clones PB 235 and RRIM 600 as shown in Fig. 6 and 7. The highest levels of MVA kinase for PB 235 was at 1800 h while that of RRIM 600 was observed at 2200 h., respectively. At the peak of MVA kinase level, an increase of 11.4 fold above basal level was detected while the increase on petiole rubber value was only 2.1 fold. The broad differences of the two values seemed to bear no significance on correlation being investigated. The relevance of MVA kinase needed to be compared to other enzymes for further evaluation.

A.3.3 Diurnal Variation of Leaf HMGR Activity and Its Relation to Petiole Rubber Value

A study on diurnal variations of HMGR activity in leaves and its relation to petiole rubber value was similarly performed. The leaves of two *Hevea* clonal types were used in the study, RRIM 600 and PB 5/51. The results of the correlations were shown in Fig 8 and 9. The peak of HMGR levels was observed at 1800 h for both clonal types (Fig. 8). Similarly, the petiole rubber value peak was also observed at 1800 h for both (Fig.9). The display of highly positive correlation observed in this study was very significant. It was seen

that there was a high degree of positive correlation between leaf HMGR activity and petiole rubber value in both clonal types. Both HMGR activity and petiole rubber values were shown to reach their maximum levels at 1800 h. The HMGR peak level showed 5.5 fold increase while the peak level of rubber value was increased by 3.5 fold. The parallel increases for both parameters were highly correlated and correspondingly. Even though a small difference existed, the high degree of positive correlation did indicate the importance of HMGR in controlling the rubber biosynthesis. This pointed out a close relationship between HMGR activity and petiole rubber value levels. The findings could be of practical use in early screening protocol since the results were obtained with the young seedlings in this series of studies.

A.4 Comparison Studies on Diurnal Correlations obtained Between Enzymes in Rubber Biosynthetic Pathway and Rubber Yield in the Latex obtained from Various *Hevea* Clonal Types

The results reported in part A.2 and A.3 revealed that there was a positively close relationship between the leaf HMGR activity and petiole rubber value. In this part a similar study on diurnal correlations were carried out with the latex from mature rubber tree. Comparison studies of the three enzymes and their correlation to rubber contents in the latex were investigated. This would complement and verify the results obtained with the leaf sample. The latex samples from various *Hevea* clonal types were tapped and collected from mature rubber trees (approximately 20 years old, rubber plantation of the Songkla Rubber Research Center). The mature trees were tapped and latex collected at the time indicated for the study. Care was taken as to tap on the clear day in a uniform manner in order to minimize errors due to climatic changes and other variable factors.

A.4.1 Diurnal Correlation Between HMG-CoA Synthetase Activity and Rubber Yield in the Latex

Diurnal variation of HMG-CoA synthetase was investigated in latex collected from 3 different rubber clones (RRIM 600, GT 1, and KRS 21). Results in Fig. 10 showed patterns of diurnal variations of HMG-CoA synthetase activities in the C-serum. The HMG-CoA synthetase activities of each clone tended to vary differently during the 24 hour cycle. The difference between high and low activity levels were less dramatic as compared to those found with HMGR and MVA kinase (see above). High and low activity values were observed at 0200 and 1800, respectively. Data on diurnal variation of dry rubber content of the same latex samples used for HMG-CoA synthetase assay were shown in Fig. 11. High and low rubber contents were observed between 1400 and 1800 h, respectively. The high and low levels of HMG-CoA synthetase observed did not correspond to those of dry rubber content in this study. Inverse correlation was noticed at 1800 h for clones KRS 21 and RRIM 600 where HMG-CoA synthetase was highest and dry rubber content was lowest. The results indicated no correlation could be observed between the enzyme HMG-CoA synthesis and the dry rubber content.

A.4.2 Diurnal Correlation Between MVA Kinase Activity and Rubber Yield in the Latex

Diurnal variation of MVA kinase activities was similarly investigated in latex collected from the same 3 rubber clones (RRIM 600, GT 1, and KRS 21). Results in Fig. 12 showed variations of MVA kinase activities in the C-serum. The MVA kinase activity of each clone showed similar pattern of variations

during the 24 hour cycle. They were at high level between 1000-1400 h and at low level between 1800-2200 h. The difference of the high and low activity level was about 2 fold. Dry rubber contents in the latex samples used for MVA kinase assay showed different pattern of diurnal variations (Fig.13). The high and low level of dry rubber contents were observed at 14.00-18.00 h and at 02.00-06.00 h, respectively. The variation patterns of MVA kinase did not correspond with those of dry rubber contents in this study. There seemed to be no positive correlation that could be stated from these experiments. However, the variation patterns had to be compared to HMGR for meaningful interpretation.

A.4.3 Diurnal Correlation Between HMGR Activity and Rubber Yield in the Latex

In this HMGR correlation investigation two diurnal cycles were studied on latex HMGR and dry rubber content of 3 rubber clones (RRIM 600, GT 1 and KRS 21) The first cycle was Feb-March and the second was May-June. Five trees per clonal type were used for the study. Average results on the first two diurnal cycle (Feb-March) were shown in Fig. 13-14. The results on the second two diurnal cycles (May-June) were shown in Fig. 15-16. The results of HMGR studies showed high degree of positive correlation. As depicted in Fig. 14 and 16, HMGR activity levels 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.13,15) Concurrently the level of dry rubber content was also considerably high at this period (Fig.14,16). The corresponding positive relationship between HMGR specific activity and dry rubber content was clearly observed. This positive correlation was predominantly manifested during the dark period (1800-0600). Inverse relationship was also observed to be present around the mid-day period (between 1000-1400 h.). The existence of a highly significant positive corresponding correlation between HMGR activity and dry rubber content indicated the importance of HMGR enzyme in controlling the rubber yield. This was in contrast to the other two enzymes in which no positive correlation could be observed or deduced in the previous experiments.

A.5 Interclonal and Intraclonal Correlations Between the Whole Latex HMGR Activities and Rubber Yields

Ten different trees of clone RRIM 600 selected at random were investigated for correlations between the whole latex HMGR activities and rubber yields. Analyses of HMGR levels and rubber contents revealed significant correlation results. Good positive correlation ($\alpha = 0.01$, $r = 0.76$) was obtained in this intraclonal study as shown in Fig. 17. This was in good agreement with the previous diurnal studies. Similarly, interclonal correlation study was also carried out. Ten different clonal types were analyzed for the correlation and compared. The results obtained from 10 different clonal types selected for the study showed highly positive correlations. The correlation between HMGR activity and rubber yield among 10 different clones as depicted in Fig.18. This correlation was calculated and shown to be of highly statistical significance with values of $\alpha = 0.01$ and $r = 0.78$. Both interclonal and intraclonal correlations between HMGR activities and rubber yields strongly suggested a possible direct and important role of the HMGR enzyme in controlling rubber biosynthesis. Furthermore, this together with the previous results helped substantiate the possible use of HMGR as a useful and practical biochemical parameter for early selection of potential high yielding clones. The selection of superior clones could possibly be done early at seedling stage to prevent the planting of poor yielders. Optimization of the assay be developed for mass screening of the young seedlings. This approach could save several years in time and resources as compared to the

present evaluation practice. If the correlations could be shown to remain, stable consistent, stable and reliable from seedling throughout the tapping period. This would have a substantial impact on rubber productivity. The early selection of potential high-yielding clones with HMGR parameter could possibly become a new innovative practice and in common use in the near future.

A.5.1 Correlation Between HMGR Activities and Rubber Yields in Trees Rated as High-, Medium-, and Low-yielding Rubber Trees

In this study, the objective was to identify the high-, medium-, and low-yielding rubber trees for further experimental studies. Randomly selected RRIM 600 rubber trees (9 trees) were tapped and measured for rubber yields. Results of the average rubber yield from 5 tappings per tree were shown in Fig. 19. From the rubber yield results, the tree number 8,4, and 7 from clone RRIM 600 were rated and selected to represent low-, medium-, and high-yielding trees, respectively. The correlation studies were carried out between rubber yield and total HMGR activity as well as HMGR activity per ml latex. The results were calculated and summarized in Table 2. cumulative results showed very high degree of positive correlations. When the rankings of rubber yields and HMGR activities were compared and tabulated, they showed highly corespondent ranking positions. The high- (# 7) and low-yielder (# 8) had the highest and lowest total HMGR activities, accordingly. The same corresponding correlation were also observed with HMGR activity per ml latex. The statistical significance of these studies were ratified and ascertained with calculated r values of 0.99. These findings provided supportive evidence for the possible direct and important role of HMGR in controlling rubber biosynthetic capacity in mature rubber trees as similarly suggested from previous results with other specimens and conditions.

A.6 Indirect Approach to Show Positive Correlation Between HMGR Level and Rubber Yield may also be found as early as Seedling Stage

Three RRIM 600 trees were selected from 9 RRIM 600 trees which gave different rubber yield values per tapping as shown in Fig. 19. The tree number 8,4 and 7 were analyzed and rated as low-, medium-, and high yielding trees in this study (see part A.5.1). High branches about 7 inches in diameter and 6-8 meters above the ground on trees # 8,4 and 7 were selected and trimmed off in order to allow regeneration of new young branches (Fig. 20). Young green buds were then taken from the new generated branches and used for graftings in the experiments in this section. This bud graftings specimens from low-, medium-,and high-yielding trees (# 8,4,7) were used in the experiments that followed.

A.6.1 Studies on Activity Levels of Latex HMGR Tapped from Young Branches Generated from Low-, Medium-, and High-yielding Buds after Grafting on a Common Rootstock

The young green buds taken from RRIM 600 trees #. 8,4, and 7 were grafted on two different rootstocks in this study. One was grafted on young branches (6 month-old) generated from a common rootstock in the open field as shown in Fig. 21. The other grafting was on a two-year-old rootstock grown in an experimental control room (Fig. 22). After 3-4 months of grafting, all of the field grafted buds (from trees # 8,4 and 7) generated and developed into young branches. For the grafted bud grown in the experimental control room, only the buds taken from the low and high-yielding trees (# 8 and 7) generated and developed into young branches. Latex were collected from these young branches by tappings with the sharp (blade-like) end of a needle-syringe. The HMGR

activity levels in latex from each young branches were assayed and compared. The results of these young grated seedlings were quite similar to those obtained with mature trees. The results reported in Fig. 23. clearly indicated that HMGR activity levels in latex tapped from young budding still retained the patterns characteristics of the mature tree from which the buds were derived. The new branches generated from buds taken from low-, medium- and high-yielding trees after grafting on a common rootstock still remained low, medium and high in their HMGR activity levels. These results pointed out a very important finding which might indicate that the different on yield potential could be detected at an early stage. The parallel and corresponding correlations were thus being established in this study. The young grafted seedlings were thus demonstrated to be of a useful and practical model for early screening and election of the superior clones.

A.6.2 Studies on Activity Levels of Latex HMGR Tapped from Young Branches Generated from Bud Taken from Low-, Medium- and High-yielding Trees after Grafting on Different Seedling Rootstocks

The young green buds taken from RRIM 600 trees # 8,4, and 7 were grafted on two sets of different rootstocks. One grafting was on one-year-old (Fig. 24) and the other was on 8-month-old (Fig.25) seedling rootstocks, respectively. The latex were collected by tapping with sharp end of syringe on the young branches generated from the grafted buds for analyses. The experiments were carried out after 4 and 5 months of grafting on the one-year-old and the 8 month-old seedling rootstocks, respectively. The latex HMGR activity levels were measured and the results shown in Fig. 26b and 26c. The results of this study were different from the previous experiment and did not quite confirm to the yield character of the mature trees from which the buds were derived. Unlike results in part A.6.1, the latex HMGR levels on young branches generated from buds taken from low-, medium-, and high-yielding trees were slightly deviated from the expected yield potentials. Since they were grafted on different rootstocks, and thus might not be able to retain or keep the characters of their original yield potentials. The deviated changes in the observed yield potential might be due to many factors, especially variation on the degrees of stock influences which were from different clonal types. More experiments needed to be carried out before the results could be accepted or verified.

A.6.3 Variation of HMGR Activity Levels from Commercial Grafted Seedings

The results shown in part A.6.2 suggested that stock influence might have certain effect on rubber yield on the scion part. Therefore, the young grafted seedling should be screened in order to detect and obtain the superior yield potentials. This study was carried out using commercial grafted seedling for screening experiments. Five grafted seedlings of clones RRIM 600, PB 235, and PBM 24 were randomly chosen from the local nursery in this investigation.

The young branches on the grafted seedings were tapped by using sharp end of syringe and the latex were collected for HMGR activity level measurements. The results of this study were shown in Fig. 27. They indicated wide variations on HMGR activity levels among the commercial grafted seedlings. The clone RRIM 600 and PB 235 showed quite broad variations. However, there was a lower degree of variation for PBM 24 clone. The highest average on the HMGR activity level for clone PBM 24 was also obtained. At present, the market price for grafted seedling of clone the PBM 24 is the highest. It is generally observed and known among rubber farmers that the PBM 24 clone gives better yield per acre than the RRIM 600 or GT 1 clones. However, time is still

needed for the completion of a large-scale trial conducted by the Rubber Research Center. Therefore, it is not yet officially recommended for a large-scale plantation until conclusion can be reached and yield potential verified.

A.7 Development of ELISA for HMGR Measurement

Our results in part A.1-6 showed that HMGR level was highly correlated with rubber yield. These findings might be further developed and used as an enzyme-parameter for screening and selection of high-yield potential at young seedling stage. Since a large number of seedlings could be improved from yield screening, we therefore proposed to develop an immunochemical procedure for the purpose. This would be a simple and rapid screening of HMGR level for high-yielding rubber clones. A quick outcome in upgrading rubber yield per acre could be faster and better achieved by yield screening on the stock trees which are used to propagate buds for commercial grafting. We also aim to use HMGR parameter for screening high yield potential on thousands of new *Hevea* germplasms collected from Brazil in 1981. This would help broadening and improving the *Hevea* genes pool for better productivity. The experiments that followed were carried out in an attempt to fulfill these goals.

A.7.1 Solubilization, Purification, and Characterization of *Hevea* HMGR (see also reference # 17)

In order to make antibody to *Hevea* HMGR, a substantially purified enzyme was required for immunization. Purification of HMGR was carried out for the purpose as followed.

Preparation of Soluble HMGR : Various attempts to solubilize HMGR from the bottom fraction of fresh latex were carried out using different detergents and compared. Efforts to solubilize the enzyme with Trition X-100, Brij-35 and Tween-80 were unsatisfactory. However, we found that maximum activity could be obtained in soluble form by using Brij (polyethylene ether) W-1 with satisfactory results. Preparation of the soluble enzyme was carried out under conditions similar to those reported for etiolated radish seedlings by Bach et. al. (18). After the solubilization step with Brij W-1, all the enzyme activity was present in the soluble form in supernatant fraction. The enzyme activity remained quite stable in this solubilization medium for further purification steps.

Purification of Solubilized HMGR Enzyme : Basically, the protocol being employed was similar to the recently developed method for purification of radish HMGR (18) with some modifications. The concentration and purification of HMGR was achieved by ammonium sulfate fractionation and affinity chromatography. Affi-Gel-Blue, a biospecific affinity medium for purifying nucleotide requiring enzyme was used in the first affinity step. Agarose-hexane-HMG-CoA was used in the final affinity chromatography step to obtain homogeneous enzyme. Data for a typical preparation and purification were presented in Table 3. The specific activity of the reductase enzyme solubilized from freshly prepared bottom fraction ranged from 0.4 to 0.7 nmole $min^{-1}mg^{-1}$. Solubilization led to 1.78 fold purification so that the specific activity of the enzyme in the soluble supernatant after centrifugation was around 0.7 nmol $min^{-1}mg^{-1}$. Further purification was achieved by fractionating the supernatant with addition of saturated ammonium sulfate solution to a final concentration of 40%. The uppermost yellowish enzyme protein layer was collected by centrifugation at 25,000 g for 20 min. The ammonium sulfate fractionation resulted in about 5 fold purification of the enzyme. The enzyme was further purified by chromatography on DEAE-Sephadex A-50. The elution

profile obtained was shown in Fig.28. The reductase activity was eluted from the column with buffer B containing 0.5 M KCl. The pooled fractions contained approximately 85% of the loaded activity and gave 22 fold increase in specific activity. Further purification on Affi-Gel-Blue (Fig. 29) followed by HMG-CoA-hexane agarose columns (Fig. 30) led to 77 and 257-fold purification, respectively.

Polyacrylamide Gel Electrophoresis (SDS-PAGE) : 10 ug of protein from the purified enzyme was analyzed by running in SDS-PAGE. The enzyme with specific activity of 105 nmol $min^{-1}mg^{-1}$ was applied to the gel and exhibited a single major protein band in SDS-PAGE (Fig.31). The calibrated subunit molecular weight of *Hevea* HMGR was approximately by 44,000 daltons (Fig.32.). The subunit molecular weight of HMGR from *Hevea* was similar to that reported for radish seedling (18). It was considerably lower than that reported for the liver enzyme as deduced from nucleotide sequence by Liscum et al. (31, 32). The active purified enzyme showed a single prominent band with molecular mass of 176,000 daltons in the nondenaturing gel electrophoresis. The enzyme protein was as visualized by silver staining procedure (Fig. 33,34). This value was favorably corresponded with the size of 180,000 daltons obtained for the radish enzyme calibrated from density gradient centrifugation (18). However, some proteolytic degradation during the solubilization and purification might occur. The Km value for (S) HMG-CoA was 13.3 u M with the purified *Hevea* HMGR (Fig.35). The Km value was slightly higher than those of purified yeast, radish seedlings, rat and chicken liver enzymes(18,33) and the range of Km reported for *Fusarium oxyosporum* (33). It was, however, quite lower than Km value of 56 uM reported for HMGR of crude membrane fraction obtained from the bottom fraction of the latex (10).

The stability of HMGR was generally dependent upon the presence of a reduced thiol compound (33). Early studies demonstrated that HMGR in bottom fraction was stabilized and activated by dithiothreitol with an optimal concentration of 2 mM (9). Our present study revealed that a higher concentration of dithrithreitol (20 mM) was necessary to maximize catalytic activity of the purified HMGR (Fig. 36).

The optimal pH for maximum activity for all *Hevea* HMGR assays analyzed this far was approximately 7.0. The pH optimum for the enzyme ranged from 6.5 to 7.7 (34, 35). An optimum pH of 6.8-7.0 was reported for *Hevea* HMGR in the bottom fraction suspensions (9). We found an optimum pH of 7.0 for the purified HMGR which was similar to that reported for crude HMGR (Fig. 37).

A.7.2 Preparation of Antibody Against HMGR

The antibody against HMGR was obtained from immunized rabbit. The immune serum was taken one week following the third HMGR antigen injection. The titer of rabbit antiserum was quite low, ranging from 1:1 to 1:10. While a successful development of ELISA required higher titer (approximately 1:200), therefore the antiserum thus obtained would not be ready yet be used in ELISA. The antiserum was able to precipitate HMGR in fresh latex, bottom fraction HMGR and the purified HMGR form. A single precipitin band was observed from Ouchterlony double diffusion analysis with the *Hevea* enzyme (Fig. 38). The complete inhibition of HMGR activity by the antibody was not observed with the present antiserum. The maximum inhibition of HMGR activity in the presence of various concentrations of HMGR antiserum was only 30% (Fig. 39). The antiserum obtained from modification of HMGR by denaturation with SDS to make it a better antigen was unfortunately not satisfactory. At the present it seemed *Hevea* HMGR to be a poor antigen and the reason was unknown. Further

attempt to obtain a better antiserum against HMGR is still being developed.

A.8 Screening on New *Hevea* Germplasms for High Yield Potentials Using HMGR as Yield Parameter

An attempt on preliminary yield screening on 563 new *Hevea* germplasms collected from Brazil was made by a combination of a coarse and fine screenings. The coarse screening was carried out by evaluating the amount of latex obtained upon cutting through leaflets of 3 month-old seedlings using method of Zhongyu *et. al.*(12). Leaf blade was cut parallel on both sides of midrib vein with 1 cm apart. Amount of latex being excreted from lateral veins were then visually measured. Grades A to D were given to the corresponding high to low latex yield obtained. Results on yield evaluation by estimating latex excreted from lateral veins were shown in Table 4. There were only 42 out of 563 new *Hevea* germplasms with grade A. These 42 grade A specimens were subjected to further yield evaluations by measuring their latex HMGR levels. The latex were collected by making a small tapping on the stems. Results on the latex HMGR levels of the 42 samples in comparison to that of RRIM 600 under the same treatment were shown in Table 5. The comparison results were quite scattered. Most of HMGR activity levels on the 42 new *Hevea* germplasms being measured were lower than that of RRIM 600. The clone RRIM 600 was known to be the established high yielder. There was, however, 10 new clonal types as shown in Table 6 with higher HMGR activity level than that of the RRIM 600 control. The best clone came from material source MT/C/2, clonal code 10/27. This clone (10/27) had 6 times higher in latex HMGR activity level than that of RRIM 600 control. It thus appeared that with the expansion of new *Hevea* germplasm, we might come up with a favorable superior clones by simple screening assay of HMGR level among the new seedlings.

A.9 Studies on HMGR Activator (*Hevea* calmodulin)

This part of study would provide basic knowledge on the regulation of latex HMGR and the result from the study was also published (21). Better understanding of HMGR regulation would provide clearer picture on the control of rubber biosynthesis.

A.9.1 Purification of HMG-CoA Reductase (HMGR) Activator in Latex of *Hevea brasiliensis*

It was earlier reported that heat-stable protein from the C-serum fraction was able to activate HMGR in the bottom fraction of centrifuged latex (36). In our investigation, C-serum prepared from latex of high-, medium-, and low-yielding trees of clones RRIM 600 and KRS 208 were studied. When they were used to activate HMGR in the bottom fraction, different degrees of activation were obtained. High, medium, and low percent of HMGR activation were observed with the C-serum from corresponding high, medium, and low yielding trees, respectively (Fig. 40). This suggested an important role of the C-serum in regulation of HMGR activities. Further purification and characterization of HMGR activator was carried out and reported in this section.

The HMGR activator was found to be a heat stable protein. It was purified from the C-serum fraction of centrifuged latex by modifying methods used for calmodulin purification (22,23). The purification steps involved heat treatment, DEAE-cellulose (batch binding), ammonium sulfate precipitation, and Affi-Gel Phenothiazine chromatography in the final step (Fig. 41). The HMGR activator was purified 284 fold with specific activity of 105 unit/mg as quantitated from the increased amount of HMGR activity due to its presence

(Table 7). Molecular mass of HMGR activator when analyzed by SDS-PAGE was 17,500 which was in the same range as calmodulin (Fig. 42,43). When Stains All was used for SDS-PAGE staining, the purified activator band appeared in blue color indicating its property of being Ca^{++} binding protein. The concentration-dependent rise in activity of HMGR in latex bottom fraction in the presence of HMGR activator was shown in Fig. 44. The HMGR activity increased with increasing amount of HMGR activator, reaching a maximum of 100% when 30 ug of the purified activator was added. Combining with other properties being studied, the purified HMGR activator was thus evidently identified as calmodulin.

A.9.2 Functional Properties of HMGR Activator (calmodulin)

Effects of several divalent cations on promoting HMGR activation in the presence of its activator were studied. This was to see the effect of specific cations and the results were shown in Table 8. Calcium was demonstrated to be the most effective ion in activating HMGR by its activator. Moreover the inhibition by EGTA on activation of HMGR was also demonstrated. The EGTA inhibition on its activator could be overcome by addition of Ca^{2+} to the incubation (Table 9). Trifluoperazine, a calmodulin antagonist, was found to be a potent inhibitor of the HMGR activator in activating HMGR activity (Table 10).

From results described above, several properties found in HMGR activator indicating that it was calmodulin (M_r of 17,500, heat stability, Ca^{2+} dependency, and inhibitory effect of trifluoperazine). With these properties being characterized, the purified HMGR activator was thus identified as calmodulin. Further confirmation was given by its ability to activate NAD kinases. The activations of NAD kinase in latex bottom fraction as well as commercial eukaryotic NAD kinase were observed with HMGR activator. The NAD kinase activation was similar to that found with brain calmodulin (Table 12).

It was reported earlier that ATP-Mg^{2+} could inhibit HMGR activity in the bottom fraction. The inhibition could be overcome by addition of heat-stable protein which was partially purified from the C-serum (19). Our study showed that a similar inhibition properties were also associated with the purified HMGR activator (*Hevea* calmodulin), (Table 12). The inhibition of HMGR activity due to the presence of ATP-Mg^{2+} could be overcome by addition of the purified HMGR activator. However, the overcome effect did not occur when HMGR activator was presence together with NaF. F^- was known to be a potent inhibitor of phosphatase enzyme. This result suggested a requirement of phosphatase activity for HMGR activation by the HMGR activator. HMGR activator may indirectly activate HMGR activity in bottom fraction via regulation other enzymes present in bottom fraction. These enzyme were possibly involved in phosphorylation and dephosphorylation of *Hevea* HMGR similar to those suggested for mammalian HMGR (37-39).

