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PROGRESS REPORT NO. 4

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

A RESEARCH PROJECT

USAID/PSTC PROGRAM

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

Submitted by

Dr.Rapepun Wititsuwannakul

Project Leader

Department of Biochemistry

Faculty of Science

Prince of Songkla University

Hat-Yai 90112, Thailand.

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Project Profile

Country	: Thailand
Grant No.	: 936-5542-G-00-6054-00
Program	: Program on Science and Technology Cooperation
Project Title	: Biotechnology Application for Characterization and Selection of Better-Yielding Rubber Clones.
Project Leader	: Dr. Rapepun Wititsuwannakul
Organization	: Dept. of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai 90112, Thailand.
Co-investigators	: Dr. Wallie Suvachitanont, Dr. Rapiporn Sotthibandhu, Dr. Dhirayos Wititsuwannakul, Mr. Apichai Shuprisha, Mr. Wisut Sukonrat, Dr.Nunta Churngchow.
Authorized Officer	: Dr. Phasook Kullavanij (Rector)
Total Project Budget	: US $ 150,000
Project Duration	: 3 years (27 June-1986-31 July 1989)
Reporting Period	: January-30 June 1988
Budget Allocation for This Period	: US $ 114,299

1. Background/Introductions :

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

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

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

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

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

2. Objectives :

A. To develop a reliable method for identification and selection of higher yielding rubber clones at an early stage, while the plants are still at seedling stage. This would help circumventing the time and energy needed in conventional approaches.

B. To initiate the study of disease resistant rubber clones at the level of tissue culture stage in conjunction to the high-yielding potential screening as outlined below.

Yield screening at seedling stage (Part A)

Potential low yielder (discard) | Potential high yielder

Disease resistance screening at tissue culture stage (Part B)

susceptible clone | resistant clone

Adopted as possible high yielding disease resistant clones.

3. Materials and methods :

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

Chemicals :

All chemicals and reagents used were of analytical grade. NADPH, HMG-CoA, polyoxyethylene ether W-1 (Brij W-1), mevalonolactone, NAD, NAD kinase, DTT, DEAE cellulose, alkaline and acid phosphotase and stain all were from Sigma. 3-^{14}C HMG-CoA was from Amersham England. Affi-Gel-Blue and Affi-Gel Phenothiazine were from Bio-Rad. Agarose-hexane-HMG-CoA was from P-L Biochemicals.

Collection and fractionation of latex :

Latex was obtained from several clones of *Hevea brasiliensis* as indicated in the text. The trees are regularly tapped, half spirally on every other day (S.1/D.2). All clones are grown categorized at the Rubber Research Center, Hat-Yai. Rubber trees were normally tapped at 0600 h or otherwise as indicated. After tapping the fresh latex was collected into an ice-chilled beaker. The latex from each clone was pooled and fractionated into 3 major fractions by centrifugation in a microfuge at 15,000 rpm for 60 min. They are the rubber fraction, the C-serum (latex cytoplasm) and the bottom fractions, respectively. The bottom fractions, where 95% of HMG-CoA reductase (HMGR) was located (4), were separated by cutting the bottom part of the microtube and used for assay of HMGR and NAD kinase activities. The C-serum was used as source of HMGR activator purification and characterization.

Assay of HMG-CoA reductase (HMGR) activity :

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

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

Purification of HMGR activator :

The procedure used for calmodulin purification was modified and applied for *Hevea* HMGR activator (calmodulin) purification (6,7). 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 N NaCl, 1 mM $CaCl_2$ and 1 mM mercaptoethanol. The dialysate was apply to Affi-Gel Phenothiazine column, washed with 3 N NaCl and eluted with 10 mM EGTA, respectively.

Assay of NAD kinase :

The method for NAD kinase assay in the latex bottom fraction (8)

was adopted to assay both commercial NAD kinase purified form 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 $MgCl_2$, 0.05 mM $CaCl_2$ 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.(9).

Gel electrophoresis :

Polyacrylamide gel electrophoresis (PAGE) were run either in the absence or presence of sodium dodecyl sulfate (SDS) using the method of Weber et al. (10).

Protein determination :

The protein concentration was determined by the method of Lowry et al. (11).

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

Leaf materials

Young RRIM 600 and GT-1 leaves with approximatdy 7 to 10 days old were collected from the trees and extensively washed with running tap water followed by distilled water. The leaves were taken into laminar flow and washed 3 times by dipping into 500 ml beakers containing steriled distilled water. Small leaflets were cut from petiole and layered on moist conton pad in petri-dish. All treatments were performed under aseptic conditions.

Phytopthora botryosa culturing :

Phytopthora botryosa was obtained from Plant Disease Division,

Songkla Rubber Reaearch Center. The fugus was subcultured once every two weeks in potato Dextrose Agar (PDA) medium containing (per liter) 3.9 gm PDA and 0.02 gm lecithin.

Leaf inoculations.

An agar disc (5 mm diameter) containing phytophthora was prepared by cutting through the margin of 7-10 days old phytopthera colony grown on PDA medium. Each disc was layered (upside down) on the top, middle and bottom put of the leaf lamella allowing mycelium to have direct contact with upper leaf epidermis. The inocalated leaf were incubated in moist petri dish under fluorescent light from 0800-1700 h at 28°C. Pure agar discs were prepared and used for the control samples.

4. Results/Discussions/Tablees :

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

A.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 (12). In our study, when C-serum prepared from latex of high, medium and low yielding trees of clones RRIM 600 and KRS 208 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 (Table I). This suggests an important role of the C-serum in regulation of HMGR activity. Further purification and characterization of HMGR activator is pursued in this report.

The HMGR activator was purified from the C-serum fraction of centrifuged latex by modifying methods used for calmodulin purification (6,7). The purification steps involved heat treatment, DEAE-cellulose batch binding, ammonium sulfate precipitation and Affi-Gel Phenothiazine chromatography (Fig.1). The HMGR activatior 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 II). Molecular mass of HMGR activator when analyzed by SDS-PAGE is 17,500 which is in the same range as calmodulin (Fig 2,3). When Stain all was used for SDS-PAGE staining, the purified activation band appeared in blue color indication its propr ty 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. 4). The HMGR activity increases with increasing amount of HMGR activator, reaching a maximum of 100% when 30 µg of purified activator was used.

A.2 Functional properties of HMGR activator (calmodulin)

Effects of several divalent cations on promoting HMGR activation in the presence of its activitor were also studied and the results are shown in table IV. Calcium was demonstrated to be the best supportive ion in activating HMGR by its activator. Moreover the inhibition by EGTA on activation of HMGR by its activator.can be overcome by addition of Ca^{2+} (Table V). Trifluoperazine, a calmodulin antagonist, was also found to be a potent inhibitor of the HMGR activator in activating HMGR activity (Table VI).

From results described above, several properties found in HMGR activator are similar to those of calmodulin i.e. M_r, heat stability, Ca^{2+} dependence and inhibitory effect of trifluoperazine. It is, therefore, likely to be calomodulin. Further confirmation is given by its ability to activate

NAD kinases. The activations of NAD kinase in latex bottom fraction and commercial eukaryotic NAD kinase were observed with HMGR activator similar to that found with brain calmodulin (Table VII).