Supportive evidence came from our subsequent study on effects of calmodulin and phosphatase on HMGR activity (Table 13). The *Hevea* calmodulin failed to activate HMGR in the solubilized bottom fraction as well as the purified HMGR. The enzyme alkaline phosphatase was, however, able to activate the purified HMGR. It could therefore be suggested that the activation of HMGR activity observed in the bottom fraction was due to indirect involvement of calmodulin on the HMGR enzyme. The purified enzyme might be regulated by phosphorylation and dephosphorylation as suggested for the mammalian HMGR. Although our preliminary results suggested the indirect role of calmodulin in HMGR activation, further investigation on the cascade mechanism of calmodulin

activation of HMGR as well as other enzymes involve in rubber biosynthesis is needed. This will lead to a better understanding on the roles of HMGR and other factors in controlling the rubber biosynthesis.

Part B. Disease Resistance Screening of *Hevea* at Tissue Culture Stage

B.1 Callus Culture from *Hevea* Male Flowers

During the flowering season (March-April and Aug.-Oct.) male flowers (Fig.2) were collected from several *Hevea* clonal types. We were able to initiate callus from male flowers of several *Hevea* clonal types (Table 14) by using MB media described for anther culture by Chen *et. al.*(27). Callus initiated from several clonal types (PR 255, RRIM 600, and RRIC 6) at different developmental stage, were shown in Fig. 45-49. Most of the callus changed color from yellowish green into brownish callus after 4 weeks of initiation and did following subculturing (Table 14). Fastest callus growth was observed with RRIC 6 clone compared to the others. The RRIC 6 clone was further used for studying the effect of auxin and cytokinin on callus initiation (Table 15). The RRIC callus could be initiated in a hormone-free medium. Subculturing of callus after one week of inoculation into new medium containing low concentration of auxin and cytokinin gave better callus growth rate than after two weeks of inoculation. The auxin and cytokinin seemed to be required in the inoculation medium in order to obtain better callus growth.

B.2 Callus Culture from Shoot, Stem, and Leaf Explant

Embryo culture was also conducted in order to obtain sterile shoot, stem, and leaf explants for callus initiation (Fig.50). So far, we did not yet succeed in the attempt at this stage. There was no callus formation from neither stem, shoot, nor leaf explants (Fig. 51,52). The failure might be due to the inability of nutrient in the media to be taken up into the explant tissues. After cutting the explant tissue, rubber latex might exude into the cutting edges and clogged up the tissue surfaces. This might be the cause on preventing nutrients from reaching the growing tissue.

B.3 *Hevea* Callus Histological Studies

Histological studies of *Hevea* callus were carried out following initiation. Callus tissues were fixed in glutaraldehyde (1% in phosphate buffer, pH 7) at $4^{o}C$. They were then dehydrated through an alcohol series (30,50,70,80 and 95% absolute ethanol) and embedded in paraplast. Sectioning was done by rotary microtome (8 u). The cross-sections were affixed to slide using Hupt's adhesive. They were then dewaxed in xylene, dehydrated with alcohol series, stained in 1% toluidene blue for 2-5 minutes and washed out the excess dye with water. The sections were then dehydrated with alcohol series and mounted in eurapol. Photographs of the *Hevea* callus sections were taken under Nikon inverted microscope. As shown in Fig. 53, the mass of callus cells seen as phenol-blackened spots (Fig. 54) were stained as reddish purple while the normal cells were greenish blue. The arrangement of cells appeared suggesting it to be non-embryogenic like structure.

B.4 Callus Culture from *Hevea* Inner Seed Tegument

Callus formation from inner seed tegument were obtained (Fig. 55, 56) after a few weeks of culturing. Faster growing callus was observed from the explants of inner seed tegument than that of male flowers.

B.5 Effect of *Phytophthora* Toxin on Callus Growth

Various concentrations of *Phytophthora* toxin extracts were added to the MB culture media and used to culture 45 day-old calli initiated from male flowers of GT-1 and RRIM 600 clones. It is shown in Table 17 that after one week of toxin inoculations, toxin at 25 ug/ml was not able to kill any type of calli. A higher toxin at 275 ug/ml was able to completely kill RRIM 600 callus. The toxin level at 275 ug/ml, however, was able to kill only 33.3% of total GT-1 calli. This result indicates that RRIM 600 callus is more susceptible to *Phytophthora* toxin than GT-1 callus and the expression on disease resistance may be detectable at callus stage. A similar test on the survival rate on callus initiation of GT-1 and RRIM 600 male flowers in media containing toxin was revealed in Table 17. It was seen that after the sixth week of flower inoculations, almost all RRIM 600 calli growth were ceased and while GT-1 calli were still alive with normal growth. Moreover, the growth rate of RRIM 600 calli before cessations were very poor when compared to that of its control.

B.6 Lesion Studies with Live Pathogen Infection on *Hevea* Leaves

Disease resistance study with live pathogen infection was performed on young *Hevea* leaves. The leaves were inoculated with agar discs containing *Phytophthora botryosa* prepared as described under Materials and Method. The degree of infection was evaluated by scoring 0 to +3 for leaf inoculation site with no lesion, 0.2 cm, 0.2-0.5 cm, and more than 0.5 cm in lesion diameter, respectively. Evaluation was assessed 24 hours following the pathogen inoculation.

After 24 hours of leaf inoculations by layering with agar discs containing *Phytophthora*, 75 out of 93 leaf inoculation sites on RRIM 600 leaves were infected. On the contrary, only 18 out of 93 sites on GT 1 leaves showed lesion signs (Fig. 57-60). The degree of infection observed with RRIM 600 leaves was more prominent than those of GT-1. After 48 hours of inoculation, 82 out of 93 leaf inoculation sites on RRIM 600 leaves were infected with 25, 52 and 22% of +1, +2, +3 lesion scores, respectively (Fig.57,58,61). Fifty out of 93 inoculation sites on GT-1 leaves were infected with 84 and 16% of +1 and +2 lesion scores, respectively (Fig. 57,58,62). It was clearly seen from these results (Fig. 57-62) that higher degree of lesion intensity and number of infection sites were observed with the RRIM 600 leaves than those of GT-1. The GT-1 leaf was, therefore, shown to be more resistant to *Phytophthora* infection than that of RRIM 600. These results suggested that RRIM 600 was more susceptible and GT-1 was more resistant to the Phytophthora infection.

No sign of infection was observed on the control leaf samples when either pure agar discs or agar discs contaminated with non pathogenic fungus were layered on GT 1 and RRIM 600 leaves (Fig. 63-66). There was no different on results observed either after 48 (Fig. 63,64) or 72 hours (Fig. 65-66) of inoculation time on GT-1 or RRIM 600 leaves.

B.7 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 were observed (Fig. 67). It was thus evidenced that under normal condition antifungal compounds against *Phytophthora* growth was

not present, especially in the resistant clone GT 1. Lesion studies with live pathogen infection on callus as well as 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 screen and select for the high-yielding disease resistant rubber clones.

B.8 Studies on HMGR Activity Level in Non-embryogenic Callus

Several calli obtained from part B.1 and B.4 were homogenized. The extracts were assay for HMGR activity. No HMGR activity were detected in the non-embryogenic callus extracts. Rubber was known to be synthesized in an organized latex vessel system. The HMGR activity in the non-organized callus mass is, therefore, undetectable. The HMGR marker test is needed to be confirmed in the embryogenic callus.

5. CONCLUSION AND REMARKS

Part A. Biochemical Parameter for Rubber Yield Evaluation in *Hevea brasiliensis*

- Our work on diurnal studies of rubber biosynthetic enzymes (HMGR, HMG-CoA synthetase and MVA kinase) and their relation to rubber contents in both latex and leaves suggested the possible implication and important role of HMGR in the rubber biosynthesis pathway. Moreover, significantly high inter- and intraclonal correlations between the latex HMGR activity level and rubber yields were observed. These results suggested the potential use of HMGR as a biochemical marker/parameter in rubber yield.

- The correlation between HMGR level and rubber yield were indirectly shown to be present as early as seedling stage. Along this indirect study approach, a great influence of rootstock on yield was identified. This finding may have great implication on the current technology which requires grafting onto seedling rootstock.

- 563 clones from Brazil were screened by using HMGR marker and ten potentially high-yielding clones were identified. However, the radioassay used for HMGR measurement is too slow and expensive.

- An ELISA development for HMGR measurement was attempted. HMGR was solubilized, purified and characterized as published (17). However, the HMGR was shown to be a very poor antigen and thus the antibody obtained was not able to be employed for the ELISA.

- Basic study on biochemical regulation of HMGR was also performed and results were published (21).

Part B. Disease Resistance Screening of *Hevea* at Tissue Culture Stage

- Callus were initiated from male flowers of several *Hevea* clonal types (RRIM 600, PB 310, RRIC 6, PR 255 and GT 1) as well as from inner seed teguments. They appeared to be non-embryogenic callus. The HMGR activity was not detectable in the callus extract. Further confirmation on the HMGR marker in the embryogenic callus is still needed to be experimented.

- Both leaves and callus of GT 1 were more resistant to *Phytophthora* toxin than those of RRIM 600.

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7. ANNEX

Fig. 1. Fractionation of fresh latex at 49,000 xg for 45 min.

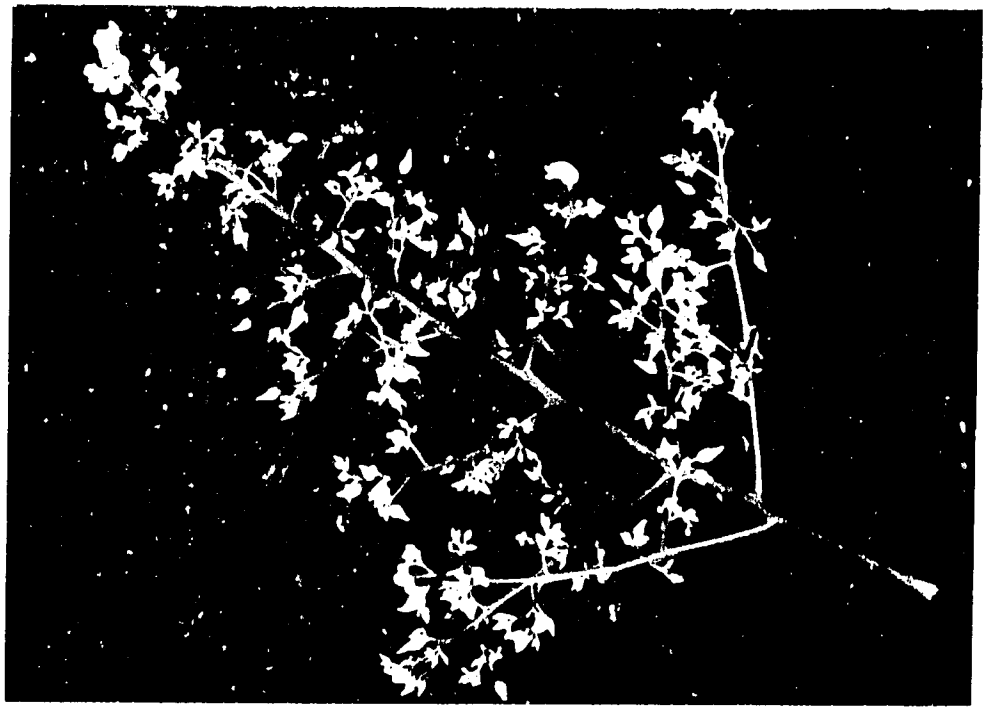

Fig. 2. *Hevea* flowers, female yellowish flowers are larger in size and located at the end of each flower branch. Male flower are smaller white buds and located below the female flowers.

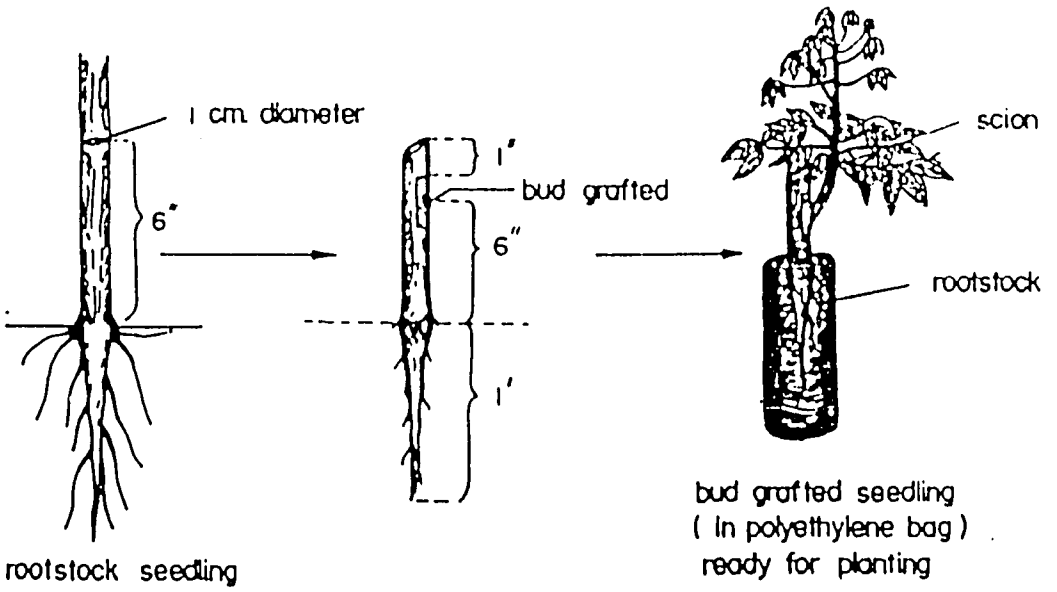

Fig. 3. Bud grafting on rootstock seedling of *Hevea brasiliensis*.

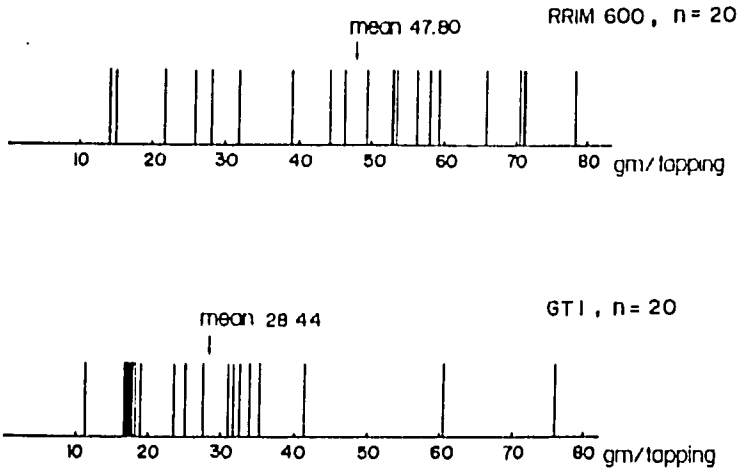

Fig. 4. Variations on rubber yield per tapping per tree. Each perpendicular line represents rubber yield per tree of clones RRIM 600 (top) and GT 1 (bottom).

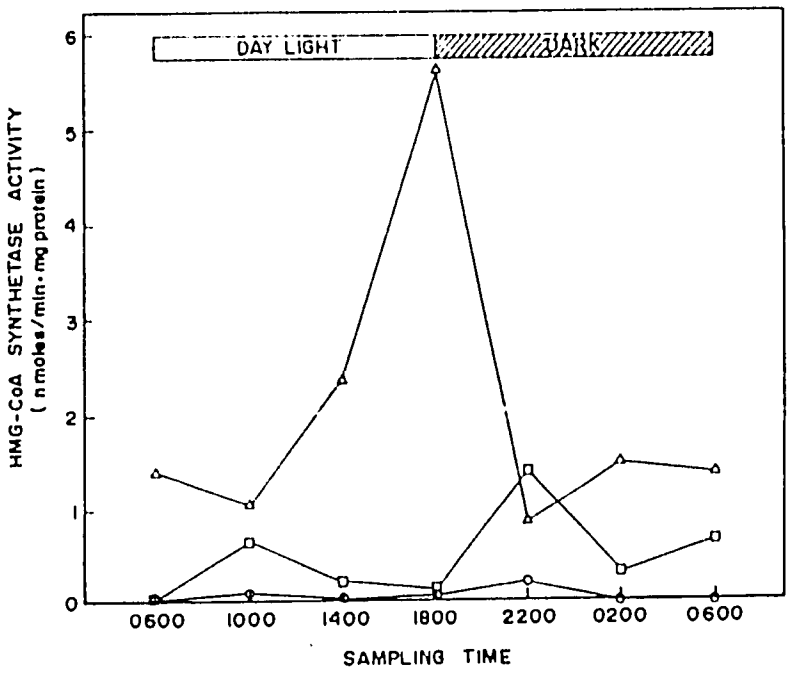

Fig. 5. Diurnal variation of HMG-CoA synthetase activity in *Hevea* leaf extracts of clones RRIM 600 (o—o), BPM 24 (Δ—Δ) and PB 235 (□—□).

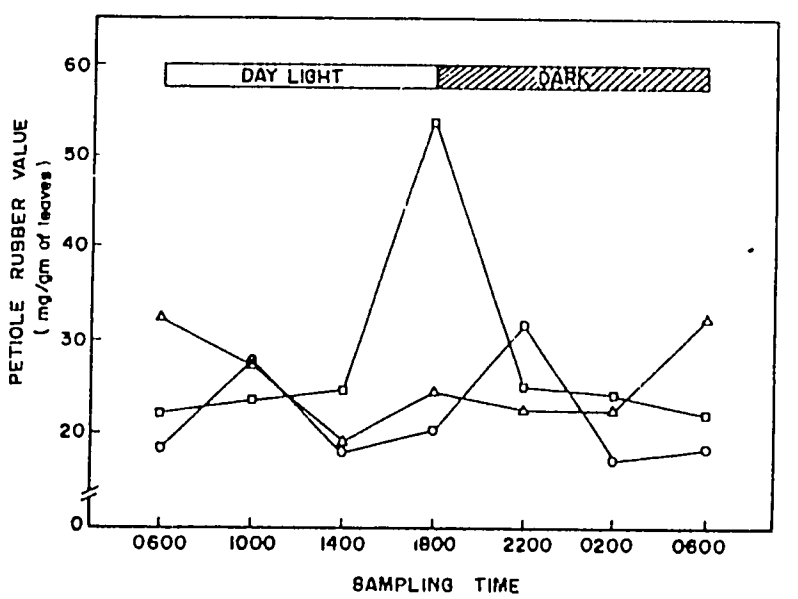

Fig. 6. Diurnal variation of petiole rubber value from *Hevea* leaves of clones RRIM 600 (o—o), BPM 24 (Δ—Δ) and PB 235 (□—□).

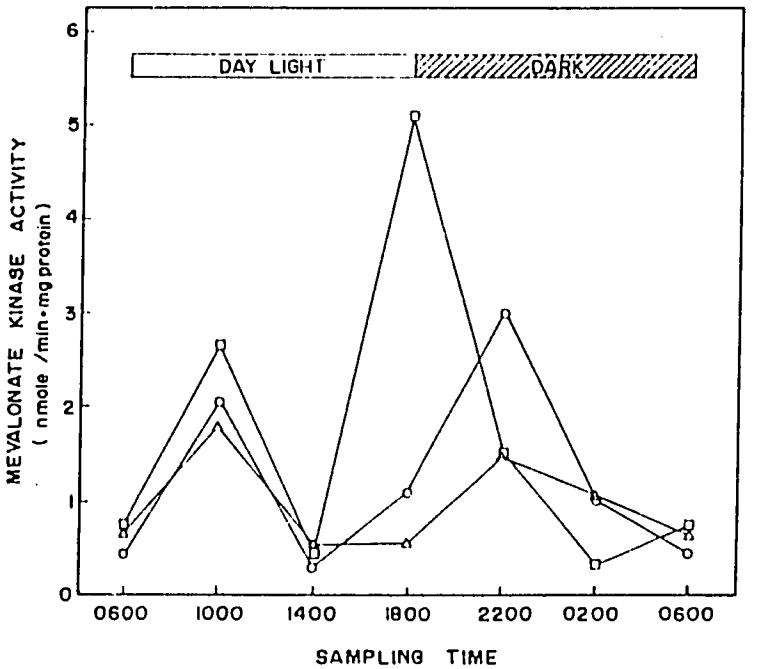

Fig. 7. Diurnal variation of MVA kinase activity in *Hevea* leaf extracts clones RRIM 600 (o—o), PBM 24 (Δ—Δ) and PB 235 (□—□).

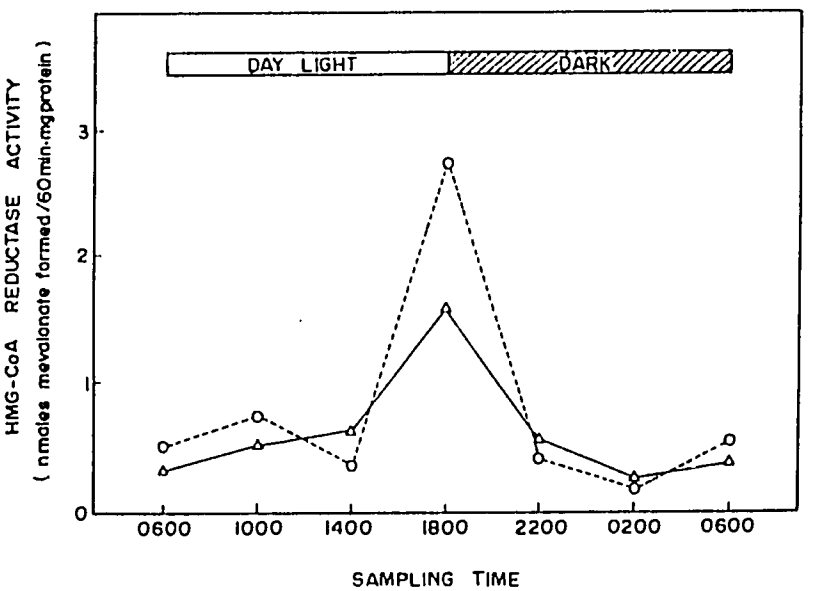

Fig. 8. Diurnal variation of HMGR activity in *Hevea* leaf extracts of clones RRIM 600 (o—o) and PB 5/51 (Δ—Δ).

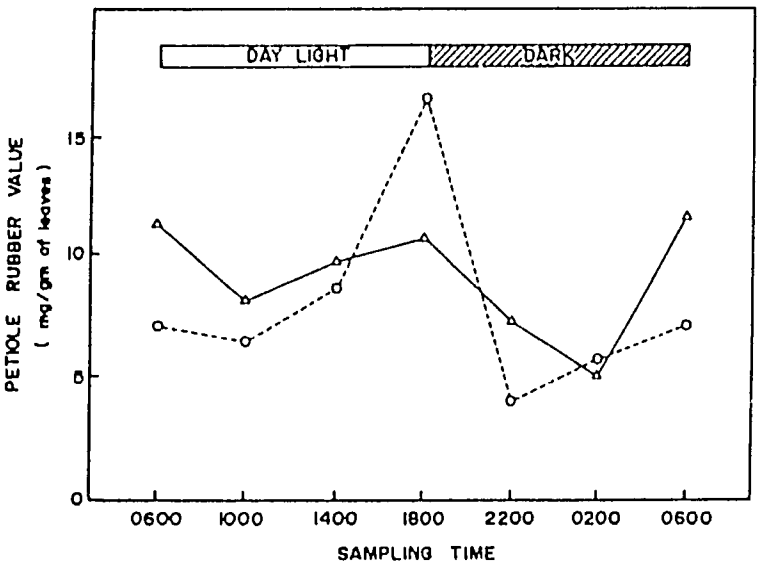

Fig. 9. Diurnal variation of petiole rubber value from *Hevea* leaves of clones RRIM 600 (O—O) and PB 5/51 (Δ—Δ).

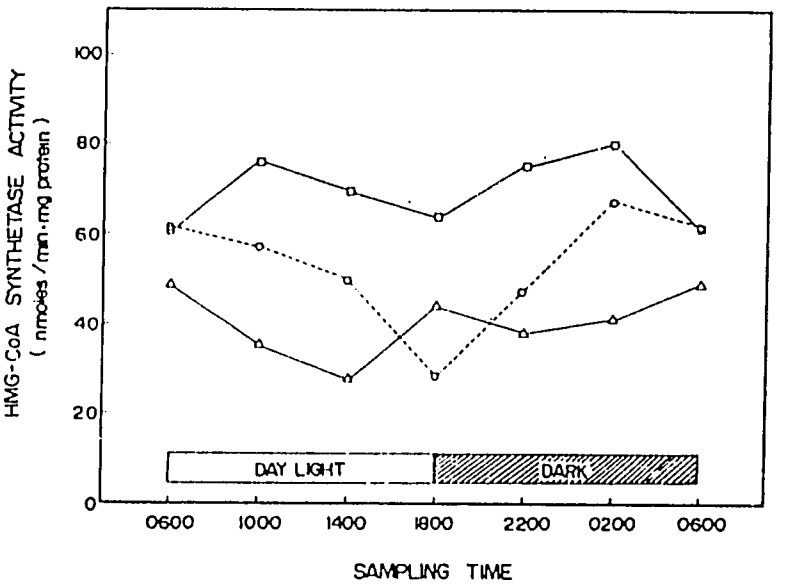

Fig. 10. Diurnal variation of HMG-CoA synthetase activity in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (Δ—Δ) and GT 1 (O—O).

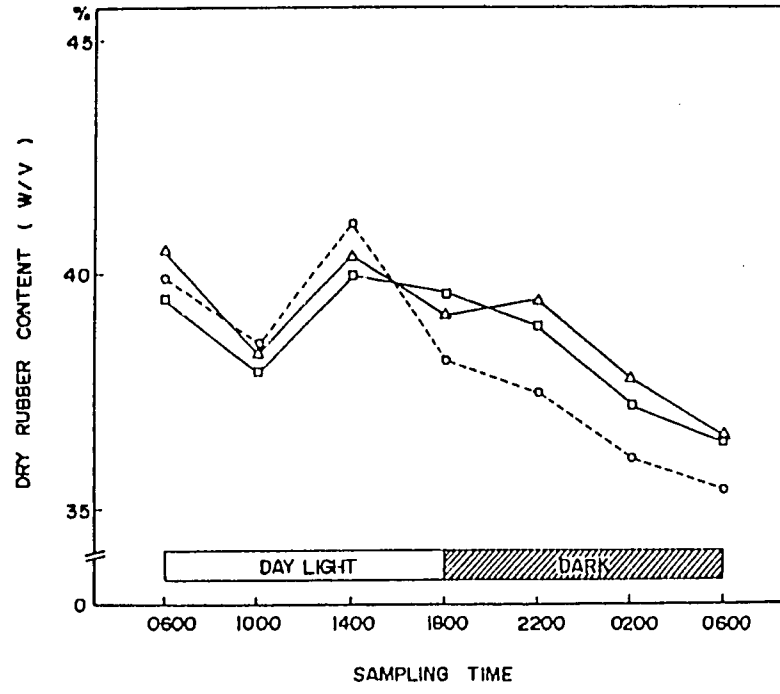

Fig. 11. Diurnal variation of dry rubber content in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○).

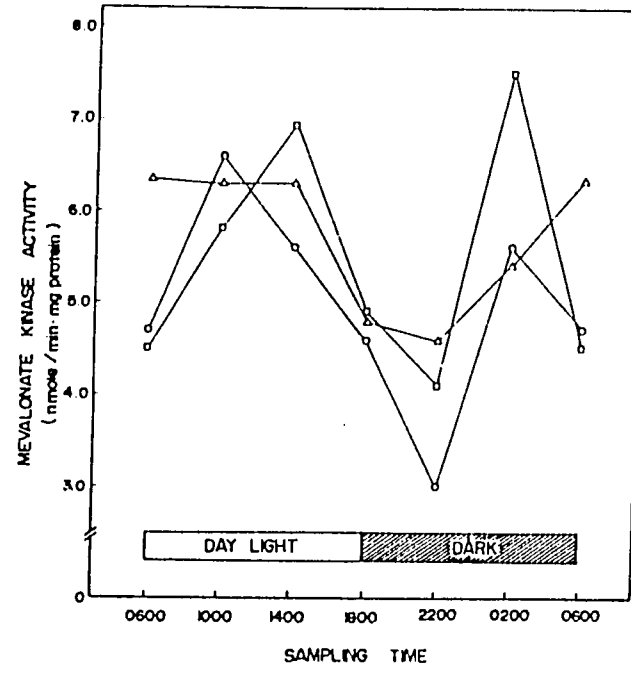

Fig. 12. Diurnal variation of MVA kinase in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○).

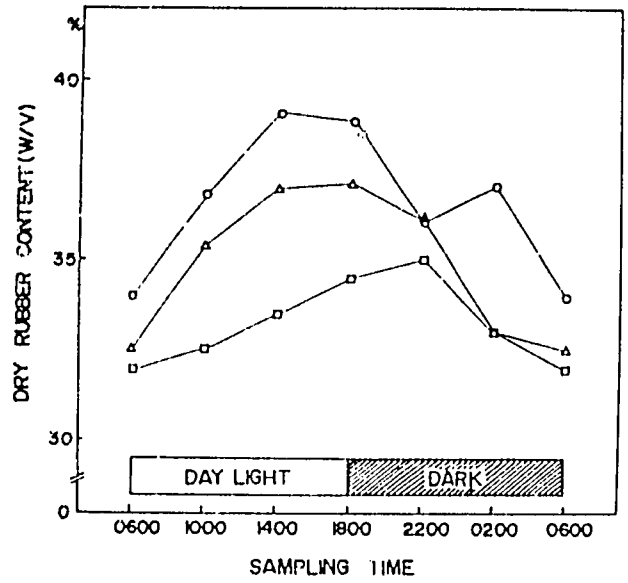

Fig. 13. Diurnal variation of dry rubber content in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○).

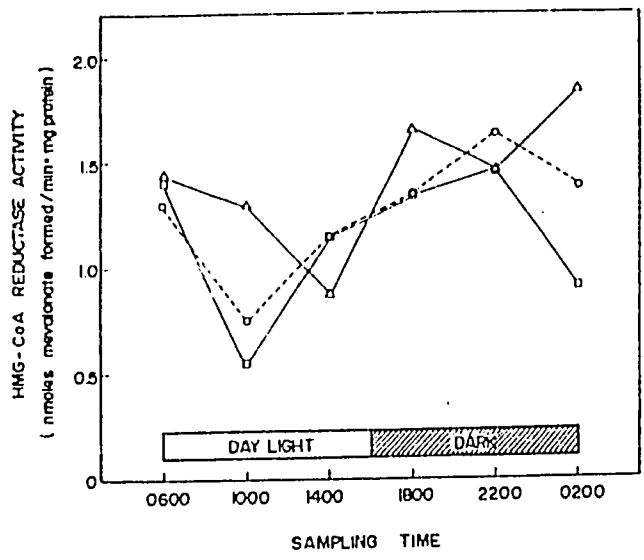

Fig. 14. Diurnal variation of HMGR activity in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○).

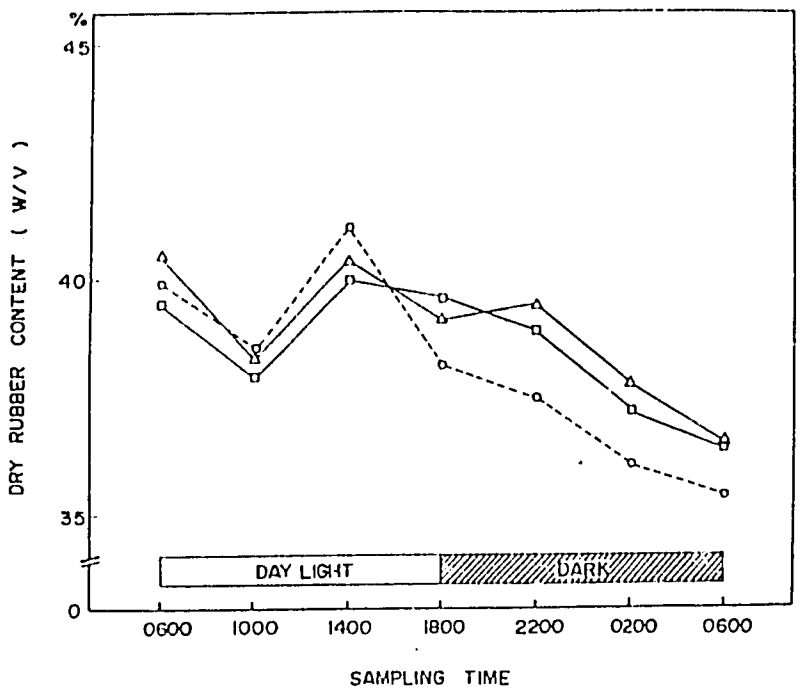

Fig. 15. Diurnal variation of dry rubber content in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○).

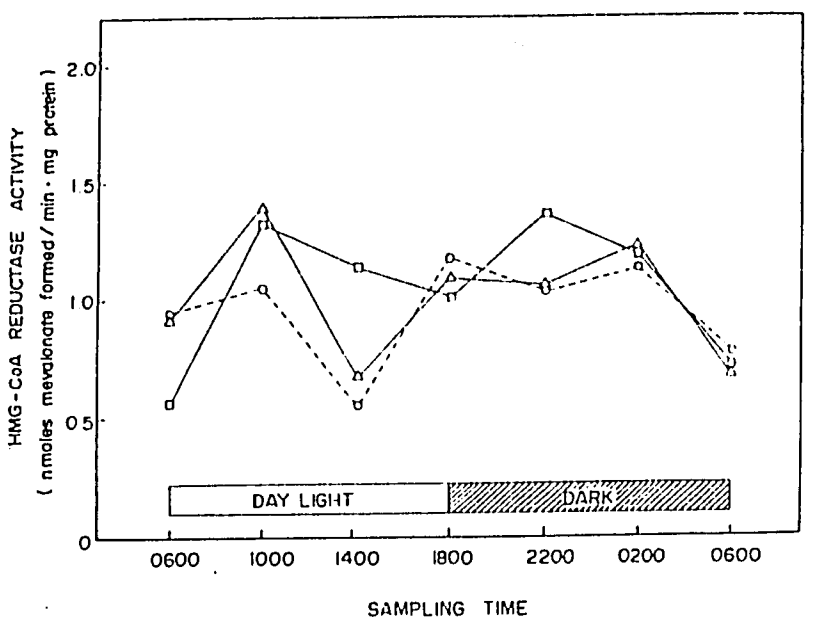

Fig. 16. Diurnal variation of HMGR activity in *Hevea* latex of clones RRIM 600 (□—□), KRS 21 (△—△) and GT 1 (○—○).

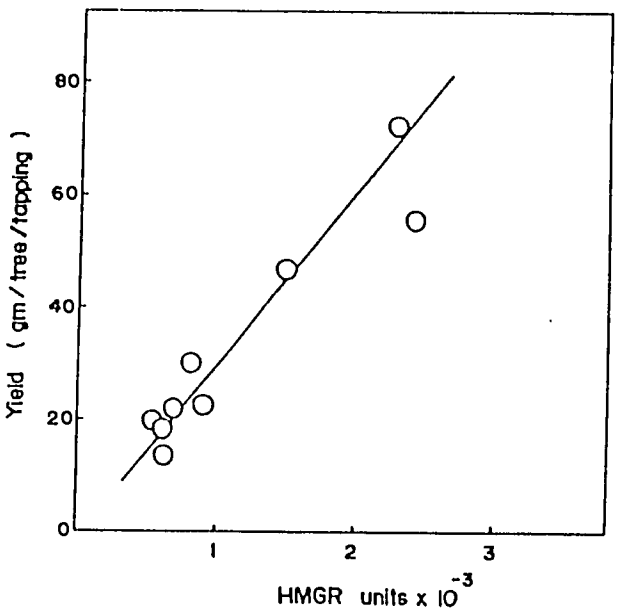

Fig. 17. Intraclonal correlation between total rubber yield and HMGR activity in 9 RRIM 600 trees. Each value is the mean of 2 tappings. The correlation is highly significant (α = 0.01, r=0.76). The unit of enzyme activity was defined as nmoles mevalonate formed per minute.

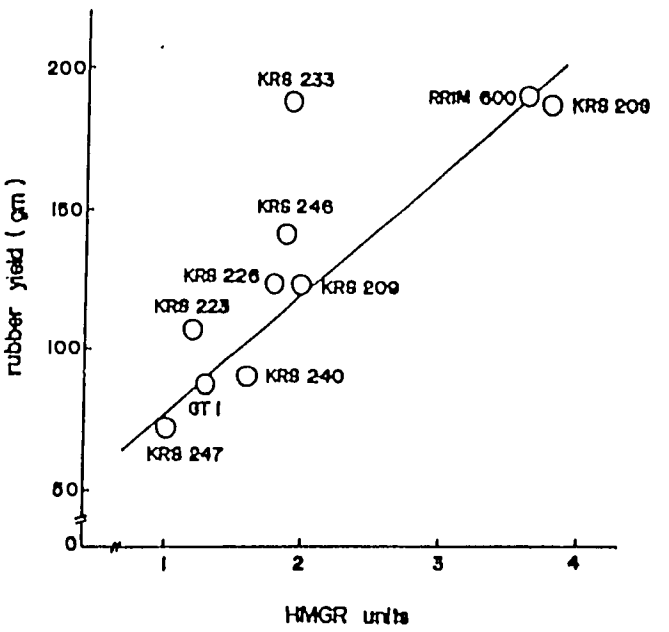

Fig. 18. Interclonal correlation between whole rubber yield and HMGR activity in ten clones. Each value is the mean of 3 tappings x 3 trees per clone. The correlation is highly significant (α = 0.01, r=0.78). The unit of enzyme activity was defined as nmoles mevalonate formed per minute.

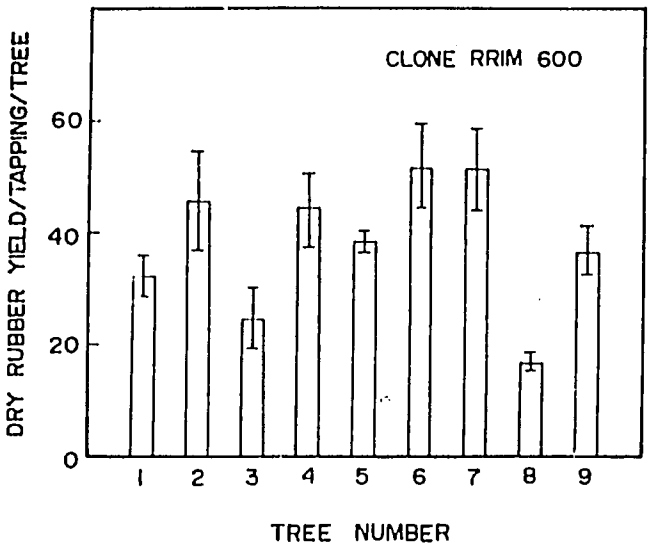

Fig. 19. Intraclonal variation of rubber yield per tapping per RRIM 600 tree. Each value is the mean ± SD of 5 tapping measurements.

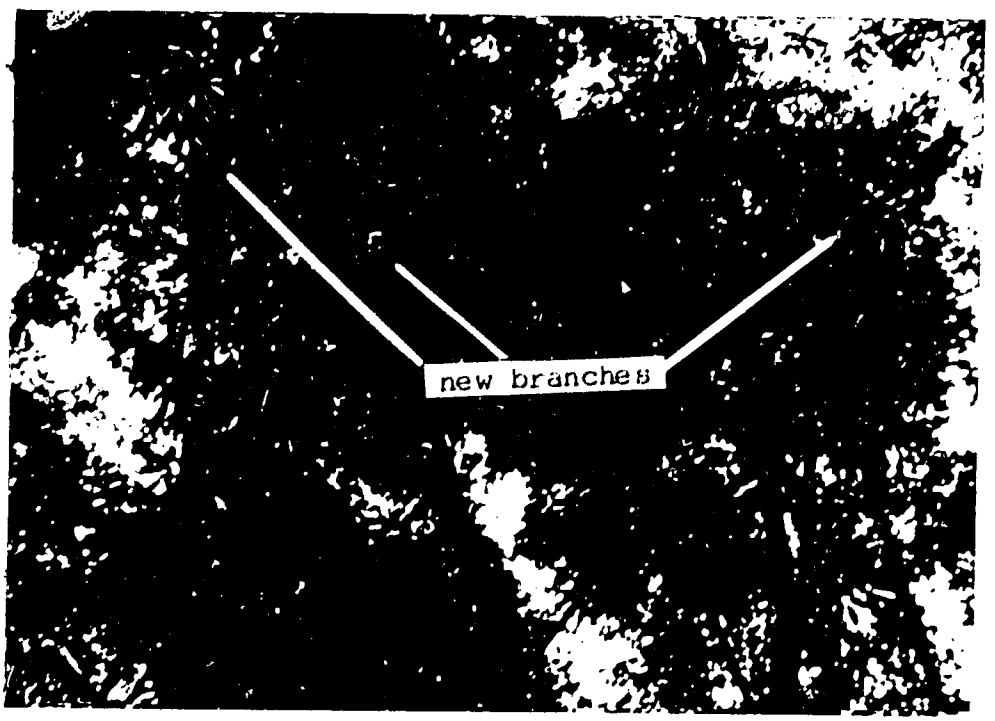

Fig. 20. New young branches were generated after trimming off old branches on RRIM 600 tree. The green buds on these young branches were used for graftings in Fig. 21-22 and Fig. 24-25.

Fig. 21. The young green buds taken from newly generated young branches (as described under Fig. 20) of RRIM 600 trees # 8,4,7 were grafted in an open field on young branch (6 month-old) generated from a common rootstock.

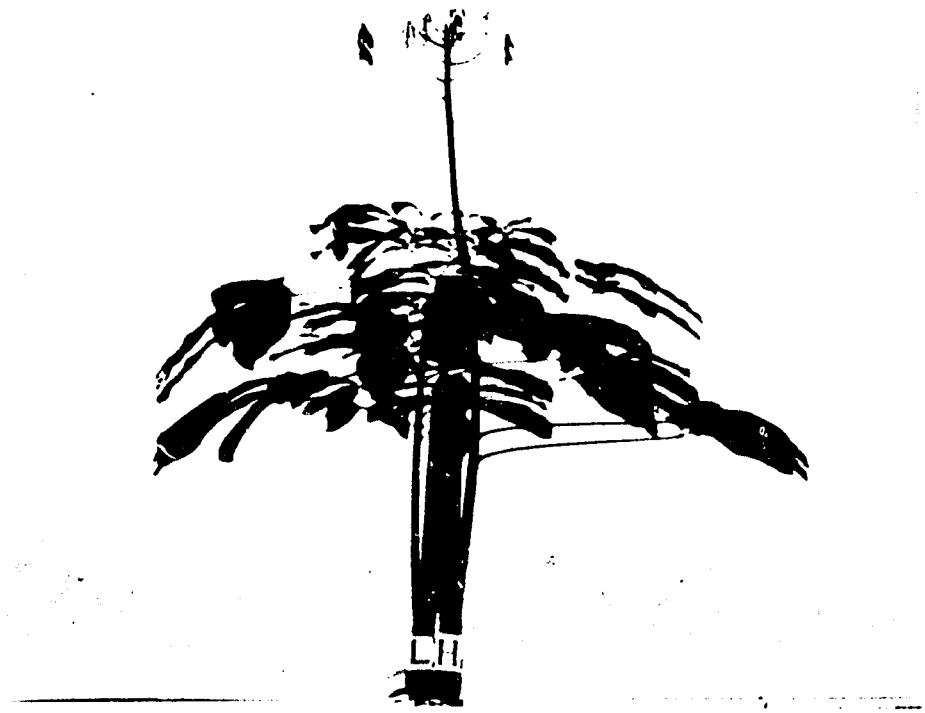

Fig. 22. The young green buds taken from newly generated young branches (as described under Fig. 20) of RRIM 600 tress # 8,4,7 were grafted on a two-year-old rootstock and grown in an experimental control room.

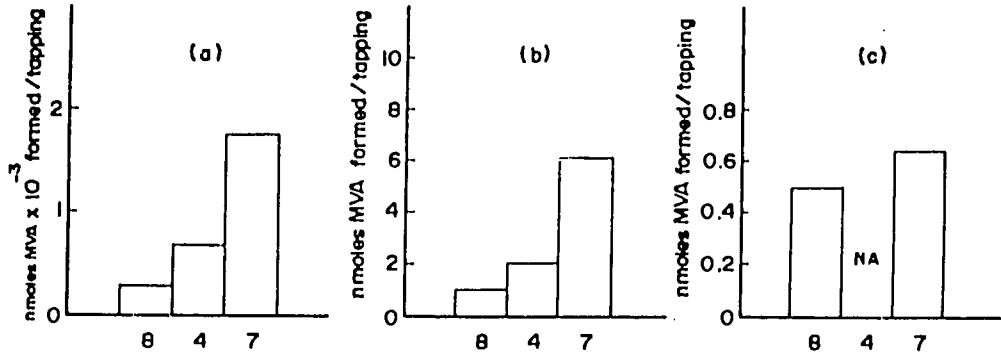

Fig. 23. Comparison study on latex HMGR activity level per tapping obtained from RRIM 600 tree # 8,4,7 (23 a), young buddings generated by graftings as described under Fig. 21 (23 b) and Fig. 22 (23 c).

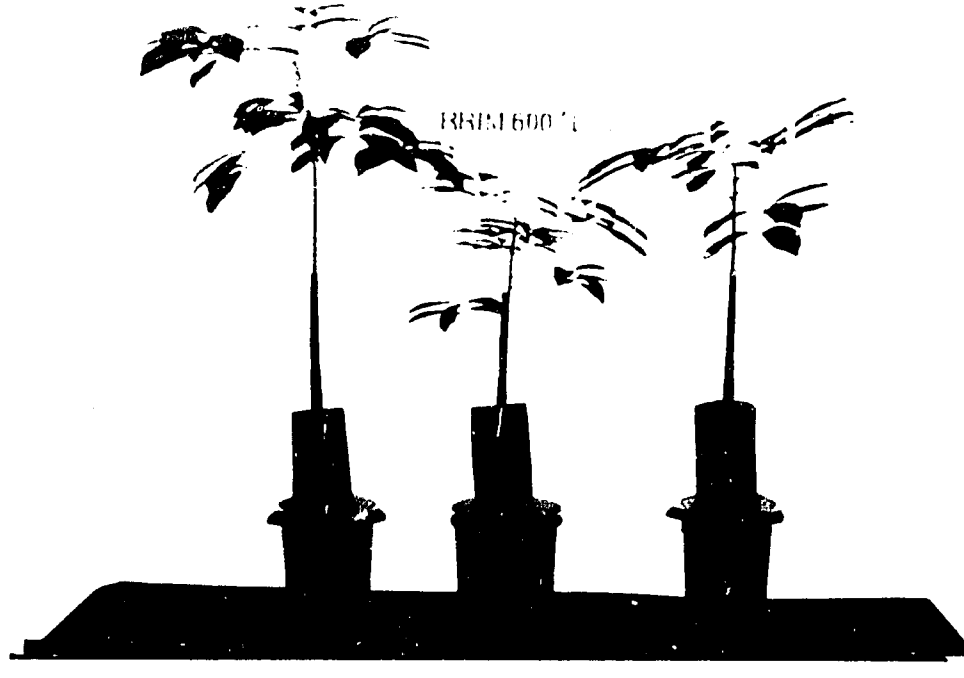

Fig. 24. The young green buds taken from newly generated young branches (as described under Fig. 20) of RRIM 600 trees # 8,4,7 were grafted on one-year-old seedling rootstocks.

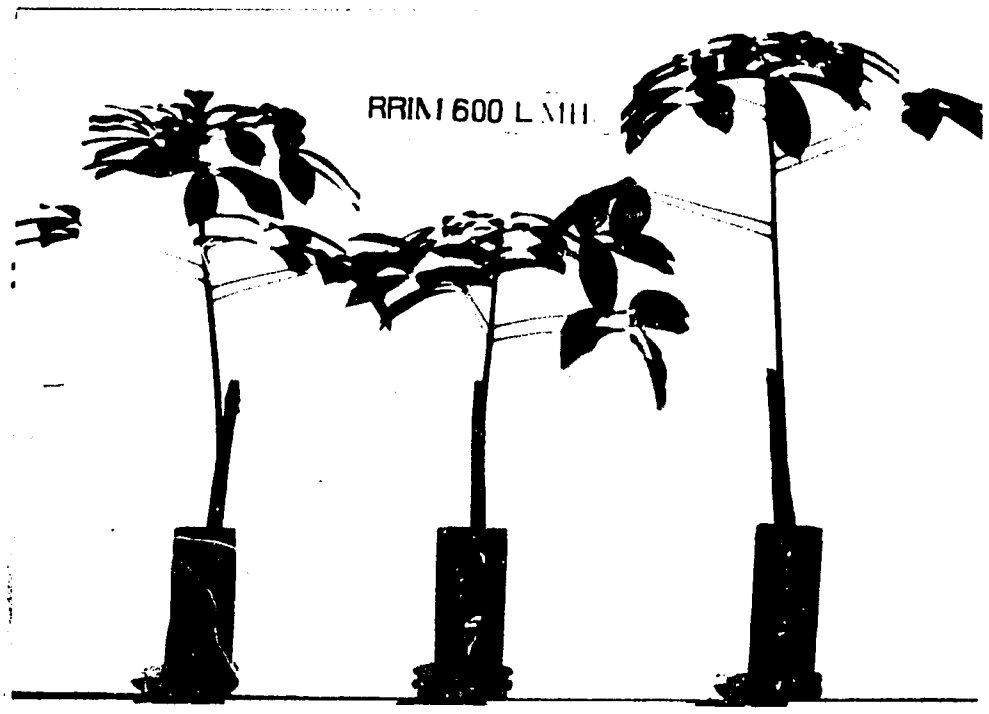

Fig. 25. The young green buds taken from newly generated young branches (as described under Fig. 20) of RRIM 600 trees # 8,4,7 were grafted on 8-month-old seedling rootstocks.