It was reported earlier that ATP-Mg^{2+} can inhibit HMGR activity in bottom fraction and the inhibition can be overcome by addition of heat-stable protein which was partially purified from C-serum (12). Our study showed that a similar inhibition properties are also associated with purified HMGR activator (Table III). The inhibition of HMGR activity due to the presence of ATP-Mg^{2+} can be overcome by addition of purified HMGR activator but not when HMGR activator is presence together with NaF. This result suggests a requirement of phosphatase activity for HMGR activation by the HMGR activator. HMGR activator may indirectly activate HMGR activity in bottom fraction via regulation of enzymes (present in bottom fraction) involved in phosphorylation and dephosphrylation of HMGR similar to those suggested for mammalian HMGR (13-15).

Supportive evidence came from our subsequent study on effects of calmodulin and phosphatase on HMGR activity (Table VIII). The calmodulin failed to activate HMGR in the solubilized bottom fraction as well as purified HMGR. The alkaline phosphatase is, however, able to activate purified HMGR. It can, therefore, be suggested that the activation of HMGR activity observed in the bottom fraction is due to the indirect involvement of calmodulin on the HMGR enzyme. The purified enzyme may be regulated by phosphorylation and dephosphorylation as suggested for the mamalian HMGR. Although our preliminary result suggest 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.

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

Disease resistance study with life pathogen infection was performed on young *Hevea* leaves. The leaves were inoculated with agar discs containing *phytophthora botryosa* prepared as described under material and method (Part B). 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 of lesion diameter respectively.

After 24 hours of leaf inoculation by layering with agar discs containing phytophthora 75 out of 93 leaf inoculation sites on RRIM 600 leaves were infected while only 18 out of 93 sites on GT-1 leaves show lesion signs (Fig. 5-8). The degree of infection observed with RRIM 600 leaves is more prominent that 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 respectivety (Fig. 5,6,9). There are 50 lesions out of 93 inoculation sites on GT-1 leaves with 84 and 16% of +1 and +2 lesion scores.(Fig. 5,6,10). It is clearly seen clearly seen from these results (Fig.5-10). That higher degree of lesion intensity and number of infection sites increase with inoculation time with the leaves of RRIM 600 than those of GT-1. The GT-1 leaf is therefore shown to be more resistant to phytophthora infection that of RRIM 600.

No sign of infection is observed on the control leaf samples when either pure agar discs or contaminated agar discs with non pathogenic fungus were layered on GT 1 and RRIM 600 leaves (Fig. 11-14). There is no different on results observed either after 48 (Fig 11,12) or 72 hours (Fig. 13,14) of inocululation time on GT-1 or RRIM 600 leaves.

5. Clonclusion remarks :

- *Hevea* HMG-CoA reductase (HMGR) may be regulated by phosphosylation and dephosphorylation as suggested for the mommalian HMGR.
- HMGR activator is likely to be *Hevea* calmodulin and appears to indirecty activate HMGR through a cascade mechanism.
- HMGR activator posses molecular weight of 17,500 daltons similar to that of calmodulin.
- HMGR activator can activate NAD kinase enzyme in the bottom fraction of centrifuged latex similar to that reported for calmodulin.
- GT-1 leaf is more resistant to *phytophthora botryosa* infection than that of RRIM 600.

6. Work plan for next period :

- Perform immonological studies on purified HMGR.
- Develop ELISA technique for HMGR measurement.
- Disease resistance assessment by using toxin extracted from suspension culture of *Phytophthora botryosa*.
- Studies on diurnal variation of MUA kinase, HMG-CoA synthetase in *Hevea* leaves.

7. References :

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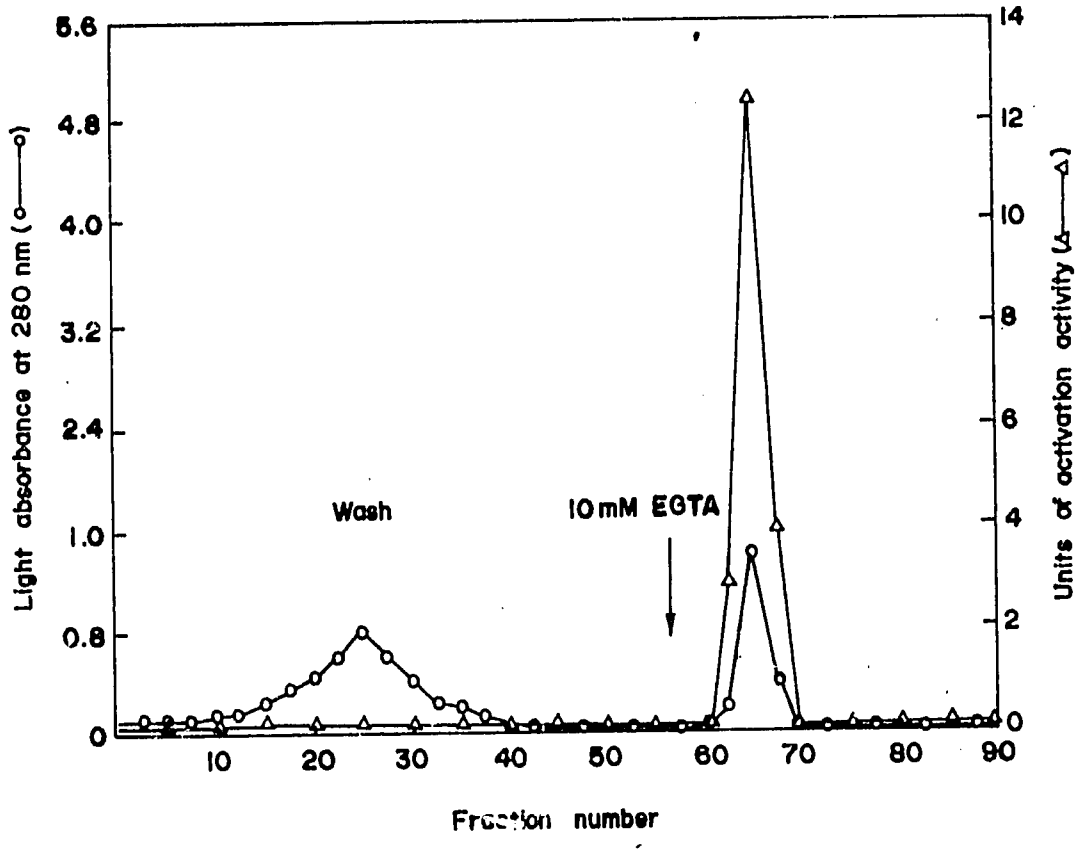


Fig. 1 Purification of *Hevea* calmodulin on Affi-gel Phenolthiazine column. The column was washed with 50 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM $CaCl_2$ and 3 N NaCl and was eluted with the same buffer containing 10 mM EGTA.