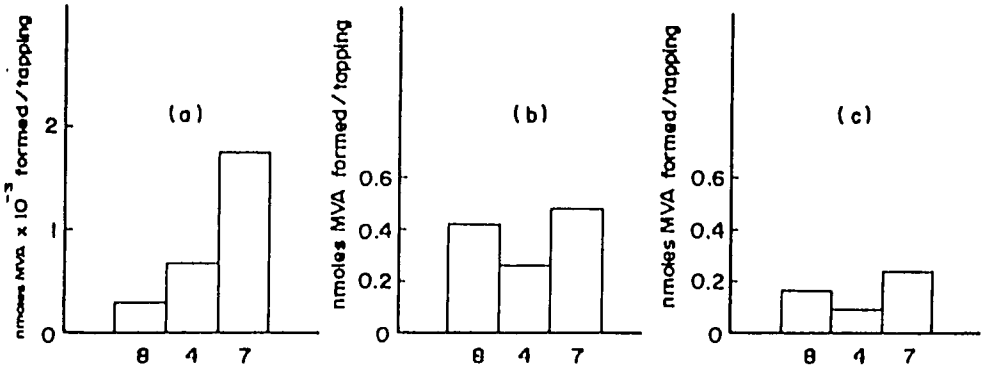

Fig. 26. Comparison study on latex HMGR activity level per tapping from RRIM 600 trees # 8,4,7 (26 a), young buddings generated from graftings as described under Fig. 24 (26 b) and Fig. 25 (26 c)

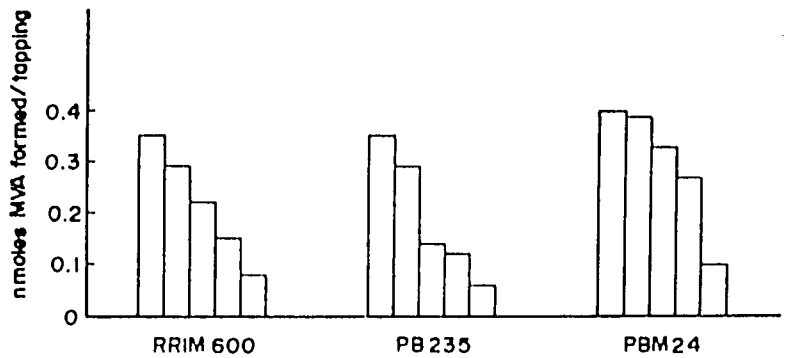

Fig. 27. Differences on activity level of latex HMGR per tapping among three clones of young grafted seedlings obtained from a local nursery.

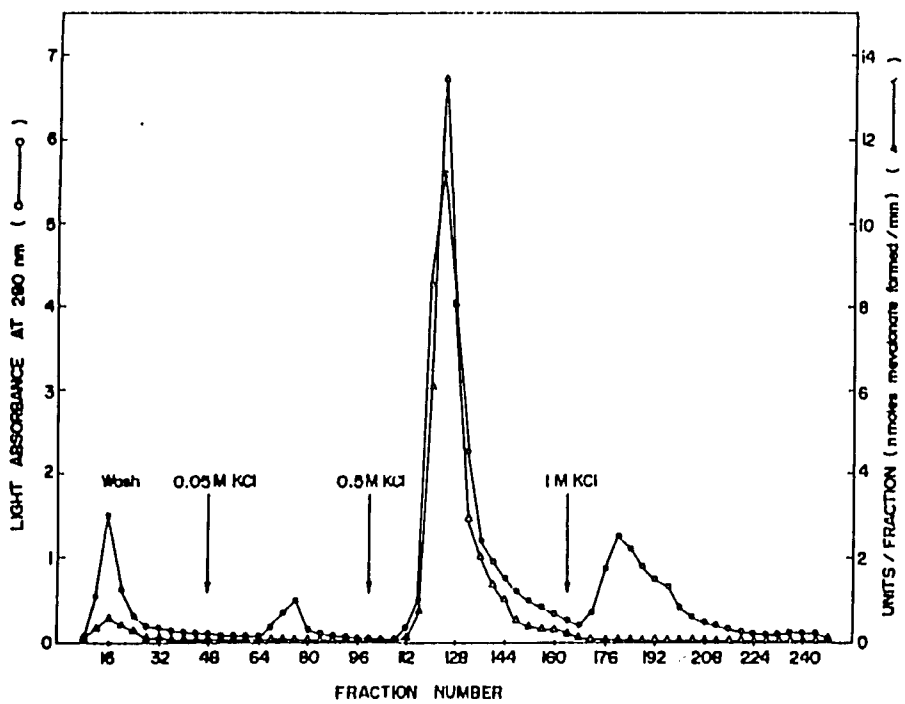

Fig. 28. Purification of detergent-solubilized HMGR on DEAE-Sephadex A 50. Position of arrows, indicating the change of salt concentrations.

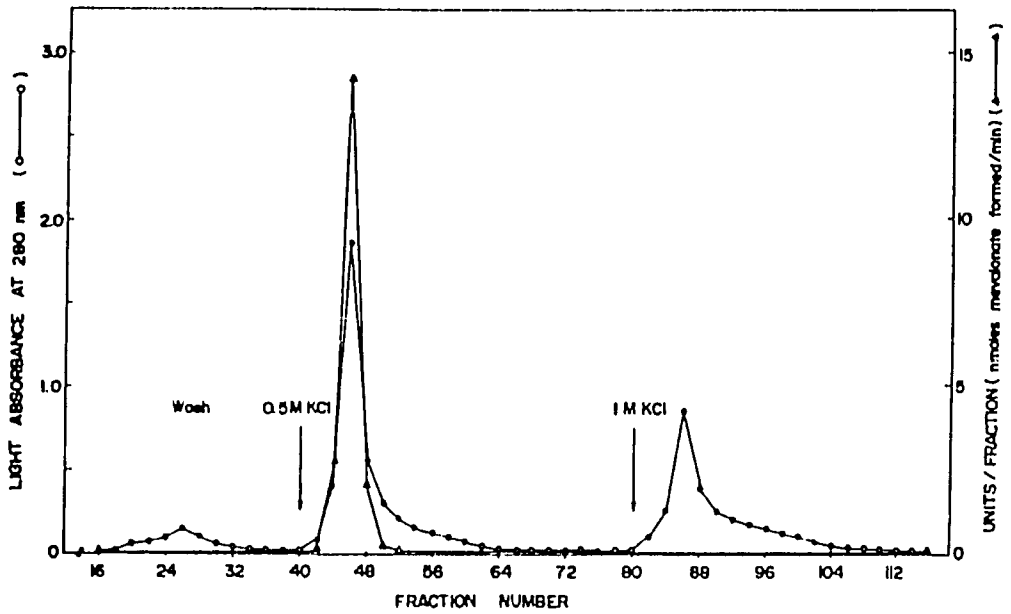

Fig. 29. Purification of active fractions obtained from the DEAE-Sephadex column on Affi-Gel Blue column. Position of arrows indicating changes of salt concentrations.

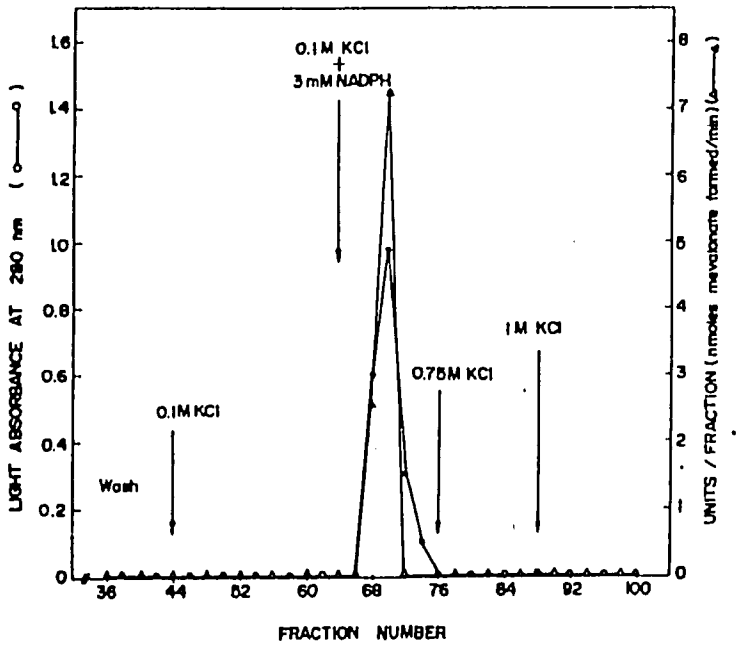

Fig. 30. Purification of HMGR on HMG-CoA-hexane-agarose. Arrows indicate change of salt concentrations with and without NADPH addition.

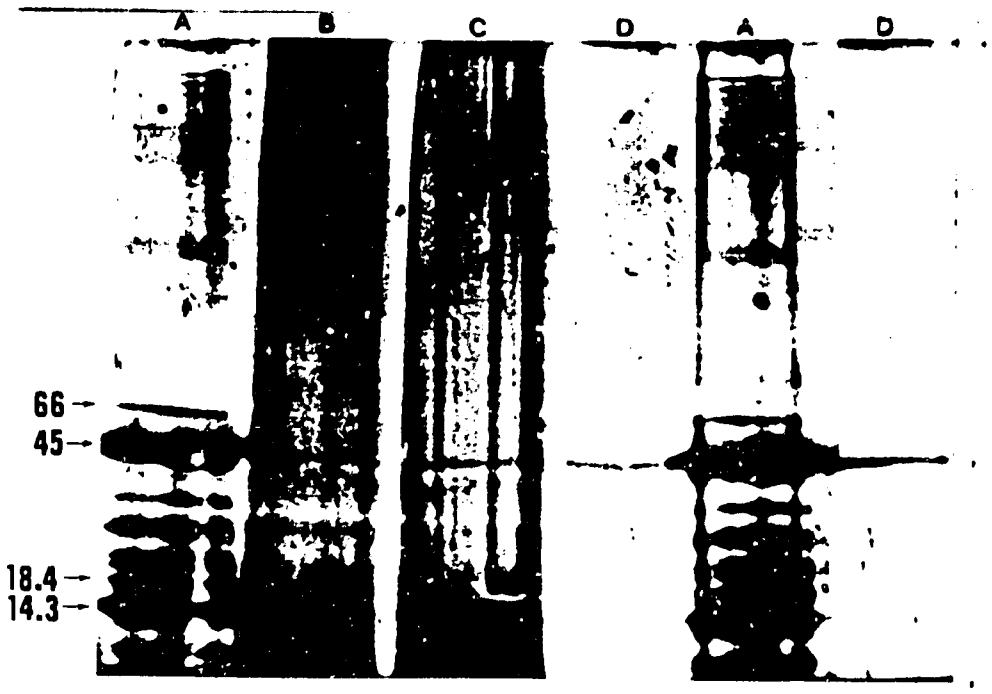

Fig. 31. SDS-PAGE of purified HMGR obtained after HMG-CoA-hexane-agarose column. The relative positions of protein standards are as indicated in lane A (K Da). Lanes B,C and D represent peak fraction proteins from DEAE Sephadex, Affi-Gel Blue and HMG-CoA-hexane-agarose columns, respectively.

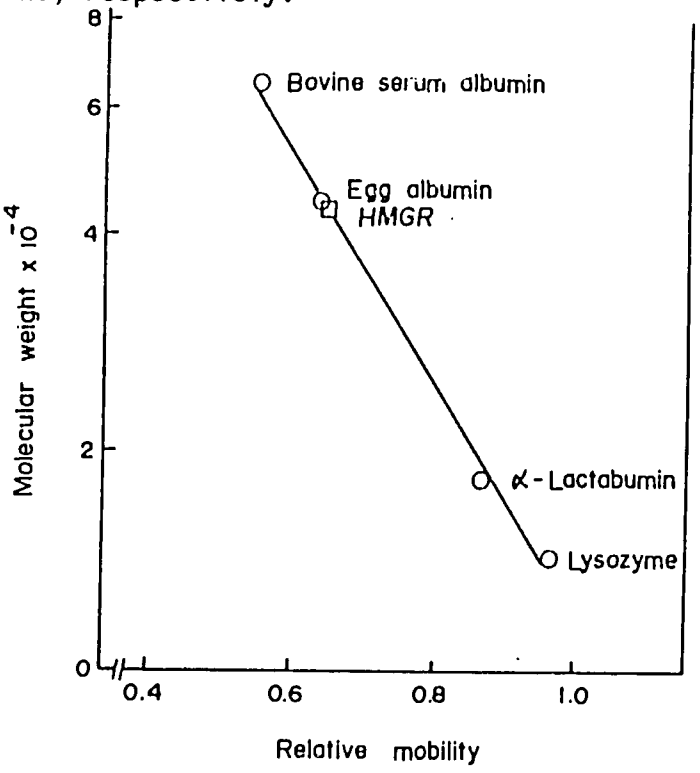

Fig. 32. Molecular weight calibration of HMGR monomeric polypeptide. Both purified enzyme and standard proteins were performed on the same slab gel under 8% SDS-PAGE.

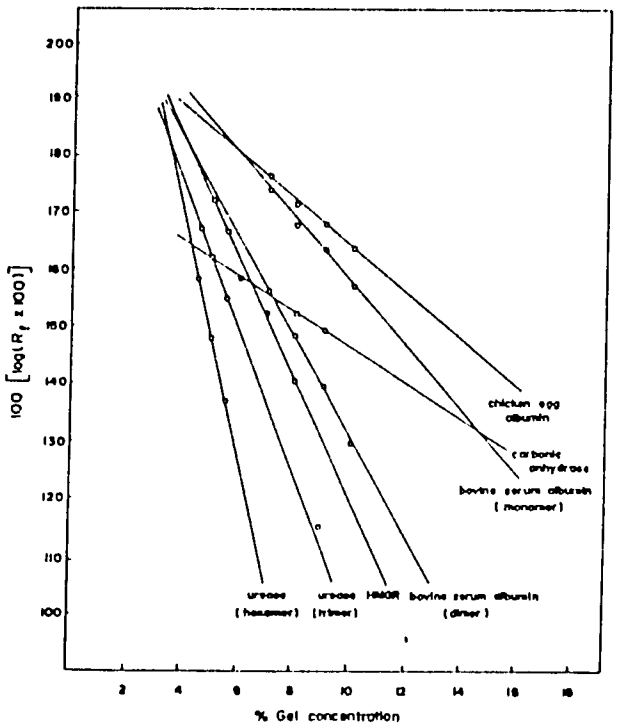

Fig. 33. Determination of retardation coefficient (slope) of standard proteins for HMGR molecular weight calibration in non-denaturing gel electrophoresis as described in Sigma Tech. Bulletin No. MRK-137 (10-86).

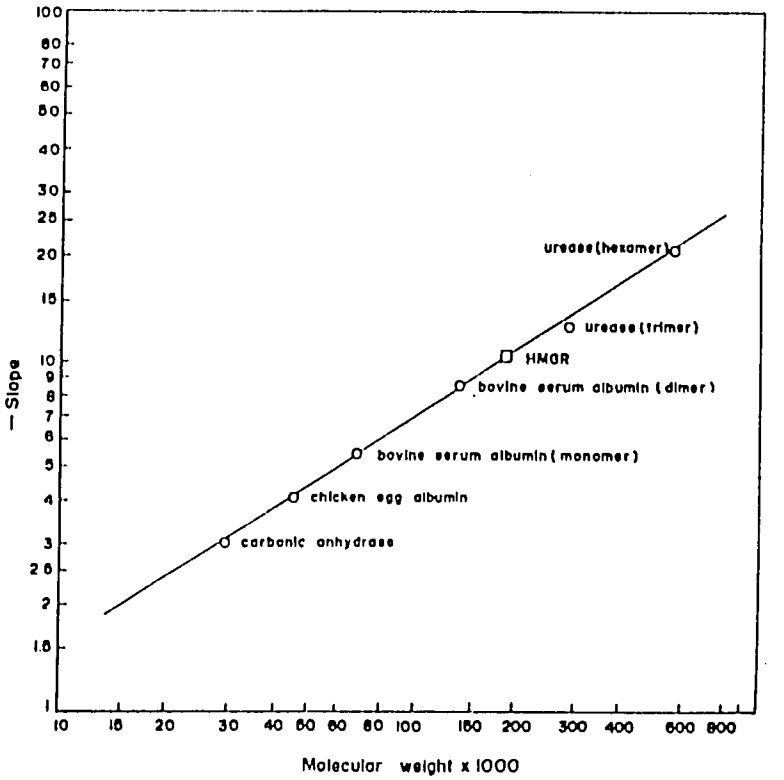

Fig. 34. Molecular weight calibration of HMGR in a non-denaturing gel electrophoresis performed according to method described in Sigma Tech. Bulletin No. MRK-137 (10-86).

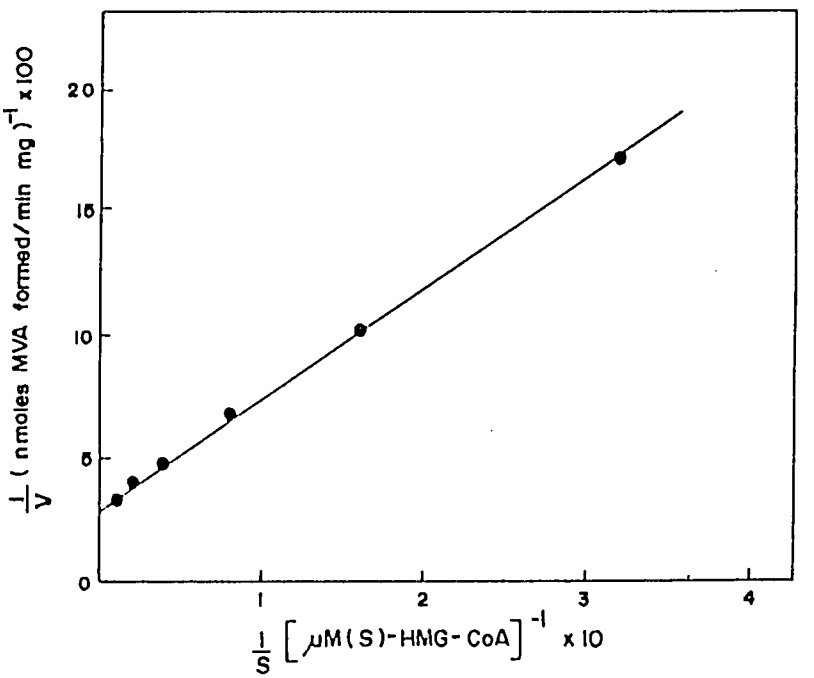

Fig. 35. Affinity of purified HMGR for (S)-HMG-CoA. The assay mixture contains 10 ug of purified HMGR, 0.4 mM NADPH 0.1 M KCl, 10 mM DTT, 5 mM EDTA in K_xPO_4 buffer, pH 7.0.

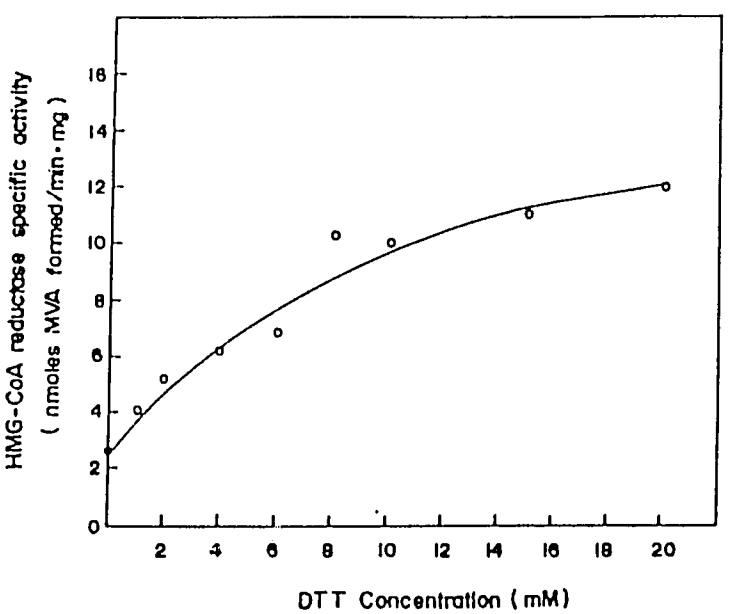

Fig. 36. Effect of dithiothreitol on purified HMGR activity. The purified HMGR was suspended in buffer (see methods) from which dithiothreitol was omitted and enzyme activity was then assayed.

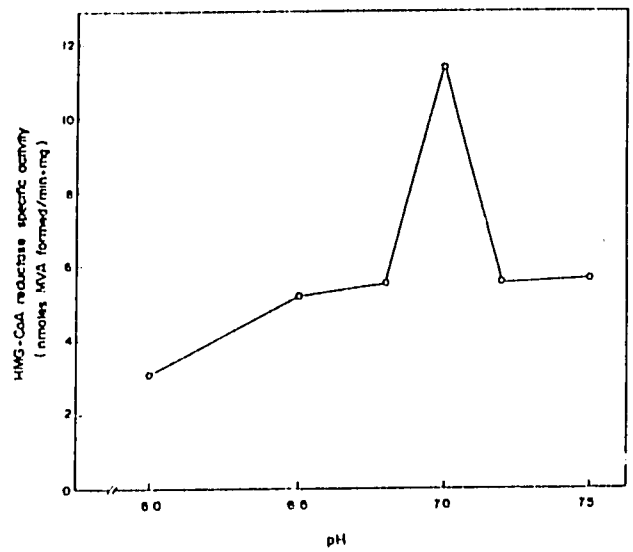

Fig. 37. Activities of purified HMGR at different pHs. The enzyme was assayed in 0.1 M K_XPO_4 buffer (pH 7) containing 0.1 M KCl, 10 mM DTT 5 mM EDTA as described under methods.

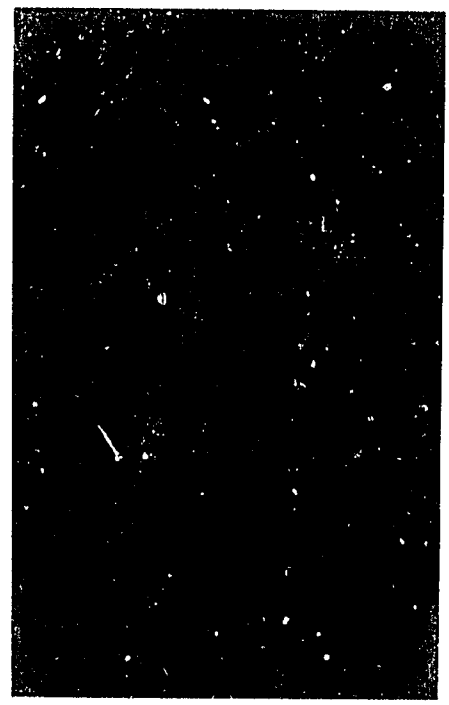

Fig. 38. Ouchtolony double diffusion in 0.9% agar, center well contained 0.87 mg of rabbit antiserum raised against HMGR, surrounding wells contained 1.2 ug of purified HMGR except for the well marked 'x' contained only buffer.

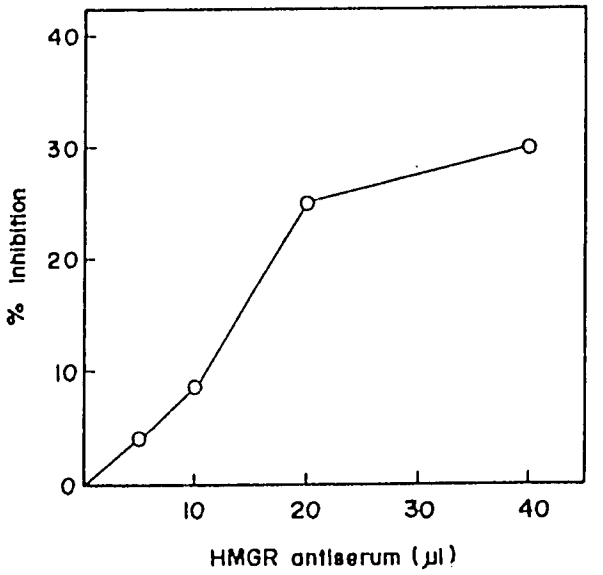

Fig. 39. Inhibition of HMGR activity by rabbit antiserum raised against HMGR

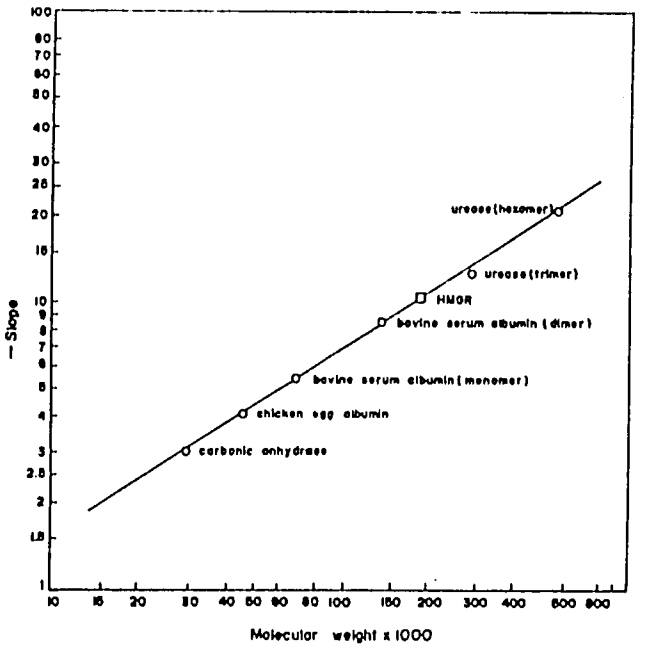

Fig. 40. Effects on activity levels of HMGR in the latex bottom fractions by the C-serum prepared from high-(empty bar), medium-(hatched bar), and low-(solid bar) yielding trees. Latex from the high-, medium-, and low-yielding trees were also used to prepare HMGR bottom fraction for assays as indicated. Control is 100% activity.

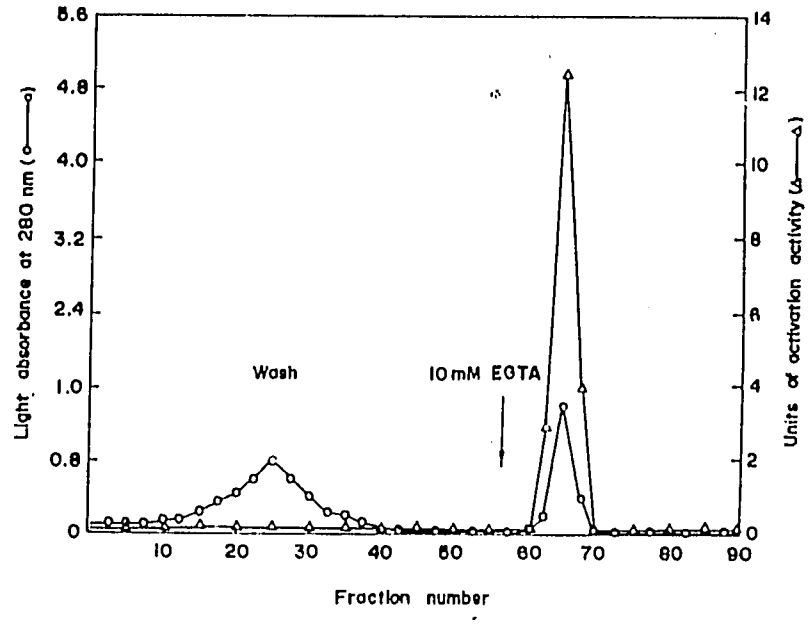

Fig. 41. Purification of *Hevea* calmodulin on Affi-Gel Phenolthiazine column. The column was washed with 50 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM $CaCl_2$ and 3 N NaCl and was eluted with the same buffer containing 10 mM EGTA.

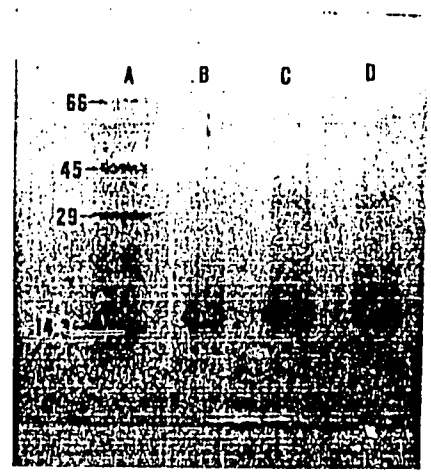

Fig. 42. SDS-PAGE of purified *Hevea* calmodulin using 10% gel. Lane A is standard proteins (KDa); Lane B,C or D is peak fraction from Affi Gel phenothiazine column at different protein concentrations.

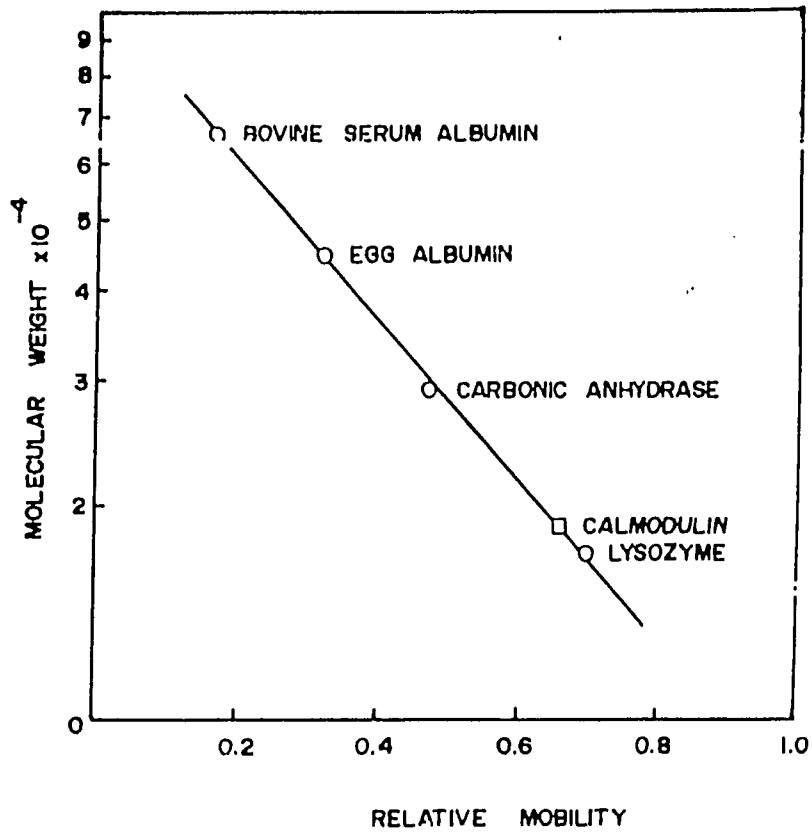

Fig. 43. Molecular weight calibration of purified *Hevea* calmodulin. Both calmodulin and standard proteins were performed on slab gel under 10% SDS-PAGE.

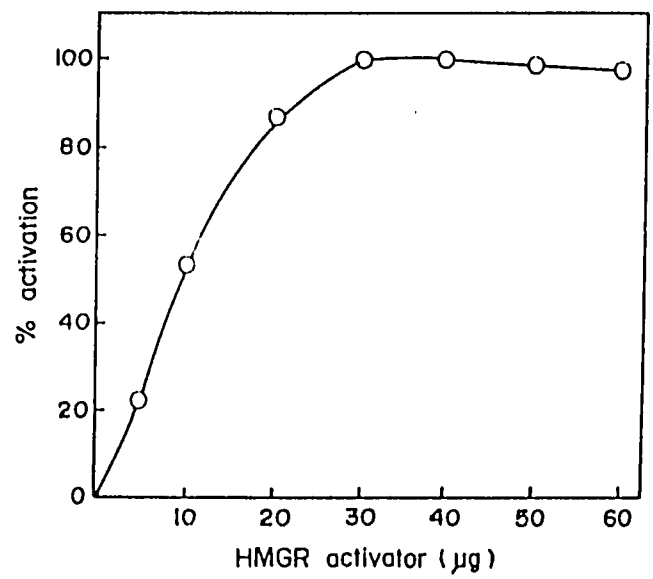

Fig. 44. Effect of varying concentrations of HMGR activator (calmodulin) on the HMGR activity in latex bottom fraction. Maximum activation was obtained when 30 ug protein was added to 1 ml reaction mixture.

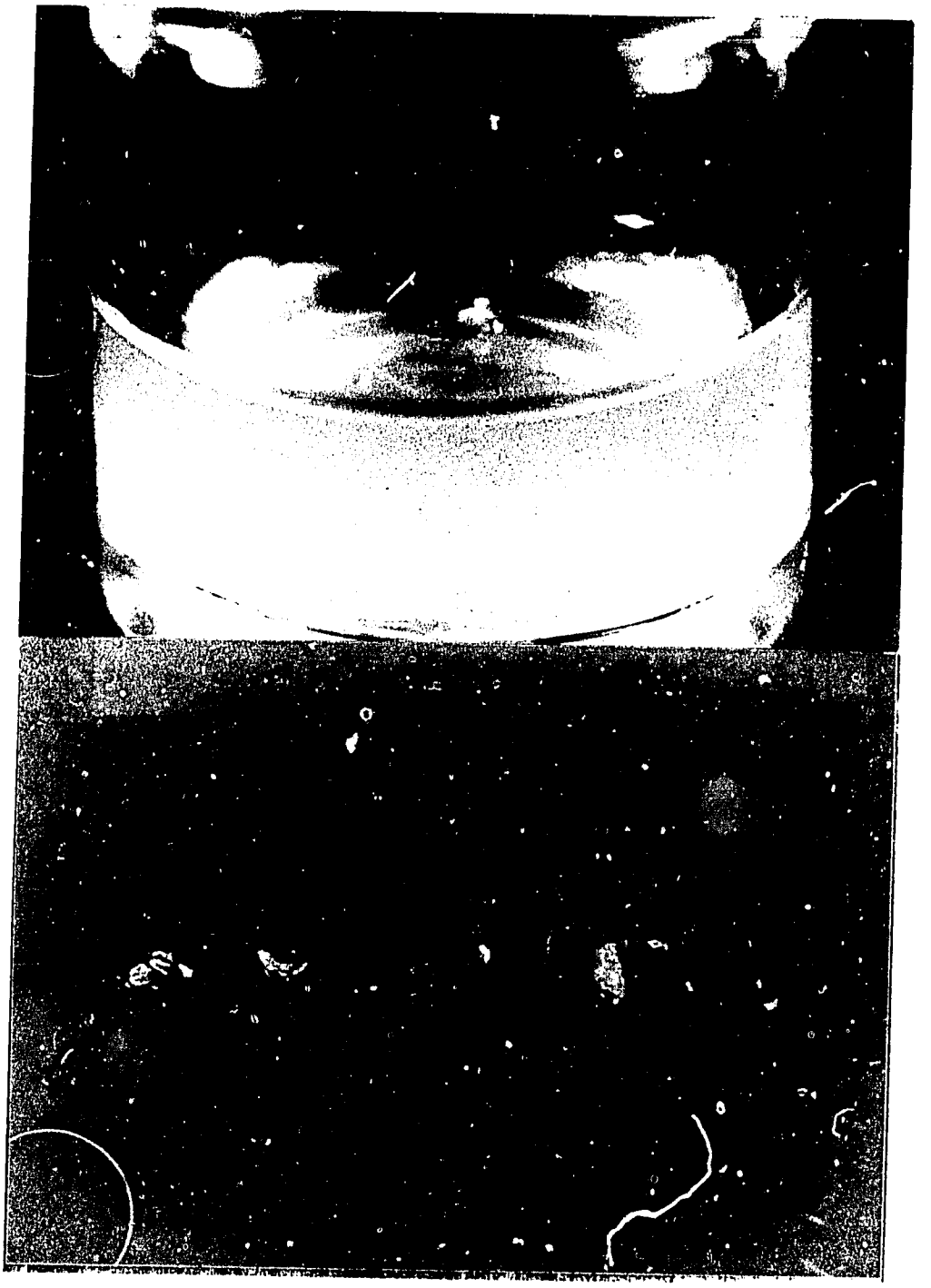

Fig. 45. Callus initiation from anther of *Hevea* male flower of clone PR 255 (top). Progress on callus growth at 1,3,4,5 weeks after initiation is also respectively shown (bottom).

Fig. 46. Callus mass from *Hevea* anther culture of clone RRIM 600 at 2 month-old. (left top), 2.5 month-old (right top) and 3 month-old (bottom)

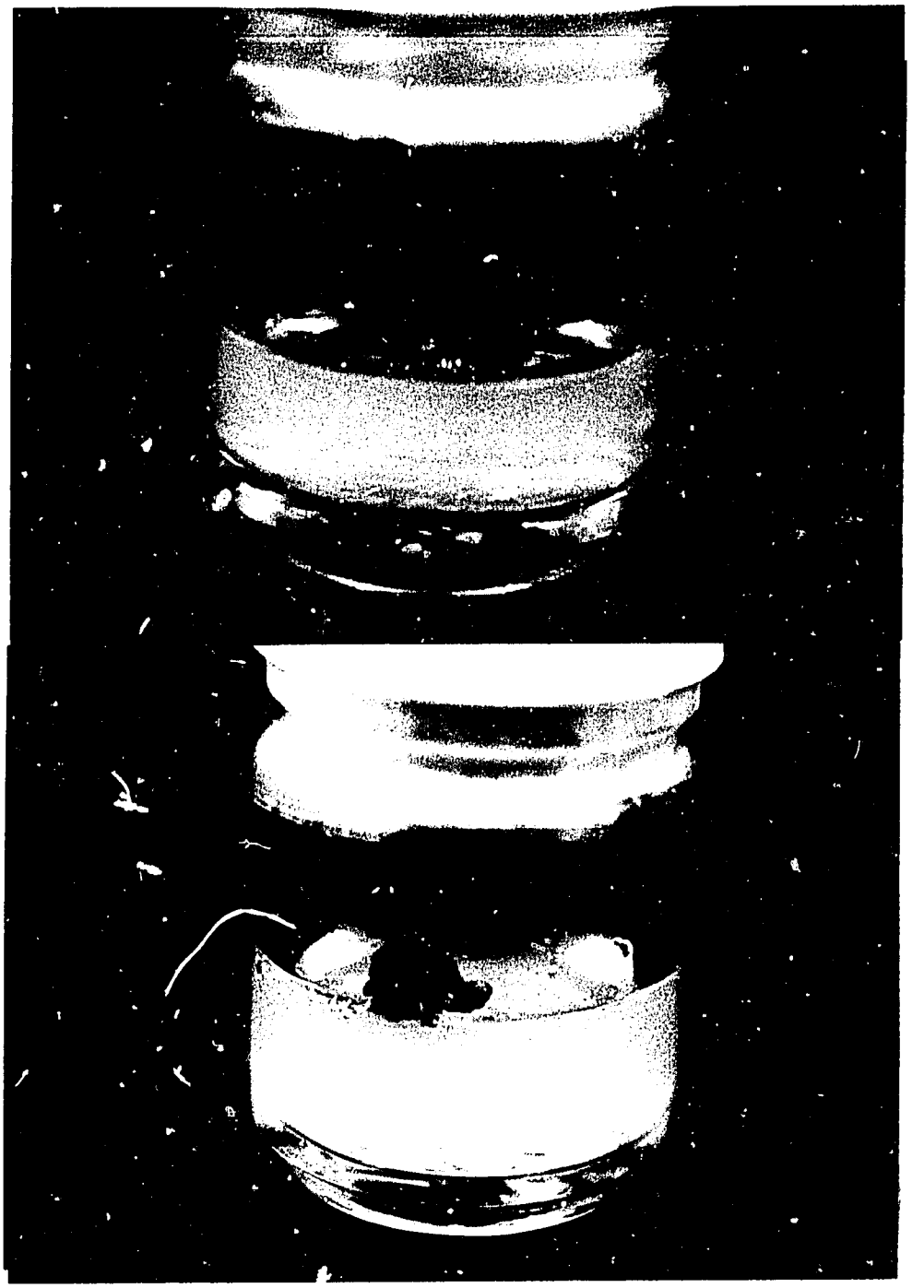

Fig. 47. Callus initiation from male flower (anther) of clone PR 255 at the age of 2 month-old.

Fig. 48. Callus derived from *Hevea* male flower of PR 255 at the age of 4 month-old.

Fig. 49. Callus mass from male flower of *Hevea* clone RRIC 6 at the age of 6 month-old.

Fig. 50. Young plantlet from embryo culture of *Hevea brasiliensis*

Fig. 51. Callus initiation of young *Hevea* shoot explant.

Fig. 52. Callus initiation of young *Hevea* stem explant.

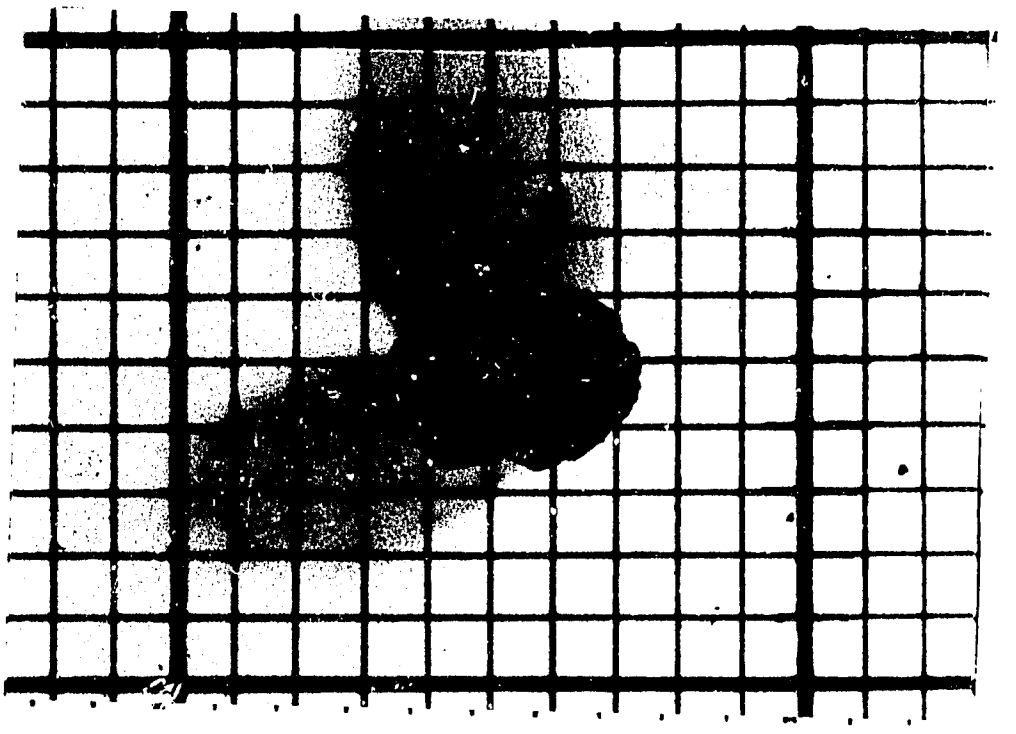

Fig. 53. An example of callus which changed color from yellowish green to brown and died following the 4th weeks as indicated by asterisks in Table 14.

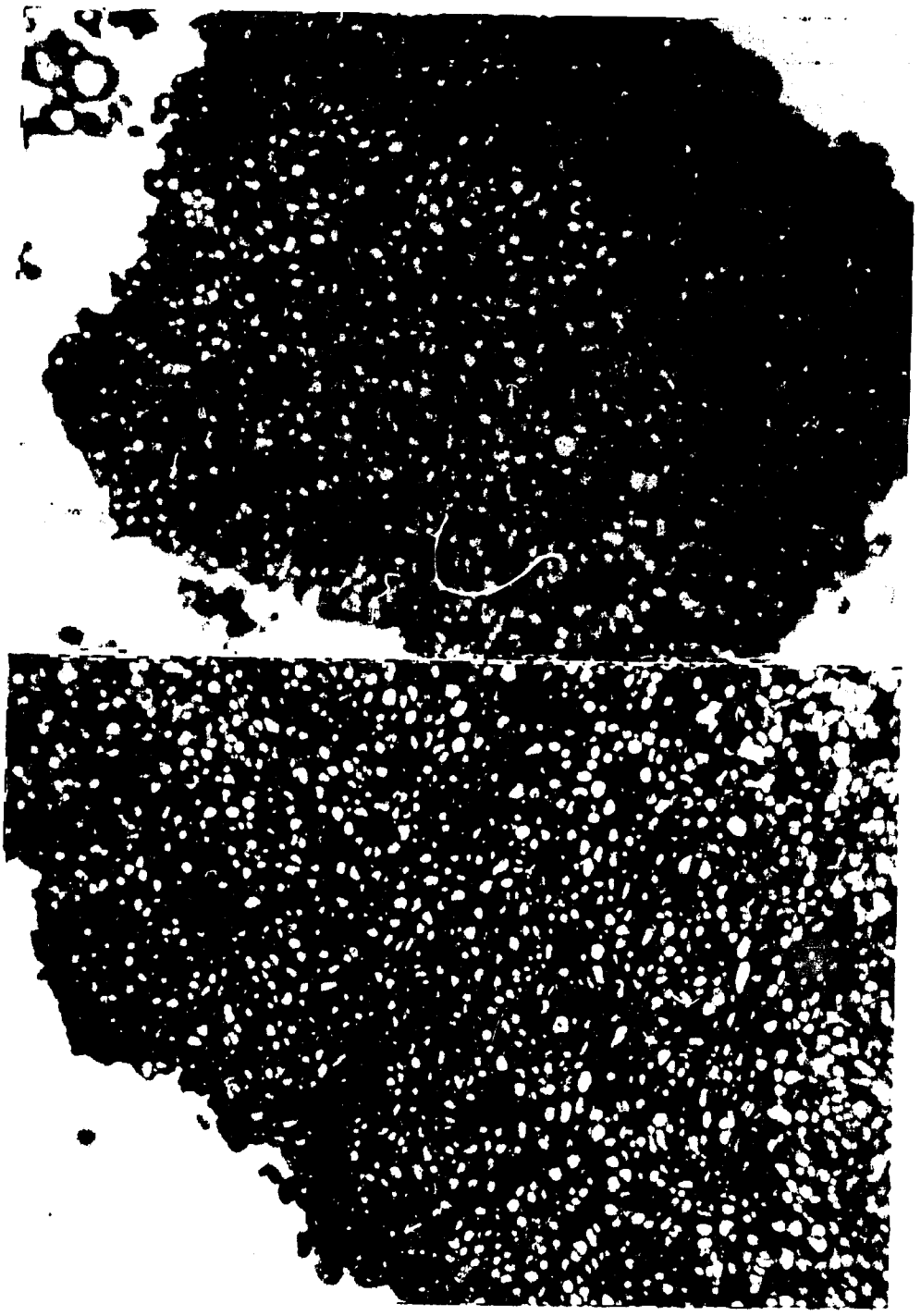

Fig. 54. Cross-section of degenerated callus as shown in Fig. 53. Color of cells arose from phenol-blackened spots on callus were stained reddish purple while normal cells were greenish blue. Callus appears to be compact but not embryogenic.

Fig. 55. Inner seed tegument slices as prepared from young immature *Hevea* seed.

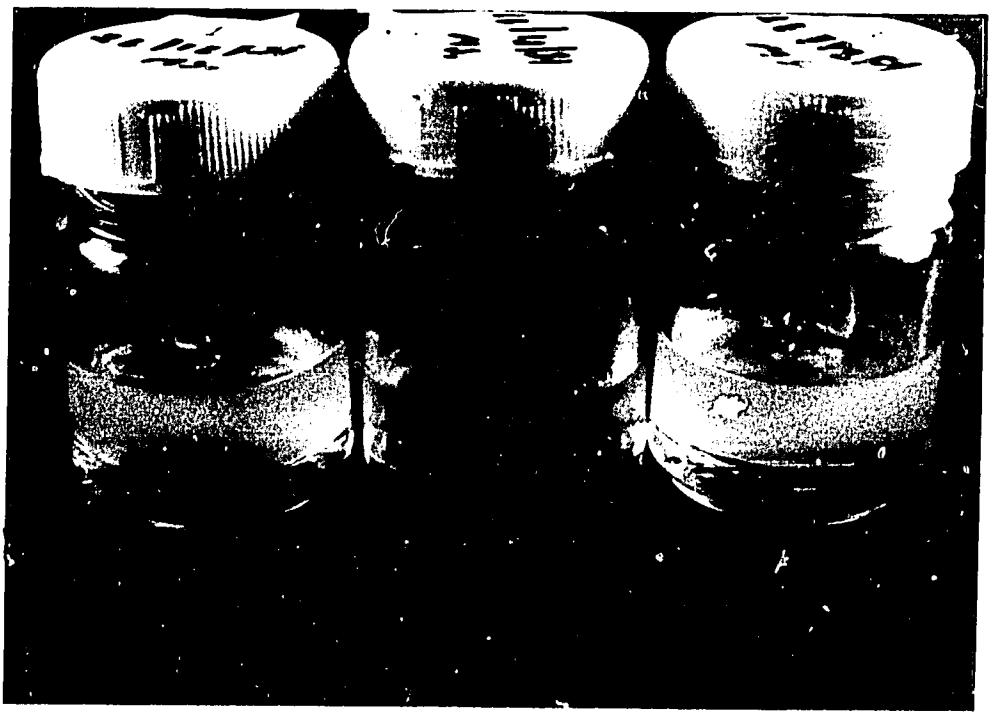

Fig. 56. Callus initiation from inner seed tegument at 2 week-old.

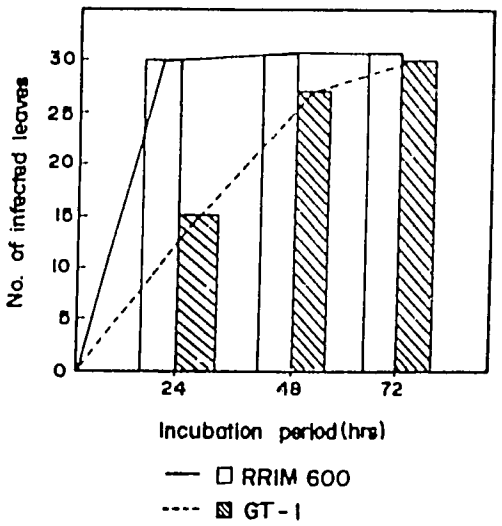

Fig. 57. Disease resistance study of life pathogen (*Phytophthora botryosa)* infection on young RRIM 600 and GT-1 leaves. The number of infected leaves (columns) were recorded at 24, 48 and 74 hours after inoculations. Solid and dotted lines represent infection rate of RRIM 600 and GT 1 clones, respectively.