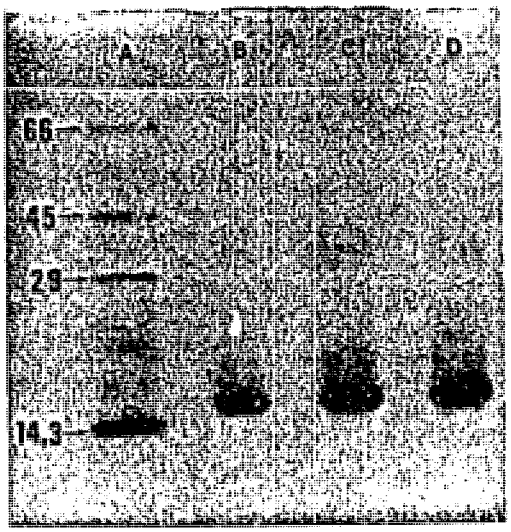


Fig. 2. Polyacrylamide gel electrophoresis of purified *Hevea* calmodulin in the presence of sodium dodecylsulfate with 10% gel. Lane A is stardard proteins (KDa); Lane B,C or D is peak fraction from Affi Gel phenothiazine column at different protein concentrations.

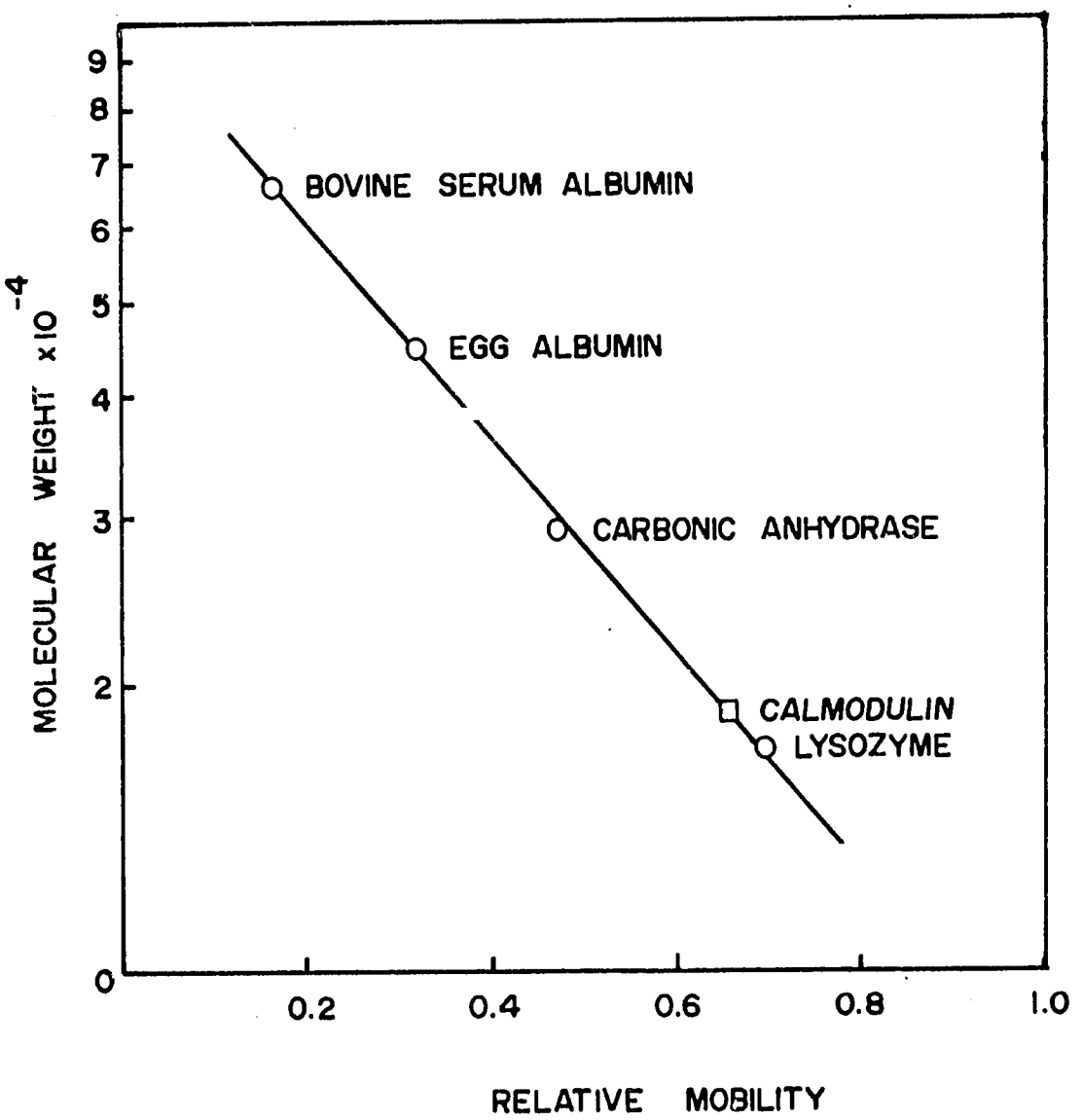
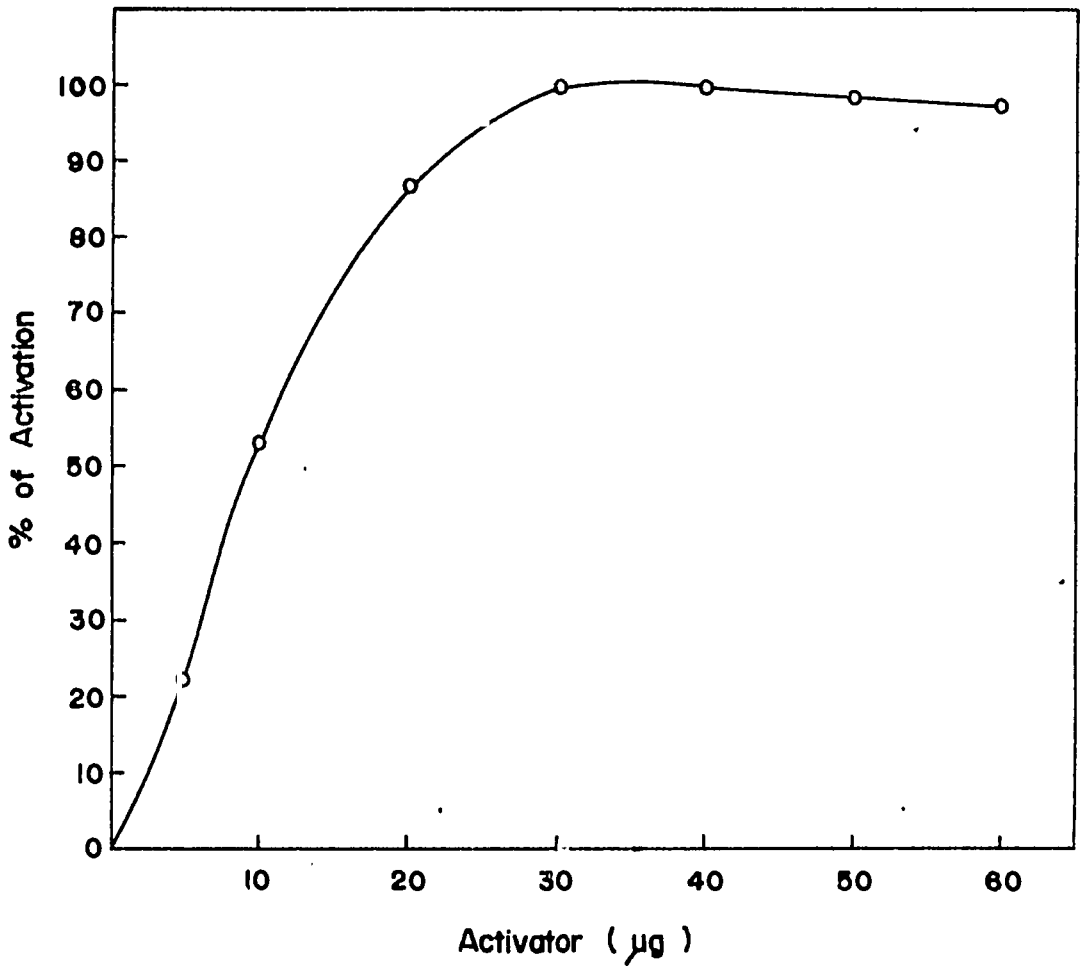


Fig. 3 Molecular weight calibration of purified *Hevea* calmodulin. Both calmodulin and standard proteins were performed on slab gel under 10% SDS-PAGE.