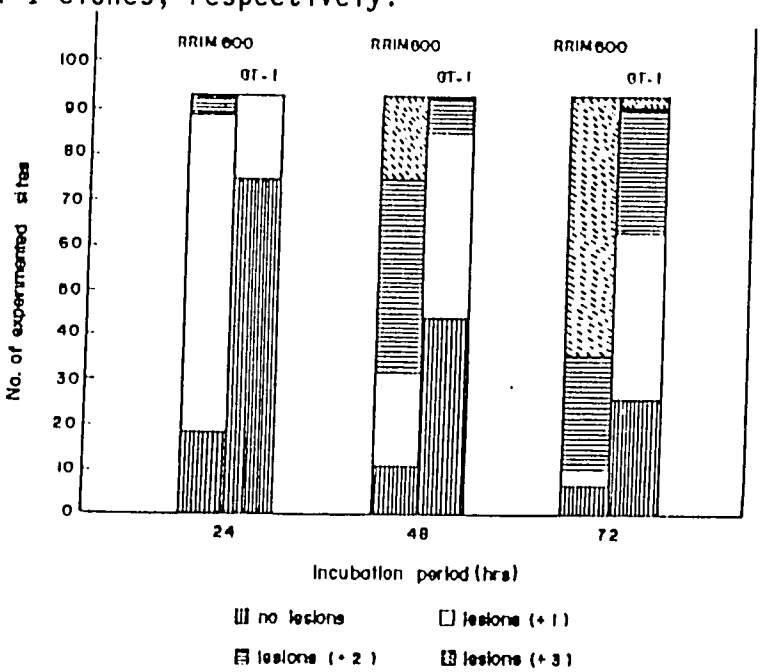

Fig. 58. Degrees of lesion intensity are compared after 24,48 and 72 hours of life pathogen inoculations on young RRIM 600 and GT 1 leaves. The empty columns represent numbers of inoculated sites that show low degree of lesion intensity with +1 score. Columns filled with axis, vertical and tilted broken lines represent numbers of inoculated sites that show no lesion, medium (+2) and high (+3) degree of lesion intensities respectively.

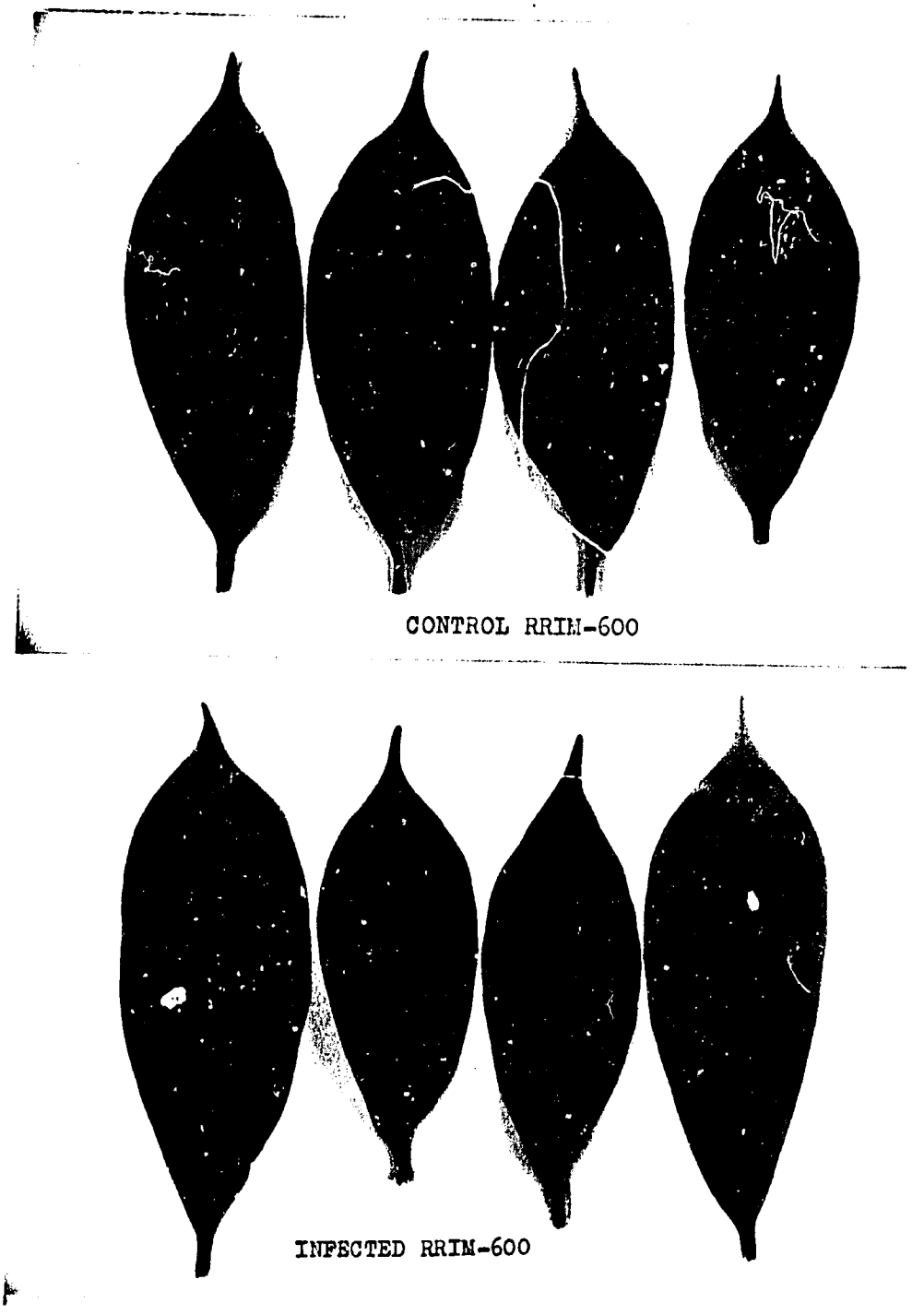

Fig. 59. Control and infected RRIM 600 leaves after 24 hours of inoculation time. Lesion scores are given on the following page.

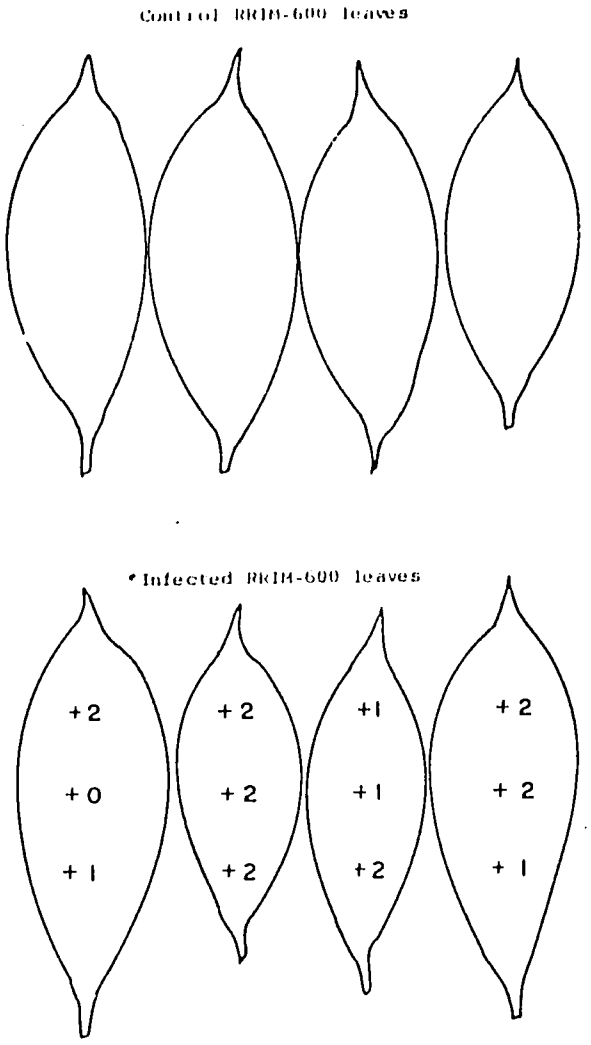

Fig. 59. Lesion intensity scores of the inoculation sites on the infected RRIM 600 leaves after 24 hour of inoculation time.

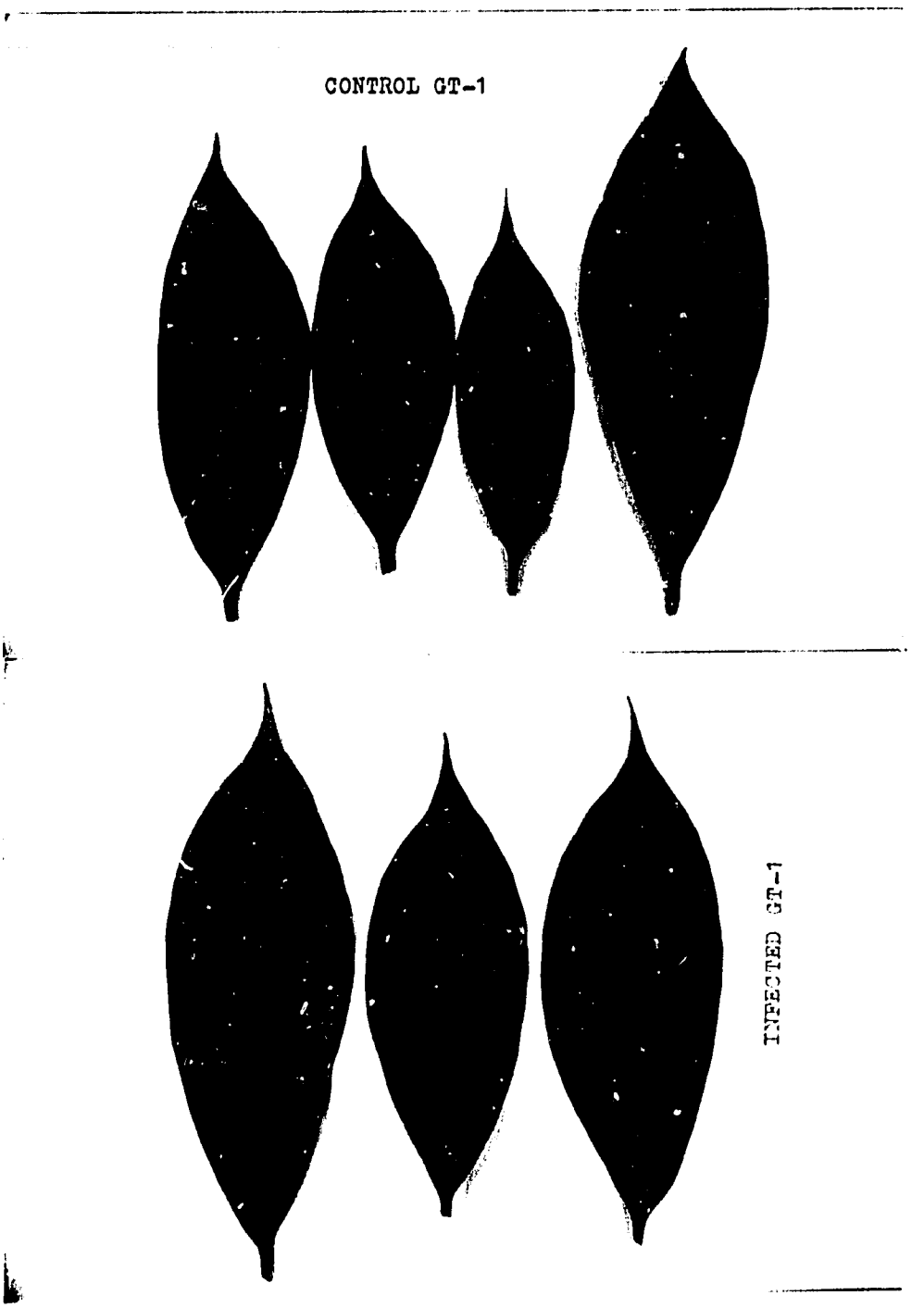

Fig. 60. Control and infected GT-1 leaves after 24 hour of inoculation time. Lesion scores are given on the following page.

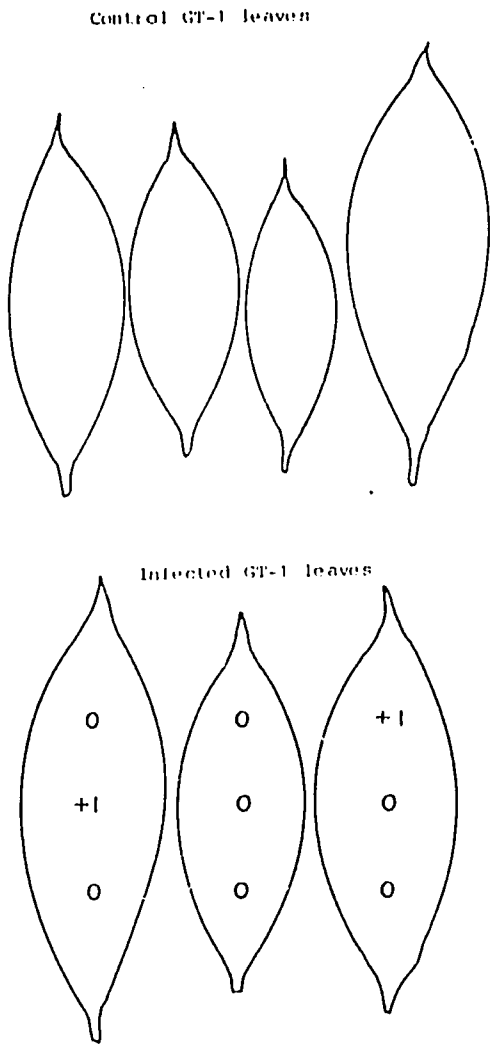

Fig. 60. Lesion intensity scores of the inoculation sites on the infected GT-1 leaves after 24 hours of inoculation time.

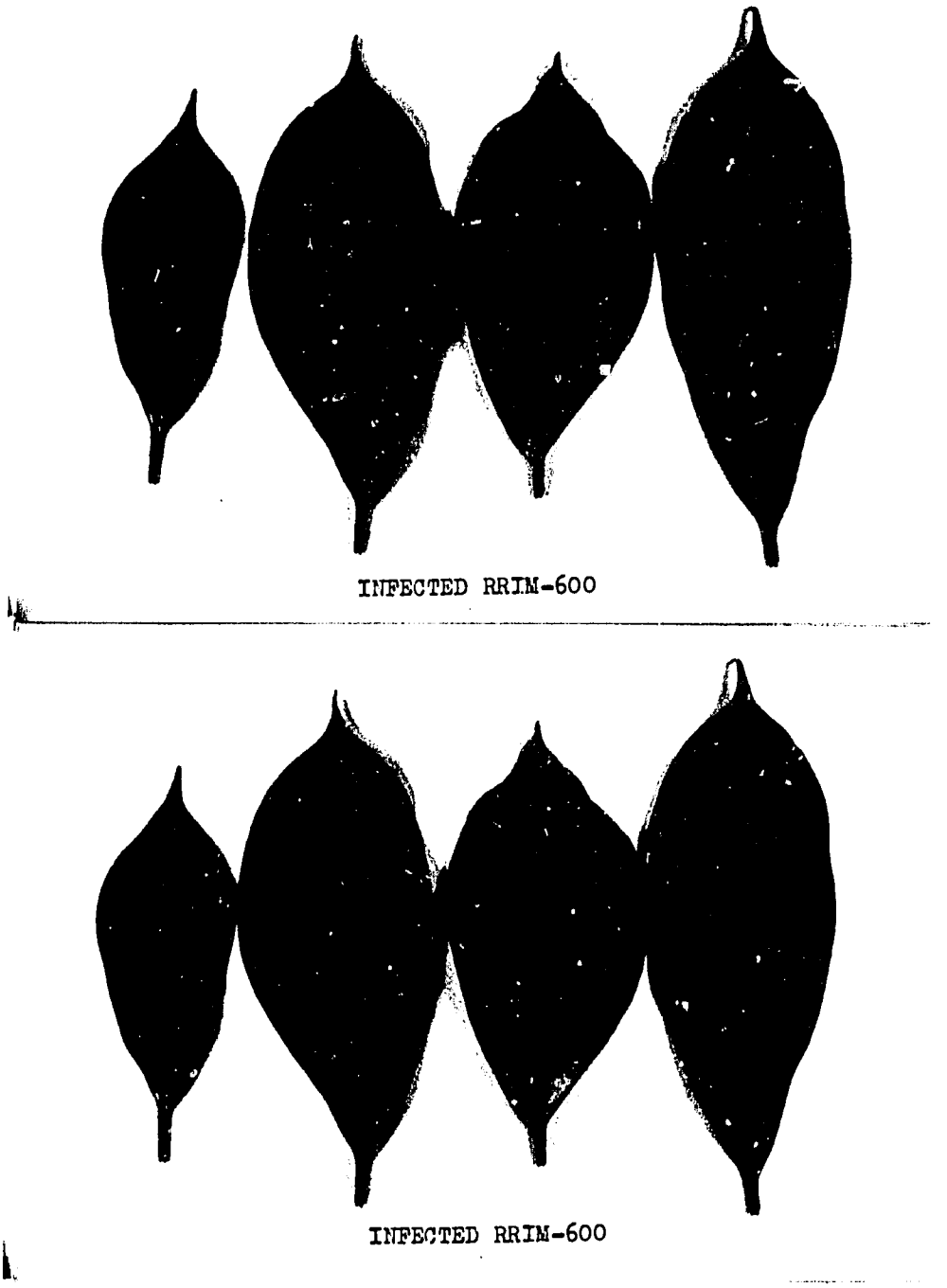

Fig. 61. Infected RRIM 600 leaves after 48 hours of inoculation as viewing before (upper picture) and after (lower picture), removal of agar discs containing pathogenic fungus (*Phytophthora botryosa*). Lesion scores are given on the following page.

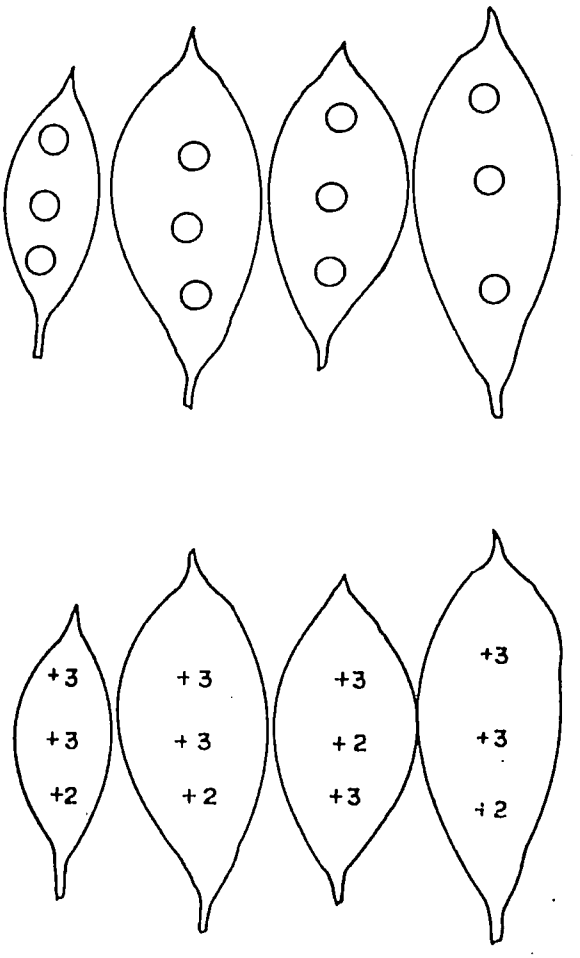

Fig. 61. Lesion intensity scores at inoculation sites of infected RRIM 600 leaves after 48 hours of inoculation time.

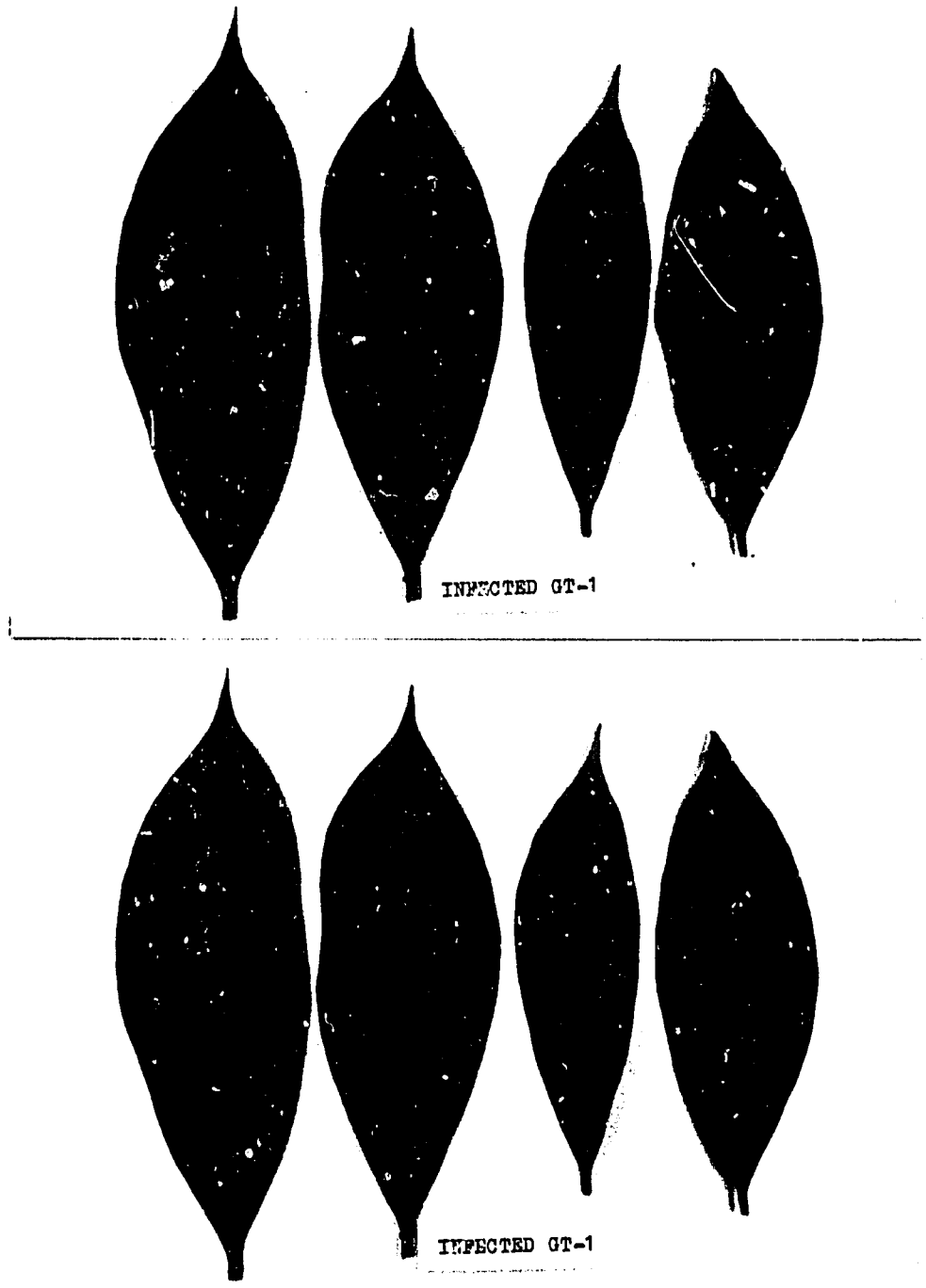

Fig. 62. Infected GT-1 leaves after 48 hours of inoculation as viewing before (upper picture) and after (lower picture) the removal of agar discs containing pathogenic fungus (*Phytophthora botryosa*). Lesion scores are given on the following page.

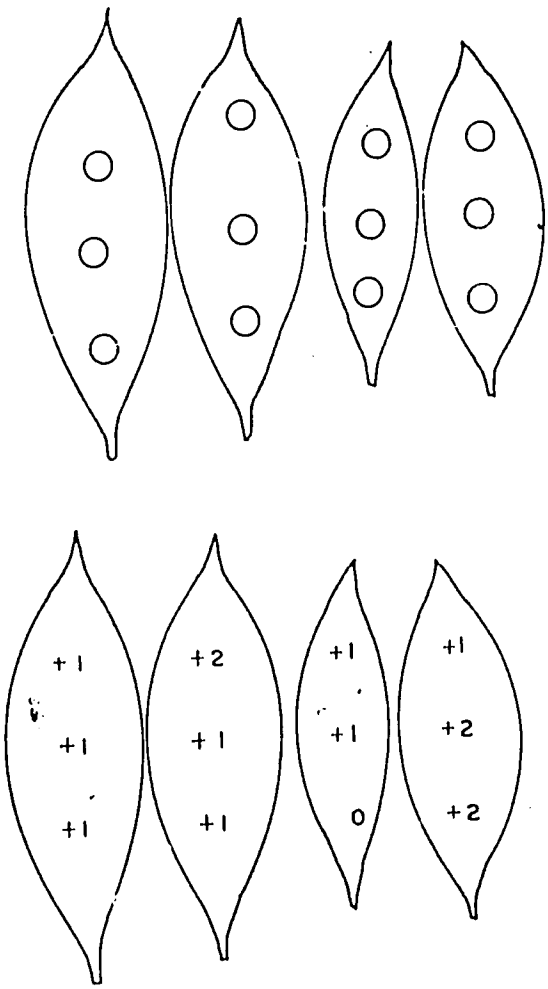

Fig. 62. Lesion intensity scores at inoculation sites of the infected GT 1 leaves after 48 hours of inoculation time.