.Fig. 4 Effect of varying concentrations of HMGR activator (calmodulin) on the HMGR activity in latex bottom fraction. Maximum activation was obtained when 30 µg protein was added to 1 ml reaction mixture.

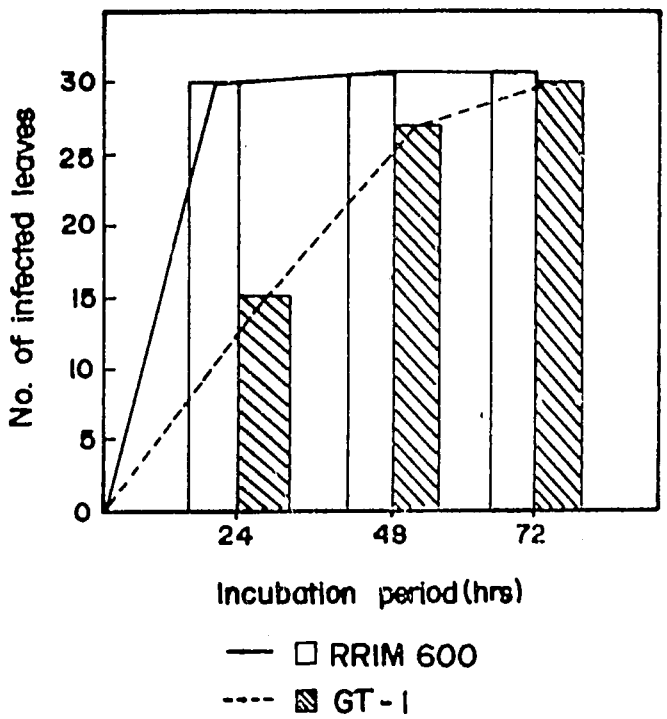


Fig.5. Disease resistance study of life pathogen *(phytophthora botryosa)* infection on young RRIM-600 and GT-1 leaves. The number of infected leaves (columns) were recorded at 24, 48 and 74 hours after inoculations. Solid and dotted lines represent infection rate of RRIM-600 and GT-1 clones respectively.

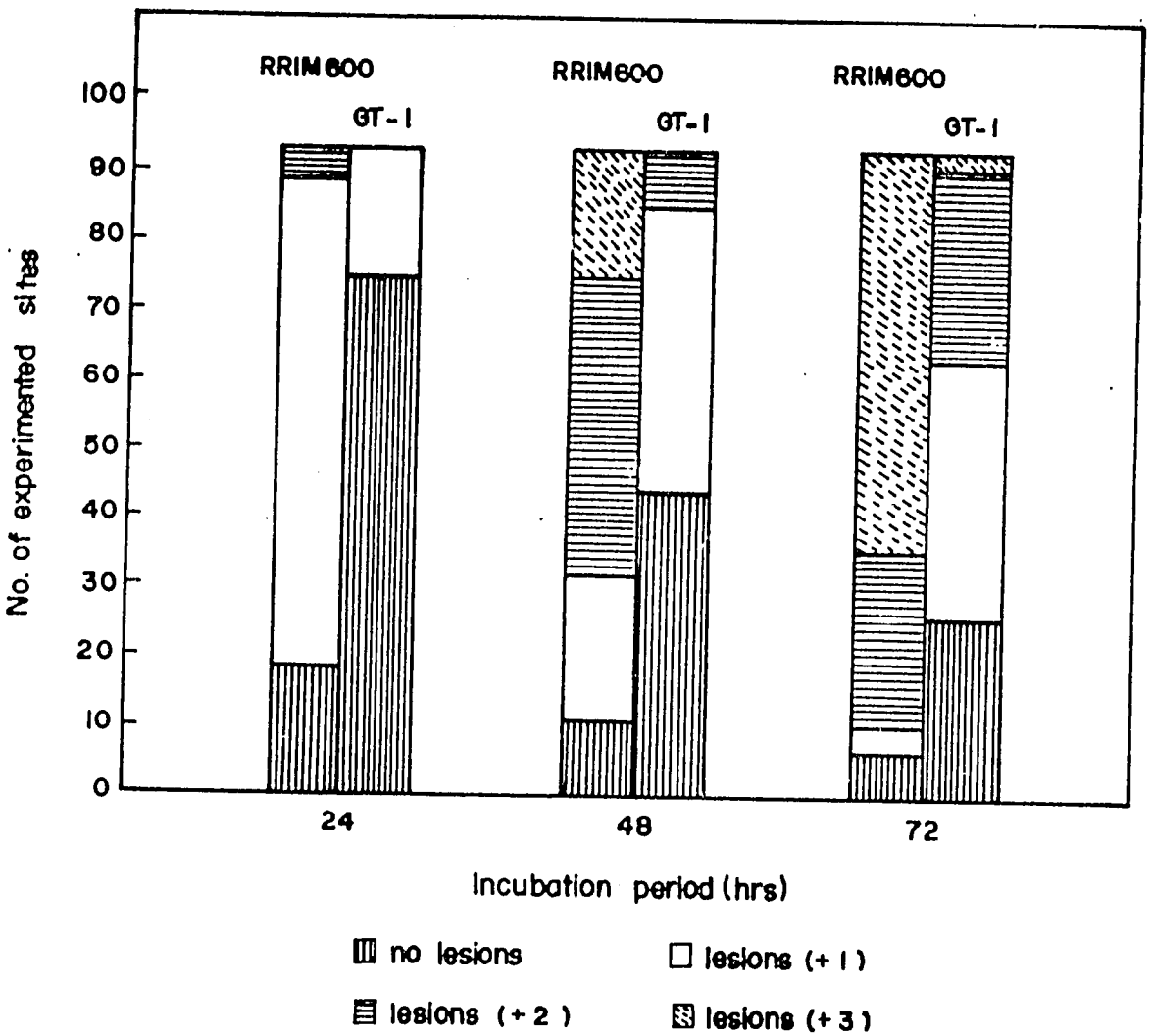


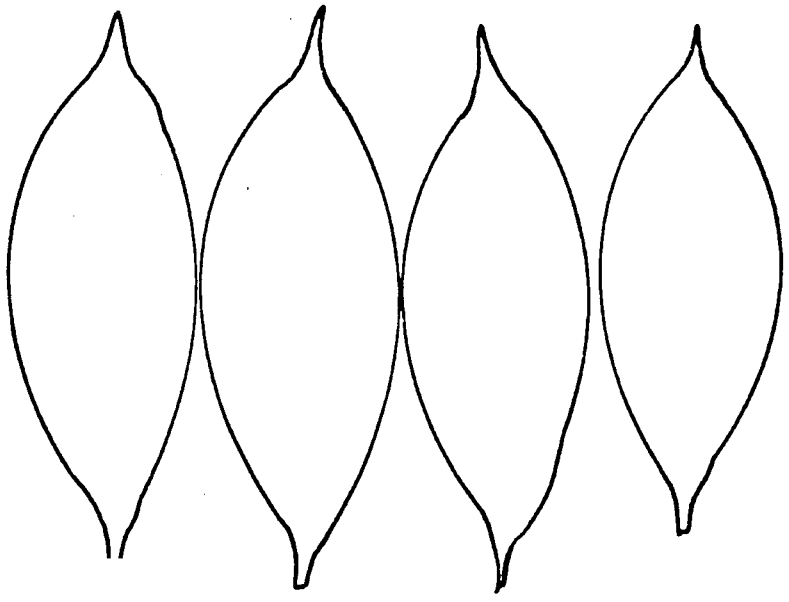
Fig. 6. Degrees of lesion intensity are compared after 24,48 and 72 hours of life pathogen inoculations on young RRIM-600 and GT-1 leaves. The empty columns represent number of inoculated sites that show low degree of lesion intensity with +1 score. Columns filled with axis, vertical and tilted broken lines represent number of inoculated sites that show no lesion, medium (+2) and high (+3) degree of lesion intensities respectively.

CONTROL RRIM-600

INFECTED RRIM-600

Fig. 7 Control and infected RRIM-600 leaves after 24 hours of inoculation time. Lesion scores are given on the following page.

Control RRIM-600 leaves



Infected RRIM-600 leaves

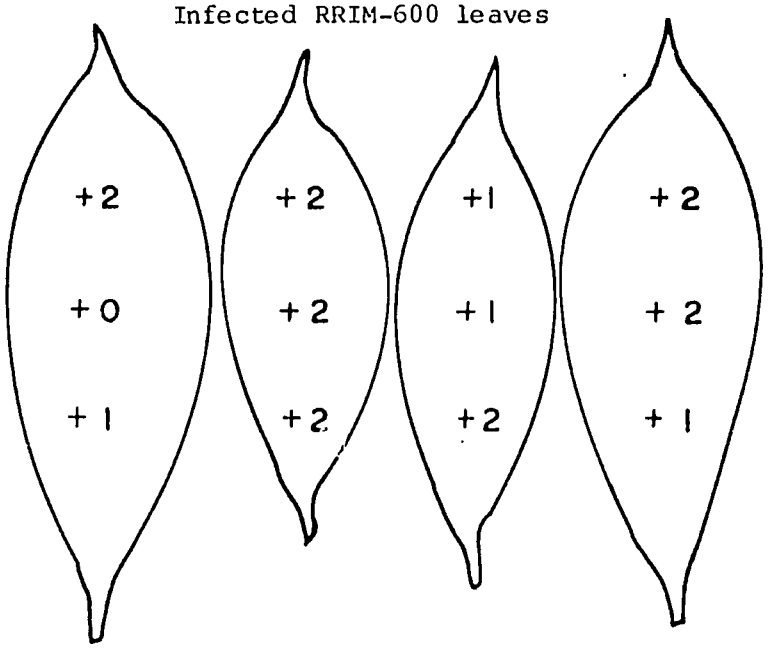


Fig. 7. Lesion intensity scores of the inoculation sites on the infected RRIM-600 leaves after 24 hour of inoculation time.

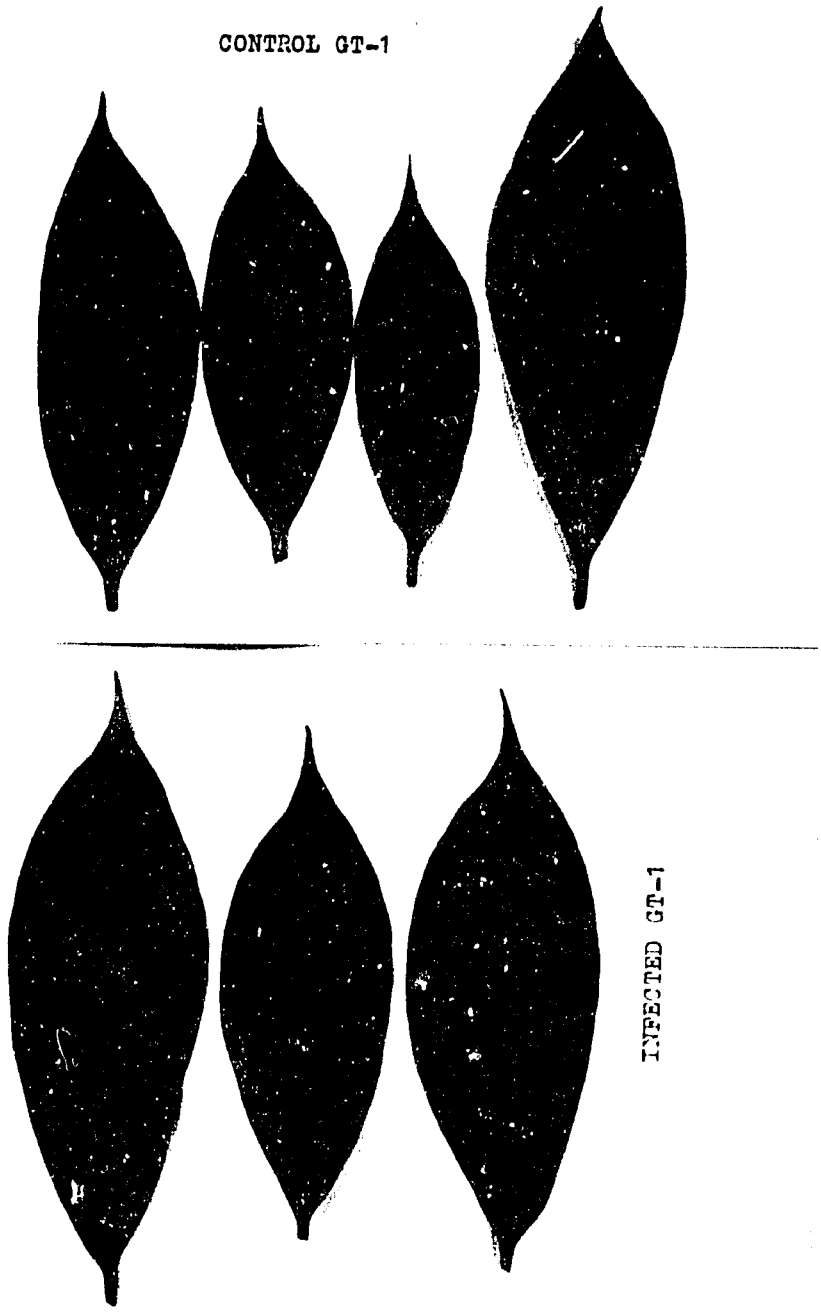
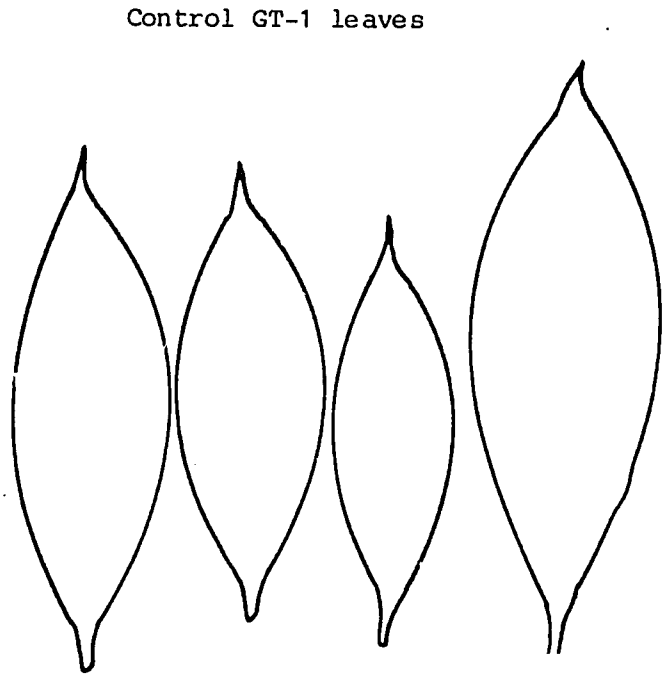