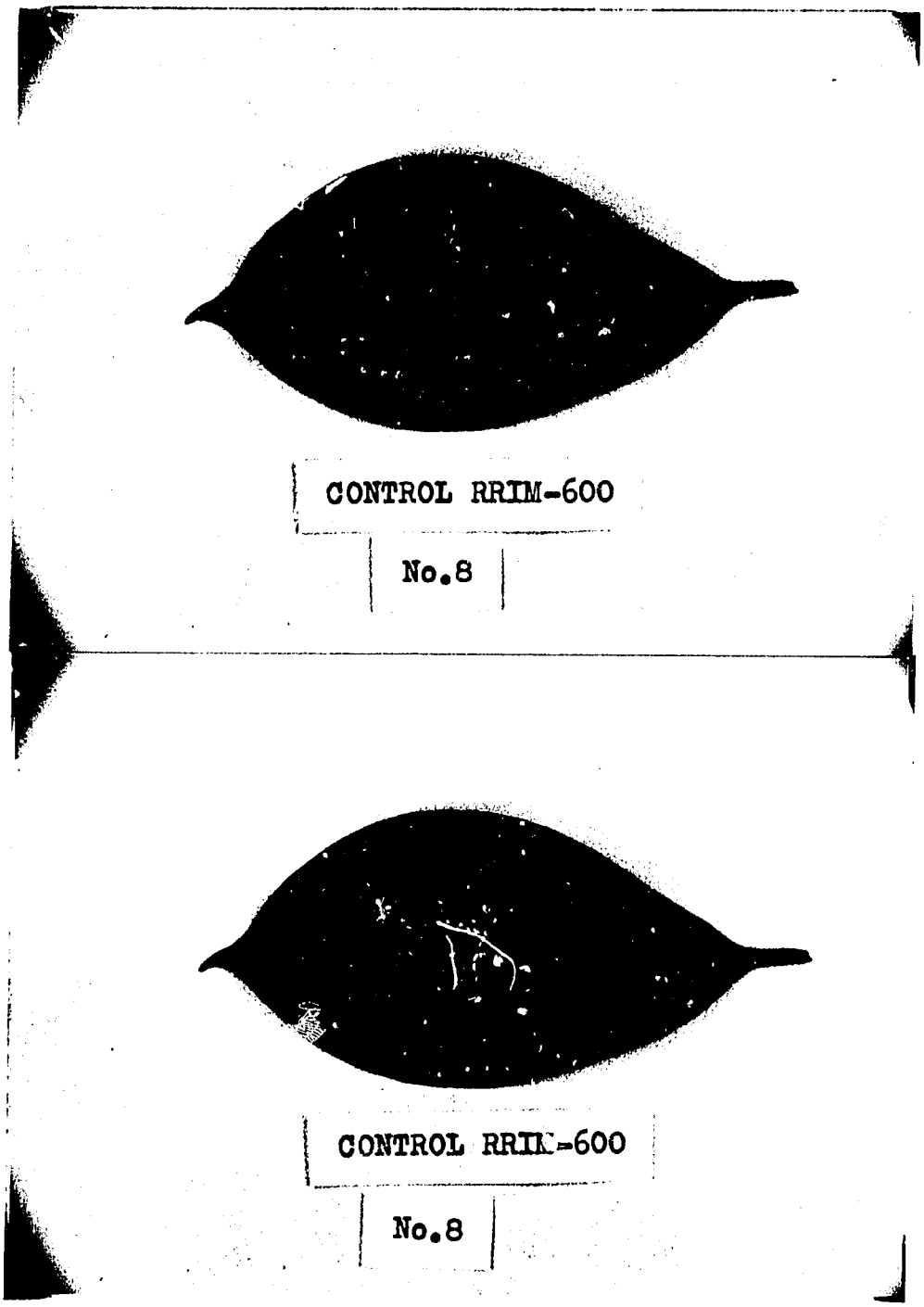

Fig. 63. Control RRIM 600 leaf after 48 hours of inoculation with uncontaminated and contaminated agar discs containing non pathogenic fungus of unidentified species before (upper picture) and after (lower picture) the removal of agar discs.

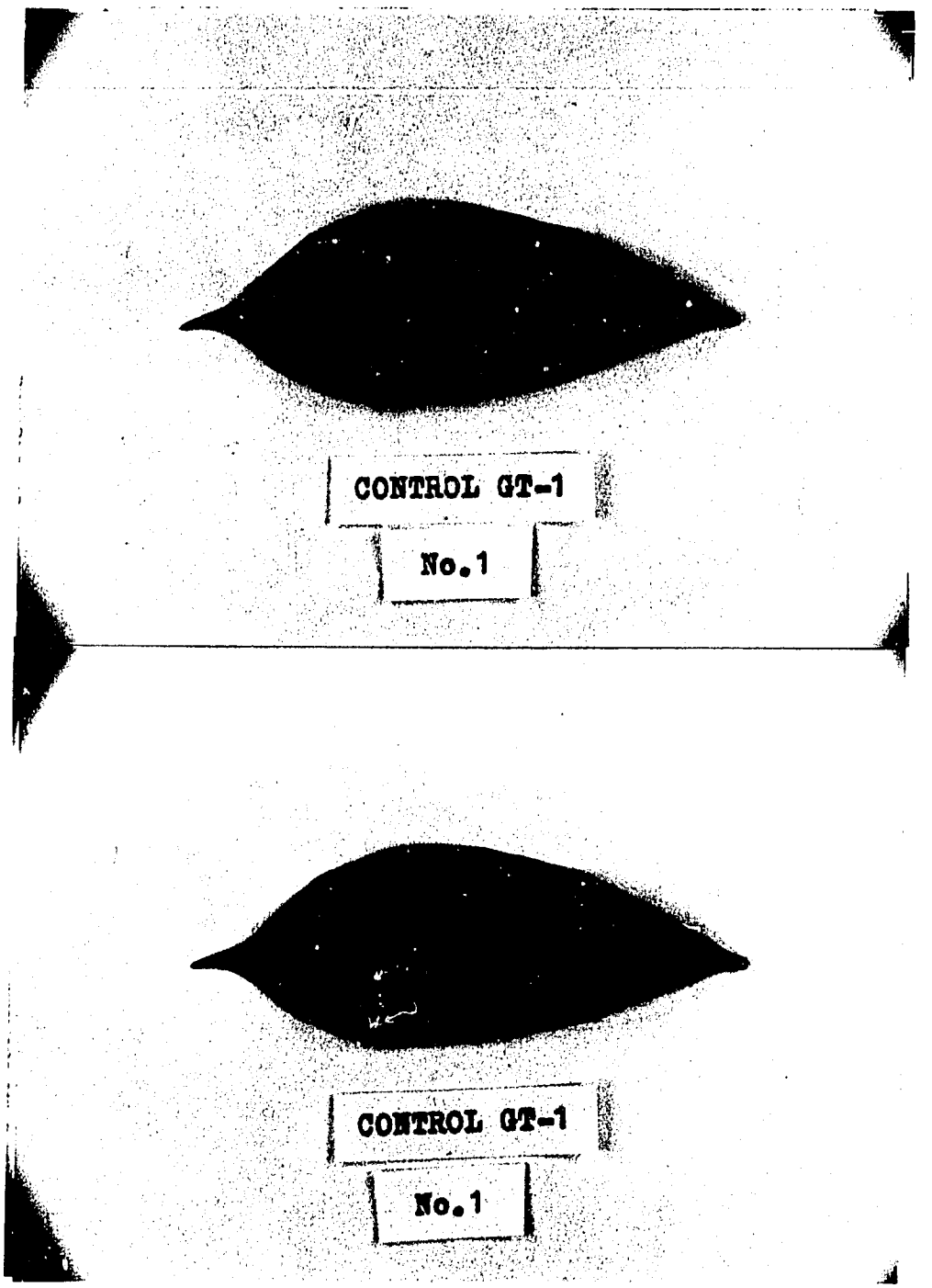

Fig. 64. Control GT 1 leaf after 48 hours of inoculation with contaminated and uncontaminated agar discs containing non pathogenic fungus of unidentified species before (upper picture) and after (lower picture) the removal of agar discs.

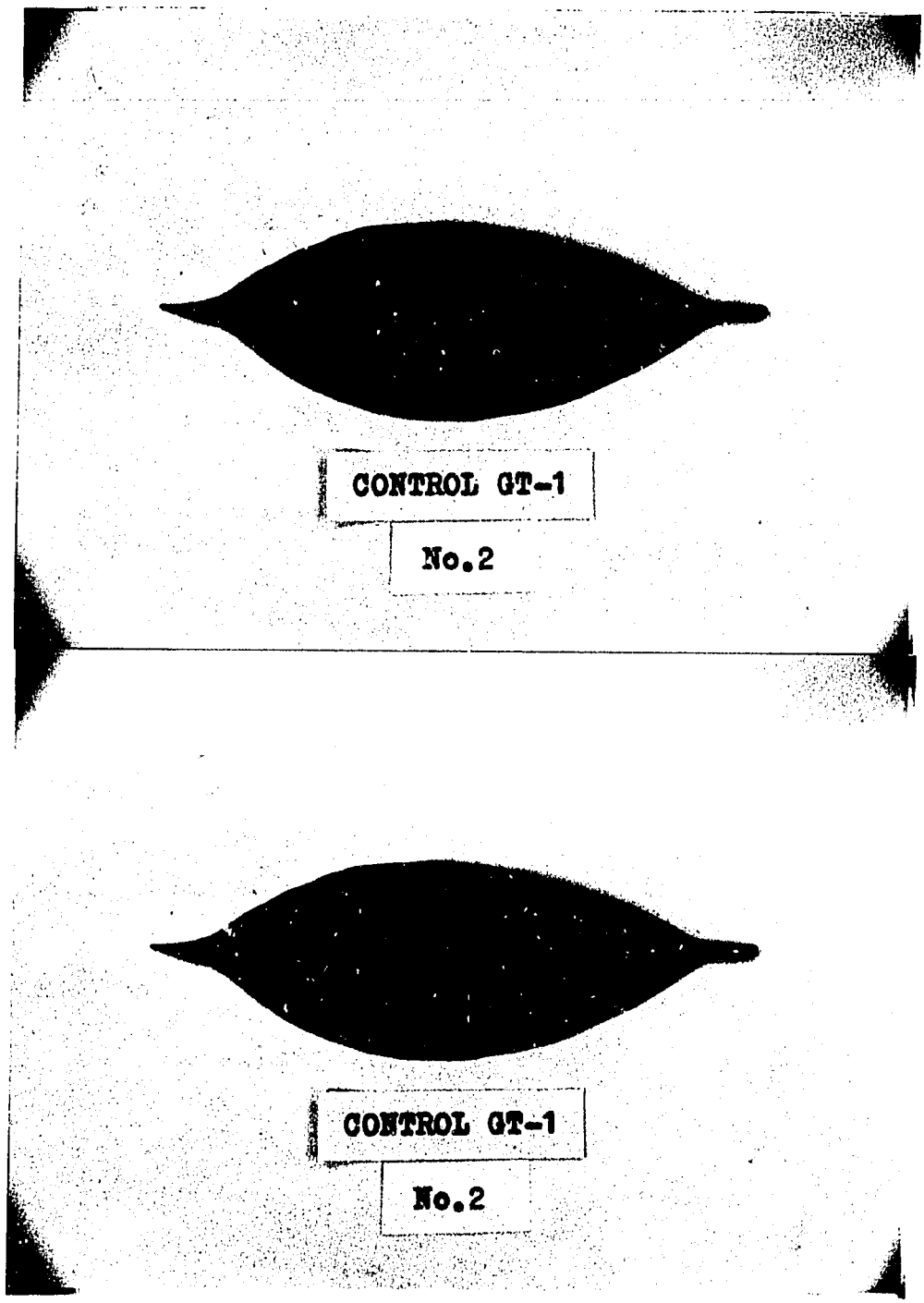

Fig. 65. Control GT 1 leaf after 72 hours of inoculations with contaminated and uncontaminated agar discs containing non pathogenic fungus of unidentified species before (upper picture) and after (lower picture) the removal of agar discs.

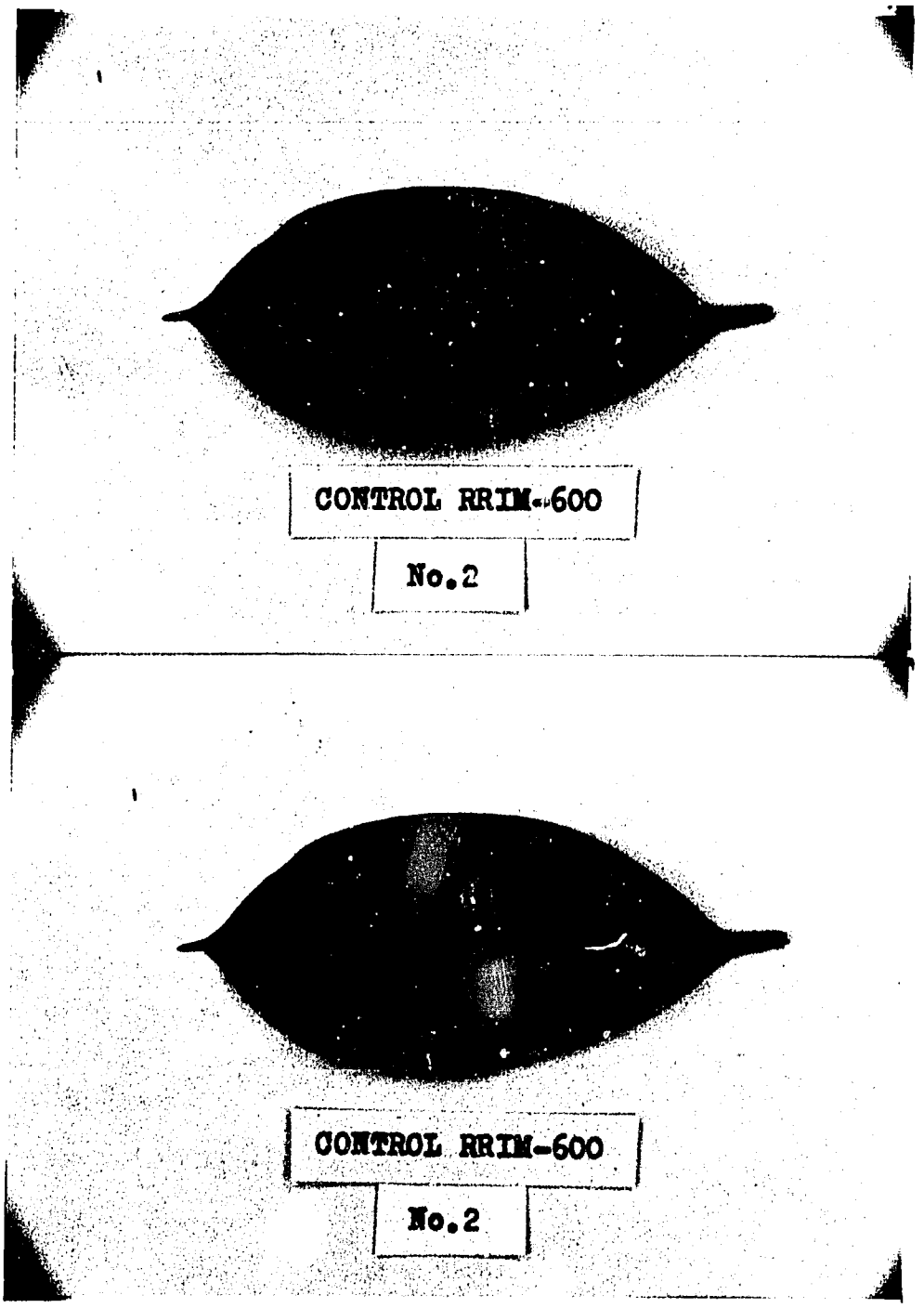

Fig. 66. Control RRIM 600 leaf after 72 hours of inoculation with contaminated agar discs containing non pathogenic fungus of unidentified species before (upper picture) and after (lower picture) the removal of agar discs.

Fig. 67. 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 ul on the left and 100 ul on the right disc respectively.

Table 1. Comparison study on activity levels of enzymes involved in rubber biosynthesis in latex of *Hevea brasiliensis*

Enzyme	nmoles product produced min/ml/latex*	nmoles substrate consumed min/ml/latex**
Acetyl-CoA synthetase	-	59
Thiolase	-	3.92
HMG-CoA synthetase	999 + 111	232
HMG-CoA reductase	13 + 4	0.078
Mevalonate kinase	428 + 64	149
P-mevalonate kinase	-	44
PP-mevalonate carboxylase	-	103
Isopentanyl-PP isomerase	-	-
Polymerase	-	22.3

* An average value obtained from two measurements using fresh latex pooled from 7 RRIM 600 trees.
** Data from reference # 5

Table 2. Correlation studies between HMGR activity and rubber yield from latex of low, intermediate, high yield RRIM 600 trees.

Experimental date	RRIM 600 tree number	Dry rubber yield[1]	Total HMGR activity[2]	Latex HMGR activity[3]	Ranking: Dry rubber yield	Ranking: Total HMGR activity	Ranking: Latex HMGR activity
14 Sept'87	8	18.06	630 ± 29	9.66 ± 0.31	L	L	L
	4	29.70	823 ± 48	9.97 ± 0.30	M	M	M
	7	72.52	2291 ± 1 (r=0.99)*	11.29 ± 0.00 (r=0.99)*	H	H	H
16 Sept'87	8	21.02	690 ± 3	10.99 ± 0.37	M	M	L
	4	19.29	555 ± 16	12.52 ± 0.32	L	L	M
	7	46.93	1507 ± 19 (r=0.99)*	13.41 ± 0.03 (r=0.86)*	H	H	H
20 Oct'87	8	13.13	623 ± 16	11.75 + 0.07	L	L	L
	4	22.62	903 ± 17	17.95 + 0.10	M	M	M
	7	55.12	2434 ± 14 (r=0.99)*	22.89 + 0.37 (r=0.99)*	H	H	H

1= gm/tapping/tree, 2=nmoles MVA formed/min/tapping, 3=nmole MVA formed/min/ml latex,
L = Low, M = Medium, H = High, ( )* = correlations calculated between corresponding rubber yield and total HMGR activity or latex HMGR activity

Table 3. Purification protocol of HMGR from *Hevea* latex

Fraction/step	Total volume (ml)	Total protein (mg)	Total activity (nmol MVA formed /min)	Specific activity (nmol MVA formed /min.mg)	Yield (%)	Purification (fold)
Bottom fraction in Buffer B						
+ Brij w-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 xg supernatant	95	570	404.7	0.71	116.4	1.78
0-40% $(NH_4)_2SO_4$	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% $(NH_4)_2SO_4$	6	7.3	00.6	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 4. Preliminary evaluation for rubber yield on new *Hevea* germplasm

Sample no.	Material source	Clonal code	Yield index*
1	7/02/81	1/22	C^+
2	RO/PB/1	2/35	D^-
3	RO/PB/1	2/36	D
4	RO/PB/1	2/38	D^+
5	RO/PB/1	2/49	C
6	RO/PB/1	2/51	C^+
7	RO/PB/1	2/59	D^+
8	RO/PB/1	2/61	C^+
9	RO/PB/1	2/68	C
10	RO/PB/1	2/69	D^-
11	RO/PB/1	2/83	D
12	RO/PB/2	3/19	D^-
13	RP/PB/1	3/27	C
14	RO/PB/1	3/70	C^-
15	RO/PB/1	3/71	B
16	RO/PB/1	3/78	C^-
17	RO/PB/1	3/87	C
18	RO/PB/1	3/95	D^+
19	RO/PB/1	3/144	C
20	RO/PB/1	3/151	B
21	RO/PB/1	3/165	C
** 22	RO/PB/1	3/201	A^+
23	RO/PB/1	3/21	C^+
24	RO/PB/1	3/23	C
25	RO/PB/1	3/26	C
26	RO/PB/1	3/28	B
27	RO/PB/1	3/301	C^+
28	RO/PB/1	3/351	E
29	RO/PB/1	3/382	D
30	AC/T/3	6/47	B^+
31	MT/C/4	7/72	B^-
32	MT/C/4	7/85	D
33	MT/C/4	7/114	-
34	MC/C/7	82	E
35	MC/C/2	10/14	D
36	MC/C/2	10/36	E
**37	MC/C/2	10/39	A^-
38	MC/C/2	10/41	E
39	MC/C/2	10/43	C
40	MC/C/2	10/48	B
41	MC/C/2	10/51	D
42	MC/C/2	10/52	D
43	MC/C/2	10/54	D
44	MC/C/2	10/59	D
45	MC/C/2	10/60	E
46	MC/C/2	10/61	C
47	MC/C/2	10/64	D^+
48	MC/C/2	10/66	B^+

Sample no.	Material source	Clonal code	Yield index*
49	MC/C/2	10/87	D
50	MC/C/2	10/92	C^+
51	MC/C/2	10/93	E
52	MC/C/2	10/94	B
53	MC/C/2	10/95	C
54	MC/C/2	10/98	D^+
55	MC/C/2	10/99	C^+
**56	MC/C/2	10/108	A
57	MC/C/2	10/109	B
58	MC/C/2	10/131	C
59	MC/C/2	10/144	B
60	MC/C/2	10/145	D^+
61	MC/C/2	10/172	D
62	MT/C/6	11/141	A
63	MT/C/5	12/30	D
64	MT/C/5	12/122	D
65	MT/C/5	12/124	C^+
66	MT/C/5	12/126	E
67	AC/T/4	13/5	C^+
68	AC/T/4	13/120	B
69	MT/C/68	14/12	B^-
70	MT/C/3	16/41	B^+
**71	MT/C/1	18/5	A
72	MT/C/1	18/7	C^+
73	MT/C/1	18/8	C
74	MT/C/1	18/79	B
75	MT/C/1	18/95	C^+
**76	MT/C/1	18/99	A
77	MT/C/1	18/101	B^+
78	MT/C/1	18/116	C^-
79	RO/OP/4	20/103	D
80	RO/J/5	33/42	E
81	RO/J/5	33/45	E
82	RO/J/5	33/48	C^+
83	RO/J/5	33/49	D
84	RO/J/5	33/51	-
85	RO/J/5	33/52	C
86	RO/J/5	33/53	D^-
87	RO/J/5	33/54	D
88	RO/J/5	33/55	D
89	RO/J/5	33/56	D
90	RO/J/5	33/57	E
91	RO/J/5	33/58	C
92	RO/C/9	23/110	B
93	RO/C/9	23/110	B^+
94	RO/C/9	23/120	C
95	RO/C/9	23/121	C
96	RO/C/9	23/129	C^-
97	RO/C/9	23/142	B^+
98	RO/C/9	23/150	C
99	RO/C/9	23/152	B

Sample no.	Material source	Clonal code	Yield index*
100	RO/C/9	23/154	A
**101	RO/C/9	23/158	C+
102	RO/C/9	23/160	C+
103	RO/C/9	23/163	C+
104	RO/C/9	23/167	-
105	RO/C/9	23/174	B
**106	RO/C/9	23/177	A
107	RO/C/9	23/180	B-
108	RO/C/9	23/182	C+
109	RO/C/9	23/183	E
110	RO/C/9	23/184	C
111	RO/C/9	23/194	C-
112	RO/C/9	23/199	B
113	RO/C/9	23/202	C
114	RO/C/9	23/206	B+
115	RO/C/9	23/212	B-
116	RO/C/9	23/216	D
117	RO/C/9	23/219	C+
118	RO/C/9	23/225	D
119	RO/C/9	23/234	C
120	RO/C/9	23/236	C
121	RO/C/9	23/239	D
122	RO/C/9	23/242	D
123	RO/C/9	23/267	E
124	RO/C/9	23/277	E
**125	RO/C/9	23/294	A-
**126	RO/C/9	23/301	C
127	RO/C/9	23/318	-
128	RO/C/9	23/329	C+
129	RO/C/9	23/346	-
130	RO/C/9	23/353	-
131	RO/C/9	23/382	C
**132	RO/C/9	23/386	A
133	RO/C/9	23/393	B+
134	RO/C/9	23/396	C+
135	RO/C/9	23/398	C
136	RO/C/9	23/405	A-
137	RO/C/9	23/411	C+
138	RO/C/9	23/413	C-
139	RO/C/9	23/416	-
140	RO/C/9	23/423	C
141	MT/VB/25A	57/2	C
142	MT/VB/25A	57/4	C-
143	MT/VB/25A	57/5	E
144	MT/VB/25A	57/8	E
145	MT/VB/25A	57/14	D
146	MT/VB/25A	57/32	C
147	MT/VB/25A	57/37	C-
148	MT/VB/25A	57/44	C+
149	MT/VB/25A	57/45	D