Fig. 8. Control and infected GT-1 leaves after 24 hour of inoculation time. Lesion scores are given on the following page.



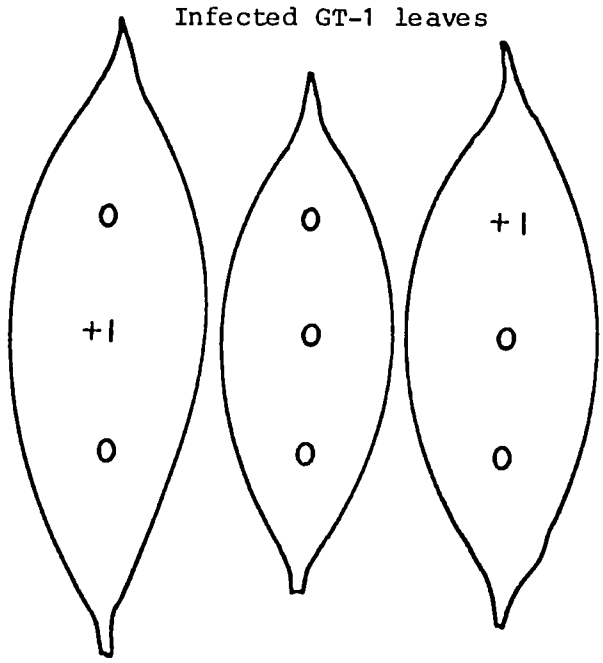
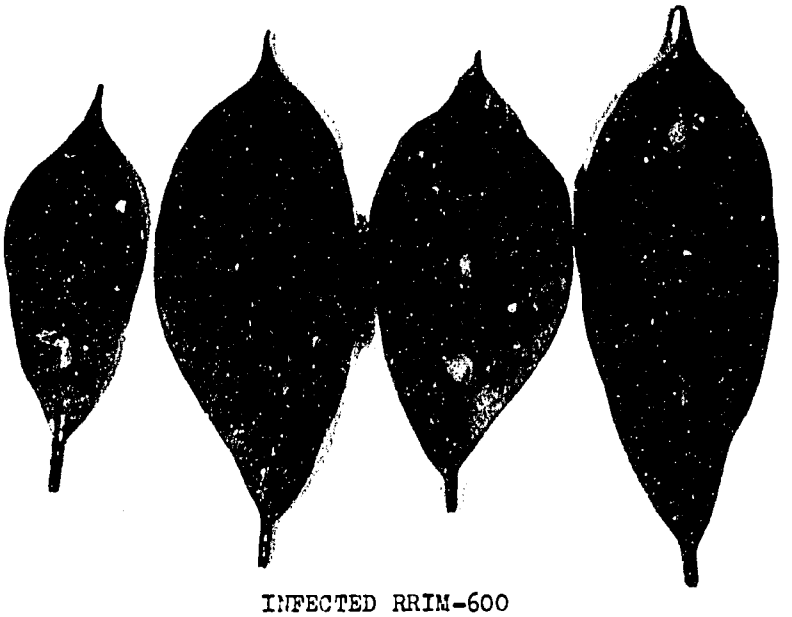
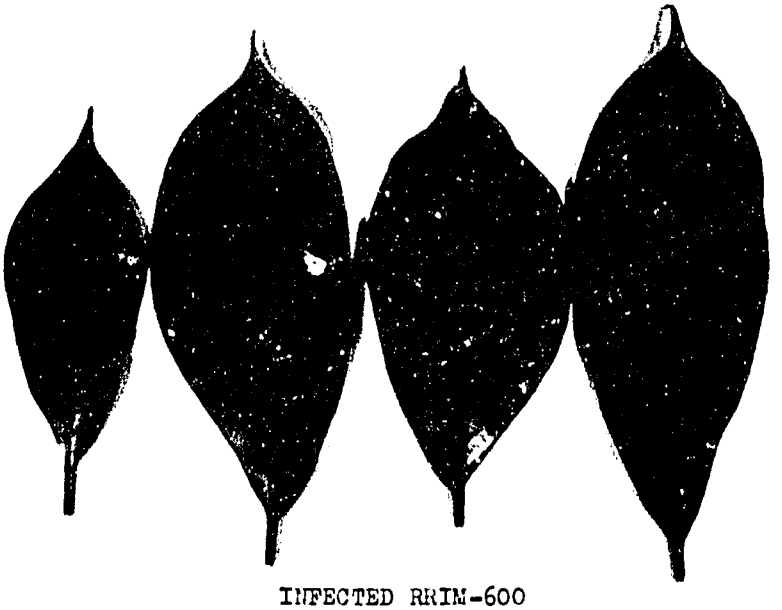


Fig. 8. Lesion intensity scores of the inoculation sites on the infected GT-1 leaves after 24 hours of inoculation time.



INFECTED RRIM-600



INFECTED RRIM-600

Fig. 9. Infected RRIM-600 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.

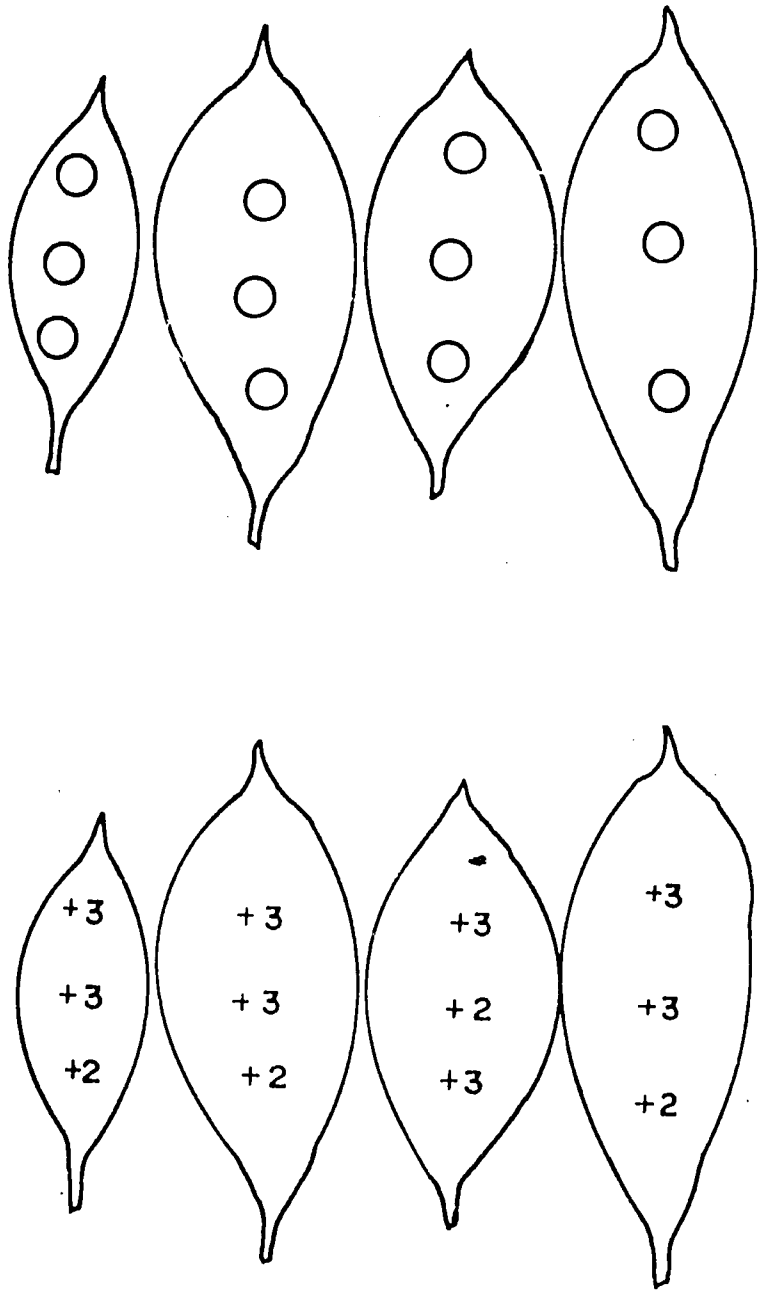


Fig. 9. Lesion intensity scores at inoculation sites of the infected RRIM-600 leaves after 48 hours of inoculation time.

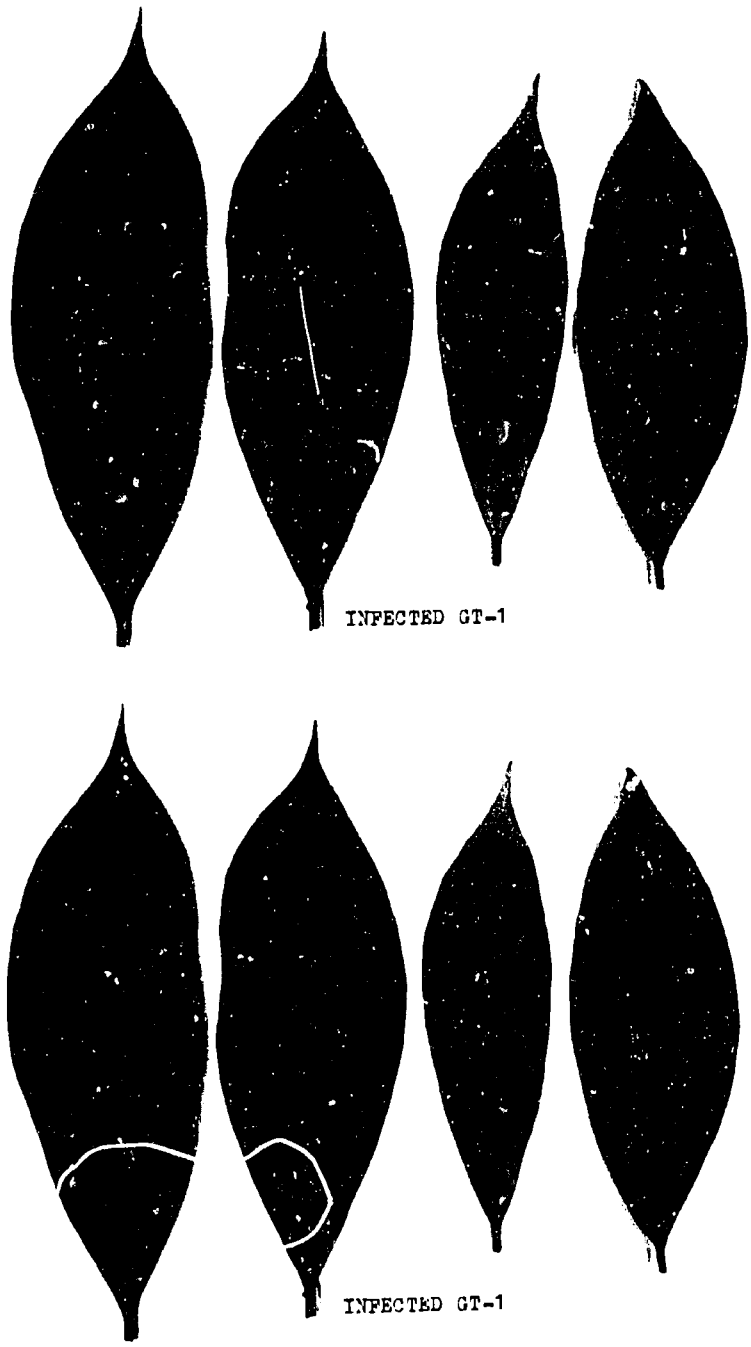


Fig. 10. Infected GT-1 leaves after 48 hours of inoculation as viewing before (upper picture) and after (lower picture) the removal of agar discs containing pathogenic fungus *(phytophthora botryosa)*. Lesion scores are given on the following page.