Sample no.	Material source	Clonal code	Yield index*
150	MT/VB/25A	57/50	E
151	MT/VB/25A	57/57	D
152	MT/VB/25A	57/69	D^+
153	MT/VB/25A	57/76	C^-
154	MT/VB/25A	57/81	C^-
155	MT/VB/25A	57/53	D
156	MT/VB/25A	57/58	E
157	MT/VB/25A	57/71	D
158	MT/VB/25A	57/19	C^-
159	RO/C/8	24/340	D
160	RO/C/8	24/341	C
161	RO/C/8	24/342	C^+
162	RO/C/8	24/344	C
163	RO/C/8	24/345	C^+
164	RO/C/8	24/358	C
165	RO/C/8	24/361	C
166	RO/C/8	24/366	C^+
167	RO/C/8	24/374	D
168	RO/C/8	24/375	C
169	RO/C/8	24/376	D
170	RO/C/8	24/382	D
171	RO/C/8	24/403	C^+
172	RO/C/8	24/409	C
173	RO/C/8	24/411	-
174	RO/C/8	24/412	D
**175	RO/C/8	24/415	A^-
176	RO/C/8	24/418	C^+
177	RO/C/8	24/419	D
178	RO/C/8	24/423	D
189	RO/C/8	24/425	D
180	RO/C/8	24/435	C
181	RO/C/8	24/437	D
182	RO/C/8	24/439	C^+
183	RO/C/8	24/446	C
184	RO/C/8	24/453	C^+
185	RO/C/8	24/454	C
**186	RO/C/8	24/457	A^+
187	RO/C/8	24/458	C+
188	RO/C/8	24/459	C
189	MT/IT/12	26/1	D
190	MT/IT/12	26/2	C^+
191	MT/IT/12	26/9	C
192	MT/IT/12	26/12	D
193	MT/IT/12	26/14	C^+
194	MT/IT/12	26/26	D
**195	MT/IT/12	26/32	A^+
**196	MT/IT/12	26/34	A^+
**197	MT/IT/12	26/37	B^+
**198	MT/IT/12	26/49	A^-
199	MT/IT/12	26/49	A^-

Sample no.	Material source	Clonal code	Yield index*
200	MT/IT/12	26/35	C
201	MT/IT/12	26/64	D
202	MT/IT/12	26/67	C
203	MT/IT/12	26/70	D
204	MT/IT/12	26/77	D
**205	MT/IT/12	26/79	A-
206	MT/IT/12	26/81	B
207	MT/IT/12	26/84	C
208	MT/IT/12	26/86	C+
209	MT/IT/12	26/118	D
210	MT/IT/12	26/122	C+
211	MT/IT/12	25/128	D
212	MT/IT/12	26/130	C
213	MT/IT/12	26/133	D
**214	MT/I/21	24	A
215	MT/I/37A	55	D
216	MT/I/39A	38	B
217	MT/I/39B	39	A-
**218	MT/I/40A	40	A
219	RO/I/10	54	B
220	RO/I/22	60	-
221	RO/I/24	61	B
222	RO/I/44	72	-
223	RO/I/50	76	C
**224	RO/I/54	79	A
225	RO/I/56	81	C
226	RO/I/60	84	D
227	RO/I/56	87	B
228	RO/I/70	91	B
229	RO/I/81	96	B
230	RO/I/105	10	B
231	RO/I/106	110	-
**232	RO/I/109	112	A
233	MT/I/12	15	B
234	RO/I/75	94	-
235	AC/T/2	4/85	B
236	AC/T/3	6/46	B
237	MT/C/1	18/37	B
**238	RO/OP/4	20/66	A
249	RO/A/7	25/3/6	B
240	RO/A/7	25/3/7	C
241	RO/A/7	25/323	B
242	RO/A/7	25/324	D
**243	RO/A/7	25/328	A
244	RO/A/7	25/338	D
245	RO/A/7	25/339	C
246	RO/A/7	25/341	D
247	RO/A/7	25/342	C
248	RO/A/7	25/344	D
249	RO/A/7	25/349	B

Sample no.	Material source	Clonal code	Yield index*
250	RO/A/7	25/350	D
251	RO/A/7	25/355	B
252	RO/A/7	25/356	A
253	RO/A/7	25/359	D
254	RO/A/7	25/362	C
255	RO/A/7	25/367	B
256	RO/A/7	25/370	C
257	RO/A/7	25/372	C
258	RO/A/7	25/386	B
259	RO/A/7	25/388	B
260	RO/A/7	25/390	C
261	RO/A/7	25/391	D
262	RO/A/7	25/394	D
263	RO/A/7	25/395	D
264	RO/A/7	25/396	B
265	RO/A/7	25/398	B
266	RO/A/7	25/399	C
267	RO/A/7	25/401	D
268	RO/A/7	25/402	D
279	RO/A/7	25/403	C
270	RO/A/7	25/404	B
271	RO/A/7	25/405	B
272	RO/A/7	25/47	D
273	RO/A/7	25/409	D
274	RO/A/7	25/411	B
275	RO/A/7	25/412	B
276	RO/A/7	25/413	A
277	RO/A/7	25/415	C
278	RO/A/7	25/418	D
279	RO/A/7	25/420	C
280	RO/A/7	25/421	A
281	RO/A/7	25/423	B
282	RO/A/7	25/424	D
283	RO/A/7	25/425	D
284	RO/A/7	25/427	B
285	RO/A/7	25/430	C
286	RO/A/7	25/432	B
287	RO/A/7	25/433	B
288	RO/A/7	25/434	B
289	RO/A/7	25/440	D
290	RO/A/7	25/447	D
291	RO/A/7	25/448	D
292	RO/A/7	25/451	B
293	RO/A/7	25/452	B+
294	RO/A/7	25/455	B
**295	RO/C/8	24/94	A
296	RO/C/8	24/152	B
297	RO/C/8	24/159	D
298	RO/C/8	24/173	C
299	RO/C/8	24/182	D

Sample no.	Material source	Clonal code	Yield index*
300	RO/C/8	24/217	B
301	RO/C/8	24/316	B
302	RO/C/8	24/329	B
303	RO/C/8	24/340	D
304	RO/C/8	24/341	B
**305	RO/C/8	24/342	A
306	RO/C/8	24/344	B
307	RO/C/8	24/345	B
308	RO/C/8	24/358	C
309	RO/C/8	24/361	B
310	RO/C/8	24/366	C
311	RO/C/8	24/374	D
312	RO/C/8	24/375	D
313	RO/C/8	24/376	D
314	RO/C/8	24/382	D
315	RO/C/8	24/403	D
316	RO/C/8	24/409	D
317	RO/C/8	24/411	B
318	RO/C/8	24/421	D
319	RO/C/8	24/416	C
320	RO/C/8	24/423	D
321	RO/C/8	24/425	D
322	RO/C/8	24/435	D
323	RO/C/8	24/437	C
324	RO/C/8	24/439	B
325	RO/C/8	24/446	B
326	RO/C/8	24/453	B
327	RO/C/8	24/454	B
328	RO/C/8	24/457	C
329	RO/C/8	24/458	C
330	RO/C/8	24/459	C
331	MT/IT/1	26/1	C
332	MT/IT/1	26/7	C
333	MT/IT/1	26/9	C
334	MT/IT/1	26/12	C
335	MT/IT/1	26/14	B
336	MT/IT/1	26/26	C
337	MT/IT/1	26/32	B
338	MT/IT/1	26/37	B
339	MT/IT/1	26/45	C
340	MT/IT/1	26/49	B
341	MT/IT/1	26/55	D
342	MT/IT/1	26/64	C
343	MT/IT/1	26/67	C
344	MT/IT/1	26/70	D
345	MT/IT/1	26/77	B
346	MT/IT/1	26/79	B
347	MT/IT/1	26/81	C
348	MT/IT/1	26/84	C
349	MT/IT/1	26/86	B

Sample no.	Material source	Clonal code	Yield index*
350	MT/IT/1	26/122	B
351	MT/IT/1	26/128	C
352	MT/IT/1	26/130	B
353	MT/IT/1	26/133	B
354	RO/A/7	25/1	B
355	RO/A/7	25/5	C
356	RO/A/7	25/8	D
357	RO/A/7	25/14	C
358	RO/A/7	25/44	D
359	RO/A/7	25/45	D
360	RO/A/7	25/52	C
361	RO/A/7	25/96	C
362	RO/A/7	25/103	D
363	RO/A/7	25/111	D
364	RO/A/7	25/122	C
365	RO/A/7	25/127	C
366	RO/A/7	25/163	C
367	RO/A/7	25/166	D
368	RO/A/7	25/174	C
369	RO/A/7	25/189	C
370	RO/A/7	25/190	D
371	RO/A/7	25/197	D
372	RO/A/7	25/225	C
373	RO/A/7	25/239	C
374	RO/A/7	25/242	D
375	RO/A/7	25/249	B
376	RO/A/7	25/250	B
377	RO/A/7	25/254	D
378	RO/A/7	25/265	C
379	RO/A/7	25/269	B
380	RO/A/7	25/279	D
381	RO/A/7	25/281	C
382	RO/A/7	25/286	D
383	RO/A/7	25/292	C
384	RO/A/7	25/307	B
385	RO/A/7	25/310	C
386	RO/A/7	25/313	D
387	RO/A/7	25/464	B
388	RO/A/7	25/465	D
399	RO/A/7	25/467	D
390	RO/A/7	25/468	C
391	RO/A/7	25/471	C
392	RO/A/7	25/472	D
393	RO/A/7	25/473	D
394	RO/A/7	25/474	D
395	RO/A/7	25/477	D
396	RO/A/7	25/480	D
397	RO/A/7	25/481	D
398	RO/A/7	25/482	C
399	RO/A/7	25/487	C

Sample no.	Material source	Clonal code	Yield index*
400	RO/A/7	25/489	D
401	RO/A/7	25/491	D
402	RO/A/7	25/493	C
403	RO/A/7	25/494	C
404	RO/A/7	25/497	C
**405	RO/A/7	25/500	A
406	RO/A/7	25/505	C
407	RO/A/7	25/508	D
408	RO/A/7	25/501	D
409	RO/A/7	25/511	B
410	RO/A/7	25/516	B
**411	RO/A/7	25/519	A
412	RO/A/7	25/513	B
**413	RO/A/7	25/539	A
414	RO/A/7	25/545	B
415	RO/A/7	25/554	B
416	RO/A/7	25/576	D
417	RO/A/7	25/577	D
418	RO/A/7	25/578	D
419	RO/A/7	25/579	C
420	RO/A/7	25/580	C
421	RO/A/7	25/583	C
422	RO/A/7	25/585	D
423	RO/A/7	25/590	D
424	RO/A/7	25/592	B
425	RO/A/7	25/605	D
426	RO/A/7	25/608	C
427	RO/A/7	25/609	B
428	RO/A/7	25/610	D
429	RO/A/7	25/593	C
430	RO/A/7	25/595	B
431	RO/A/7	25/593	C
432	RO/A/7	25/595	B
433	RO/A/7	25/596	D
434	RO/A/7	25/603	D
435	RO/A/7	25/604	B
436	RO/A/7	25/611	B
**437	RO/A/7	25/613	A
438	RO/A/7	25/614	C
439	RO/A/7	25/615	C
440	RO/A/7	25/616	C
441	RO/A/7	25/619	B
442	RO/A/7	25/620	B
443	RO/A/7	25/621	C
444	RO/A/7	25/624	D
445	RO/A/7	25/70	B
446	AC/3/8	35/3	C
447	AC/3/8	35/6	C
448	AC/3/8	35/12	C

Sample no.	Material source	Clonal code	Yield index*
449	AC/3/8	35/21	D
450	AC/3/8	35/23	C
451	AC/3/8	35/42	C
452	AC/3/8	35/43	D
453	AC/3/8	35/44	D
454	AC/3/8	35/30	D
455	AC/3/8	35/31	C
456	AC/3/8	35/36	D
457	AC/3/8	35/39	D
458	AC/3/8	35/49	D
469	AC/3/8	35/51	C
460	AC/3/8	35/52	D
461	AC/3/8	35/53	D
462	AC/3/8	35/56	B
463	AC/3/8	35/62	B
464	AC/3/8	35/66	B
465	AC/3/8	35/67	B
466	AC/3/8	35/74	C
467	AC/3/8	35/84	C
468	AC/3/8	35/88	C
479	AC/3/8	35/91	B
*470	AC/3/8	35/105	B
471	AC/3/8	35/114	B
472	AC/3/8	35/120	C
*473	AC/3/8	35/134	A
*474	AC/3/8	35/136	A
475	AC/3/8	35/170	D
476	AC/3/8	35/182	C
477	AC/3/8	35/195	C
478	AC/3/8	35/197	B
489	AC/3/8	35/203	C
480	AC/3/8	35/237	B
481	AC/3/8	35/243	B
482	AC/3/8	35/247	B
483	AC/3/8	35/281	B
484	AC/3/8	35/312	B
*485	AC/3/8	35/364	A
486	AC/3/8	35/381	C
487	AC/3/8	35/390	C
488	AC/3/8	35/404	C
489	AC/3/8	35/413	C
490	AC/3/8	35/415	D
491	AC/3/8	35/416	B
492	AC/3/8	35/418	A
493	AC/3/8	35/425	D
494	AC/3/8	35/431	B
495	AC/3/8	35/434	D
496	AC/3/8	35/436	D
497	AC/3/8	35/444	B
498	AC/3/8	35/445	D
499	AC/3/8	35/453	D

Sample no.	Material source	Clonal code	Yield index*
500	AC/3/8	35/445	D
501	AC/3/8	35/453	D
502	AC/3/8	35/458	D
503	AC/38/8	35/460	B
504	AC/3/8	35/467	D
*505	AC/3/8	35/470	A
506	AC/3/8	35/475	C
507	AC/3/8	35/479	B
508	AC/3/8	35/479	C
*509	AC/3/8	35/506	A
*510	AC/3/8	35/518	A
511	AC/3/8	35/549	B
512	AC/3/8	35/551	D
513	AC/3/8	35/552	C
514	AC/3/8	35/553	B
515	AC/3/8	5/8	C
*516	RO/A/7	25/424	A
517	MT/IT/12	26/32	B
518	AC/T/1	5/68	D
519	AC/T/1	5/88	D
520	AC/T/1	5/90	C
521	AC/T/1	5/107	D
522	AC/T/1	5/128	B
*523	AC/T/1	5/140	A
524	AC/T/1	5/166	D
525	AC/T/1	5/168	C
526	AC/T/3	6/35	D
527	AC/T/1	6/36	B
528	AC/T/3	6/46	C
529	MT/C/4	7/15	B
530	MT/C/4	7/37	B
531	MT/C/4	7/55	B
532	MT/C/4	7/113	B
**533	MT/C/4	7/135	A
534	MT/C/4	7/159	C
535	MT/C/7	8/25	B
536	MT/C/2	10/4	B
537	MT/C/2	10/10	C
538	MT/C/2	10/15	D
539	MT/C/2	10/17	B
540	MT/C/2	10/26	B
**541	MT/C/2	10/27	A
**542	MT/C/2	10/47	A
543	RO/PB/2	3/2/9	C
544	RO/PB/2	3/223	B
545	RO/PB/2	3/283	D
**546	RO/PB/2	3/283	A
547	RO/PB/2	3/3/4	B
**548	RO/PB/2	3/330	A
549	RO/PB/2	3/352	C

Sample no.	Material source	Clonal code	Yield index*
550	RO/PB/2	3/367	C
551	RO/PB/2	3/370	D
552	RO/PB/2	3/396	B
553	RO/PB/2	3/340	B
554	RO/PB/2	3/467	D
555	RO/PB/2	3/504	B
556	AC/T/1	4/15	D
557	AC/T/2	4/19	C
558	AC/T/1	5/4	B
559	AC/T/1	5/8	C
560	AC/T/1	5/10	D
561	AC/T/1	5/41	C
562	AC/T/1	5/44	D
563	AC/T/1	5/62	D

- Clones with no grade are those without latex being excreted upon cutting.

* Grading was done by visual measurements of latex being excreted from lateral veins. The leaf blade was cut parallel on both sides of midrib vein with 1 cm apart. Grades A to D were given for high to low latex yield obtained.

** Clones selected for HMGR activity measurement by radioassay and compared with the RRIM 600 control (Table 5)

Table 5. Studies on latex HMGR activity from new *Hevea* germplasms

Material sources	Clonal codes	HMGR activity* per ml latex
Wickham	RRIM 600	5.17 (Control)
RO/PB/1	3/201	NA
MT/C/2	10/39	1.46
MT/C/2	10/108	2.78
MT/C/1	18/5	6.73
MT/C/1	18/99	2.36
RO/C/9	23/158	4.42
RO/C/9	23/177	1.59
RO/C/9	23/294	1.50
RO/C/9	23/294	NA
RO/C/9	23/386	1.68
Wickham	RRIM 600	6.71 (Control)
RO/C/8	24/416	2.97
RO/C/8	24/457	4.83
MT/IT/12	26/32	4.63
MT/IT/12	26/34	7.50
MT/IT/12	26/37	3.71
MT/IT/12	26/45	4.51
Wickham	RRIM 600	3.87 (Control)
MT/I/21	24	8.94
MT/I/40A	40	1.34
RO/I/54	79	2.68
RO/OP/4	20/66	1.61
RO/A/7	25/328	1.49
RO/A/7	25/356	1.30
RO/A/7	25/413	2.07
RO/A/7	25/421	1.56
RO/A/7	24/94	2.11
RO/A/7	24/342	1.31
RO/A/7	25/500	10.87
RO/A/7	25/519	3.89
RO/A/7	25/539	6.79
RO/A/7	25/611	2.56
AC/3/8	35/91	1.74
AC/3/8	35/134	2.30
AC/3/8	35/364	2.45
AC/3/8	35/416	2.34
AC/3/8	35/470	1.94
AC/3/8	35/518	7.88
RO/A/7	25/424	2.62
AC/T/1	5/140	2.08
MT/C/4	7/135	5.02
MT/C/2	10.27	23.63
MT/C/2	10/47	3.32
RO/PB/2	3/287	9.23

* HMGR activity is expressed as nmol MVA formed /min

Table 6. Comparison study on HMGR activity level between new *Hevea* germplasm with high yield potentials and RRIM 600 control

Material source	Clonal code	Control RRIM 600 (100%)
RO/A/7	25/5/9	100.5
MT/IT/12	26/34	111.7
MT/C/1	18/5	130
MT/C/4	7/135	132
RO/A/7	25/539	175.5
AC/3/8	35/5/8	203.6
MT/I/21	24	231
RO/PB/12	3/287	238.5
RO/A/7	25/500	280
MT/C/2	10/27	610

Table 7. Purification protocol of HMGR activator (calmodulin)

Purification/step	Total volume (ml)	Total protein (mg)	Total activation activity (unit*)	Specific activation activity (unit+)	Yield (%)	Purification (fold)
C-serum	280	2800	1.05×10^3	0.37	100	1
Boiled C-serum	250	520	7.63×10^2	1.47	73	4
0.7 M KCl DEAE-cellulose	110	109	5.23×10^2	4.80	50	13
30.90% $(NH_4)So_4$	20	12	3.72×10^2	30.97	35	84
Affi-Gel phenothiazine	15	1.8	1.89×10^2	105.11	18	284

*Unit of activation activity is equivalent to amount of HMGR activity (nmole MVA formed/min) increased due to the presence of HMGR activator

+Unit of activation activity per mg protein

Table 8. Effect of divalent cations and HMGR activator on HMGR activity

Treatment	HMGR activity (nmole/min/mg	% activator (+) or inhibition (-)
Enzyme	1.42	0
Enz + activator	2.97	+109
Enz + 25 mM Ca^{2+}	1.46	+3
Enz + 25 mM Ca^{2+} + activator	3.86	+172
Enz + 25 mM Cu^{2+}	1.39	-2
Enz + 25 mM Cu^{2+} + activator	2.62	+85
Enz + 25 mM Sr^{2+}	1.49	+5
Enz + 25 mM Sr^{2+} + activator	1.76	+24
Enz + 25 mM Ba^{2+}	1.37	-4
Enz + 25 mM Ba^{2+} + activator	2.19	+54

Table 9. Effect of EGTA, Ca^{2+} and HMGR activator on HMGR activity

Treatment	HMGR activity (nmole/min/mg)	% Activator
Enzyme	0.77	0
Enz + activator	1.95	153
Enz + 5 mM EGTA	0.77	0
Enz + 5 mM EGTA + activator	0.99	28
Enz + 5 mM EGTA + activator + 5 mM Ca^{2+}	1.17	52
Enz + 5 mM EGTA + activator + 10 mM Ca^{2+}	2.26	168

Table 10. Effect of trifluoperazine and HMGR activator on HMGR activity

Treatment	HMGR activity (nmole/min/mg)	% Activation
Enzyme	0.77	0
Enz + Activator	1.95	153
Enz + 3 mM trifluoperazine	0.72	0
Enz + Activator + 3 mM trifluoperazine	0.77	3

Table 11. Effect of *Hevea* calmodulin on NAD kinase activity

Treatment	% Activation	Specific activation (unit*)
Hevea NAD kinase	0	1.6
Hevea NAD kinase + *Hevea* calmodulin	134	187
Liver NAD kinase	0	0
Liver NAD kinase + *Hevea* calmodulin	48	2.6
Liver NAD kinase + brain calmodulin	91	2.7

*Unit of specific activation is equivalent to amount of NAD kinase activity (nmoles NADP formed/min) increased per mg calmodulin.

Table 12. Effects of ATP, NaF and HMGR activator on HMGR activity

Treatment	HMGR activity (nmole/min/mg)	% activation (+) inhibition(-)
Enzyme	1.81	0
Enzyme + activator	3.23	+78
Enz + 10 mM ATP-Mg^{2+}	0.47	-74
Enz + 10 mM ATP-Mg^{2+} + activator	2.86	+58
Enz + 50 mM NaF	1.64	-10
Enz + 50 mM NaF + activator	2.25	+24
Enz + 10 mM ATP-Mg^{2+} + 50 mM NaF	0.45	-76
Enz + 10 mM ATP-Mg^{2+} + 50 mM NaF + activator	0.66	-64

Table 13. Effect of HMGR activator, phosphatase and ATP-Mg^{2+} on HMGR activity

Treatment	HMGR activity nmole/min/mg	% Activation (+) inhibition (-)
bottom	0.35	0
bottom + activator	0.87	+149
solubilized bottom	0.44	0
solubilized bottom + activator	0.44	0
purified HMGR	1.69	0
purified HMGR + activator	1.69	0
purified HMGR + alkaline phosphatase	2.71	+60
purified HMGR + Acid phosphatase	1.68	-1
purified HMGR + 5 mM ATP-Mg^{2+}	0.67	-45

Table 14. Callus culture of male flowers from various *Hevea* clonal types.

Inoculation date	Clones	Culture media	Number of samples	Callus (wks)	growth (mm)
9/10/87	RRIM 600	MB	20	3	2.50
				*4	2.70
9/18/87	RRIM 600	MB	2	2	0.51
				4	1.03
				6	1.50
				8	2.06
				10	2.57
				*12	3.00
8/20/87	PB 310	MB	40	1	1.00
				2	1.50
				*4	1.85
				9	2.50
11/4/87	PB 310	MB	24	2	0.58
				*4	1.12
11/13/87	PB 310	MZZ(MZ)	30	2	0.71
				*4	0.90
10/15/87	RRIC 6	MB	12	2	0.69
				4	1.47
				6	2.25
10/8/87	GT 1	MB	16	2	0.45
				4	0.81
				*6	1.20

* Callus began to change from yellowish green into brownish colour and died in the following weeks.

Table 15. Callus growth under different types of media.

Inoculation date	Clones	Culture media	Number of samples	Callus growth wks	Callus growth mm	1st Transfering date	Culture media	Callus growth wks	Callus growth mm
11/17/87	RRIC 6	MZZ(ZM)	10	2	0.57	12/03/87	MLLH	2	0.9
11/19/87	RRIC 6	MZZ(ZM)	25	2	0.58	12/03/87	MLLH	2	0.9
11/20/87	RRIC 6	MZZ(ZM)	38	2	0.70	12/04/87	MLLH	2	0.8
11/24/87	RRIC 6	MZZ(ZM)	24	2	0.74	12/08/87	MLLH	2	1.0
11/25/87	RRIC 6	MZZ(ZM)	20	1	0.56	12/09/87	MLLH	2	0.9
11/26/87	RRIC 6	MZZ(ZM)	32	1	0.58	12/10/87	MLLH	2	1.0
11/27/87	RRIC 6	MZZ(ZM)	40	2	0.8	12/11/87	MLLH	2	1.0
12/1/87	RRIC 6	MZZ(ZM)	47	2	0.9	12/14/87	MLLH	2	1.0

Table 16. Effect of *Phytophthora* toxin on callus growth

Clonal types	Toxin conc. (g/ml)	Total cullus samples	Callus conditions	
			dead	alive
RRIM 600	0	6	1(16.7%)	5(83.3%)
	2.5	3	-(0%)	3(10%)
	25	3	-(0%)	3(100%)
	250	3	3(100%)	-(0%)
	275	2	2(100%)	-(0%)
	300	2	2(100%)	-(0%)
GT-1	0	6	-(0%)	6(100%)
	2.5	3	-(0%)	3(100%)
	25	3	-(0%)	3(100%)
	250	3	1(33.3%)	2(66.7%)

Table 17. Effect of *Phytophthora* toxin on callus initiation

Clonal types	Toxin conc. (g/mg)	Total flower samples	Callus conditions after initiated		
			dead	alive	almost dead
RRIM 600	0	8	-	8	-
	125	4	3	-	1
	250	4	3	-	1
GT-1	0	8	-	8	-
	125	4	-	4	-
	250	4	-	4	-

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