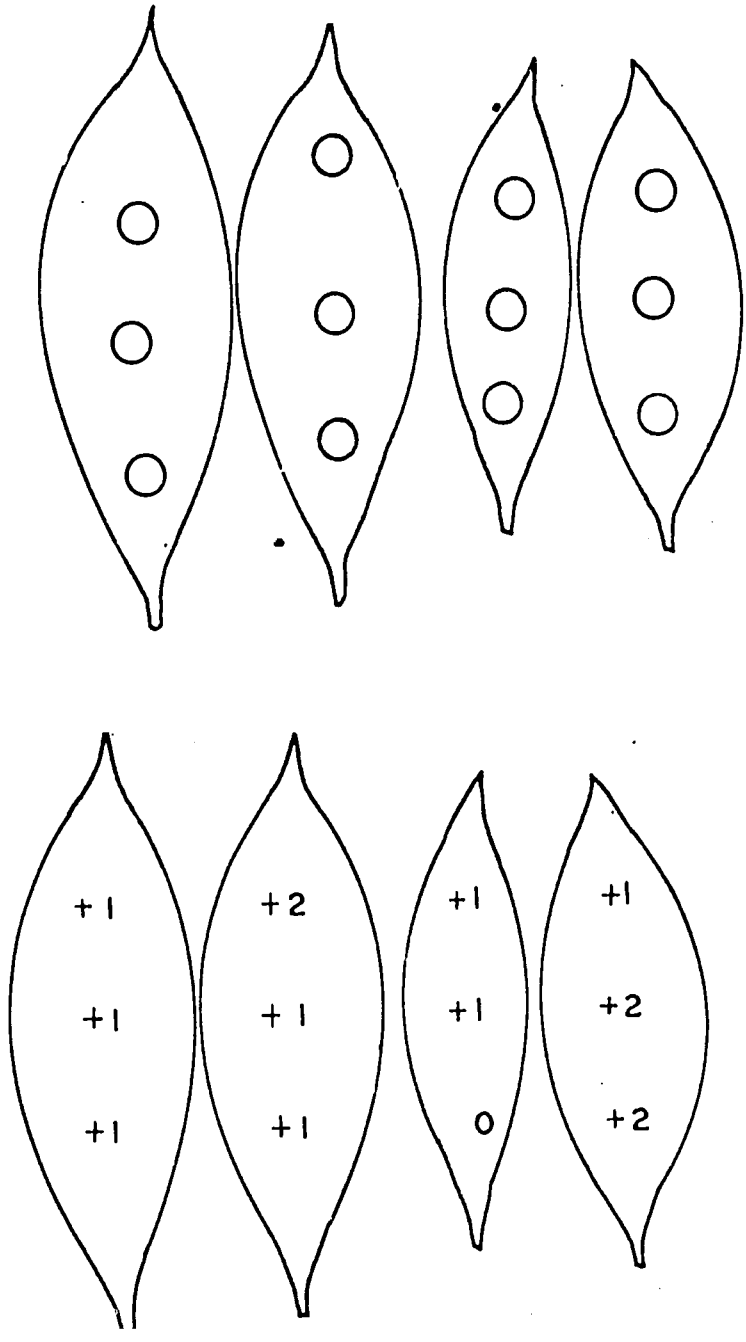
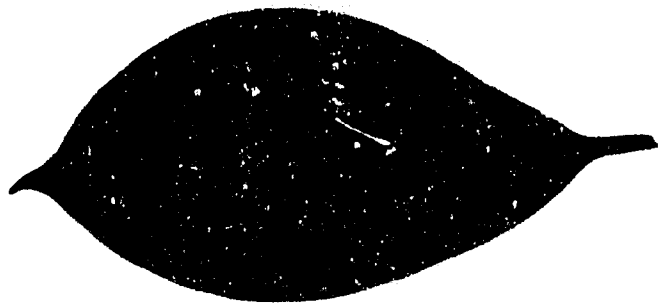
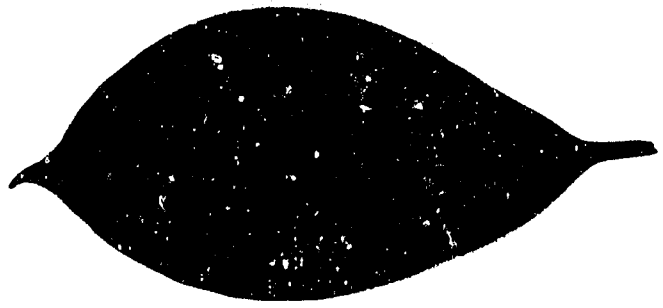


Fig. 10. Lesion intensity scores at inoculation sites of the infected GT-1 leaves after 48 hours of inoculation time.



CONTROL RRIM-600

No.8



CONTROL RRIM-600

No.8

Fig. 11. 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.

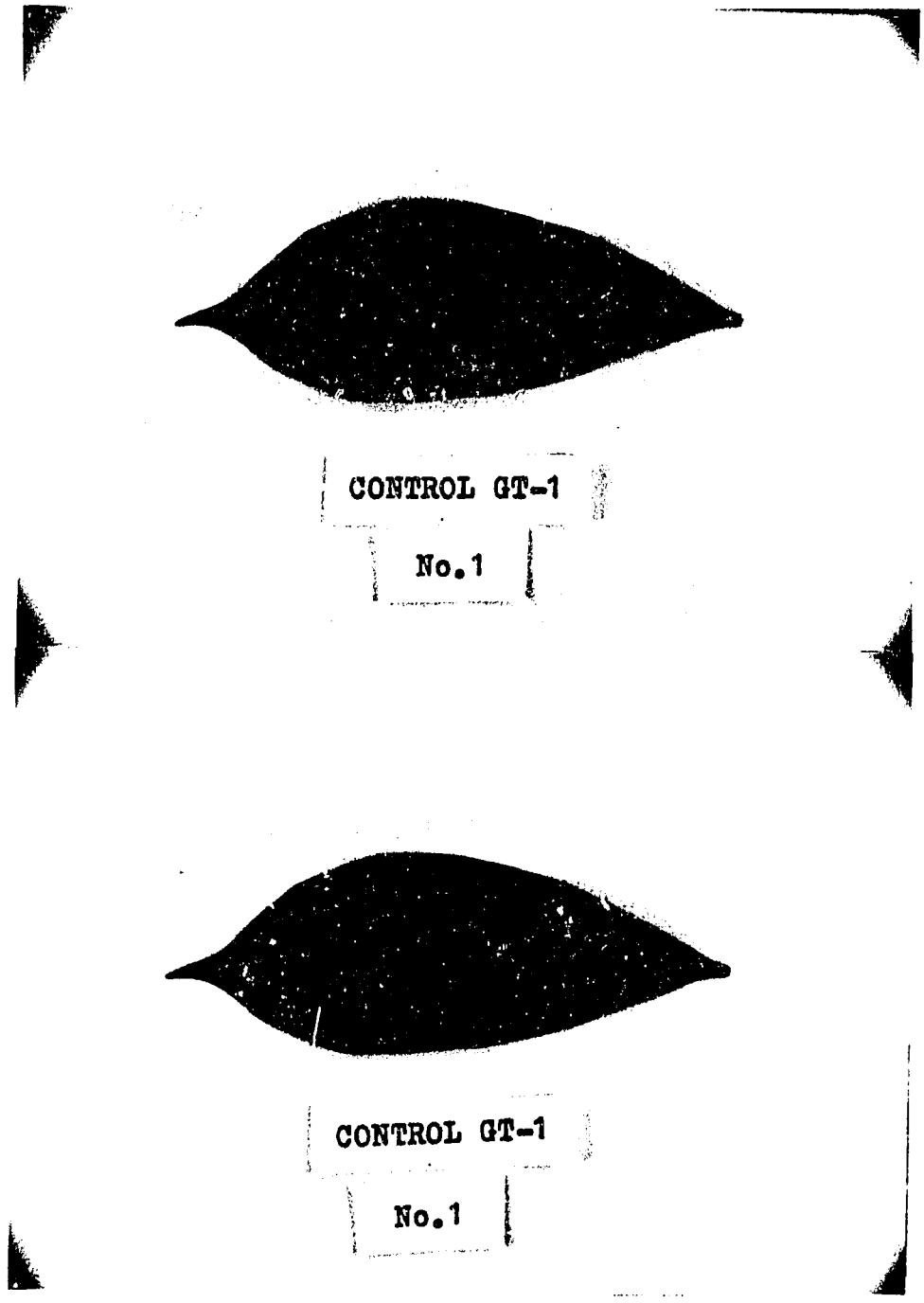


Fig. 12. Control GT-1 leaf after 48 hours of inoculation with uncontaminated and contaminated agar discs containing non pathogenic fungus of unidentified species before (upper picture) and after (lower picture) the removal of agar discs.

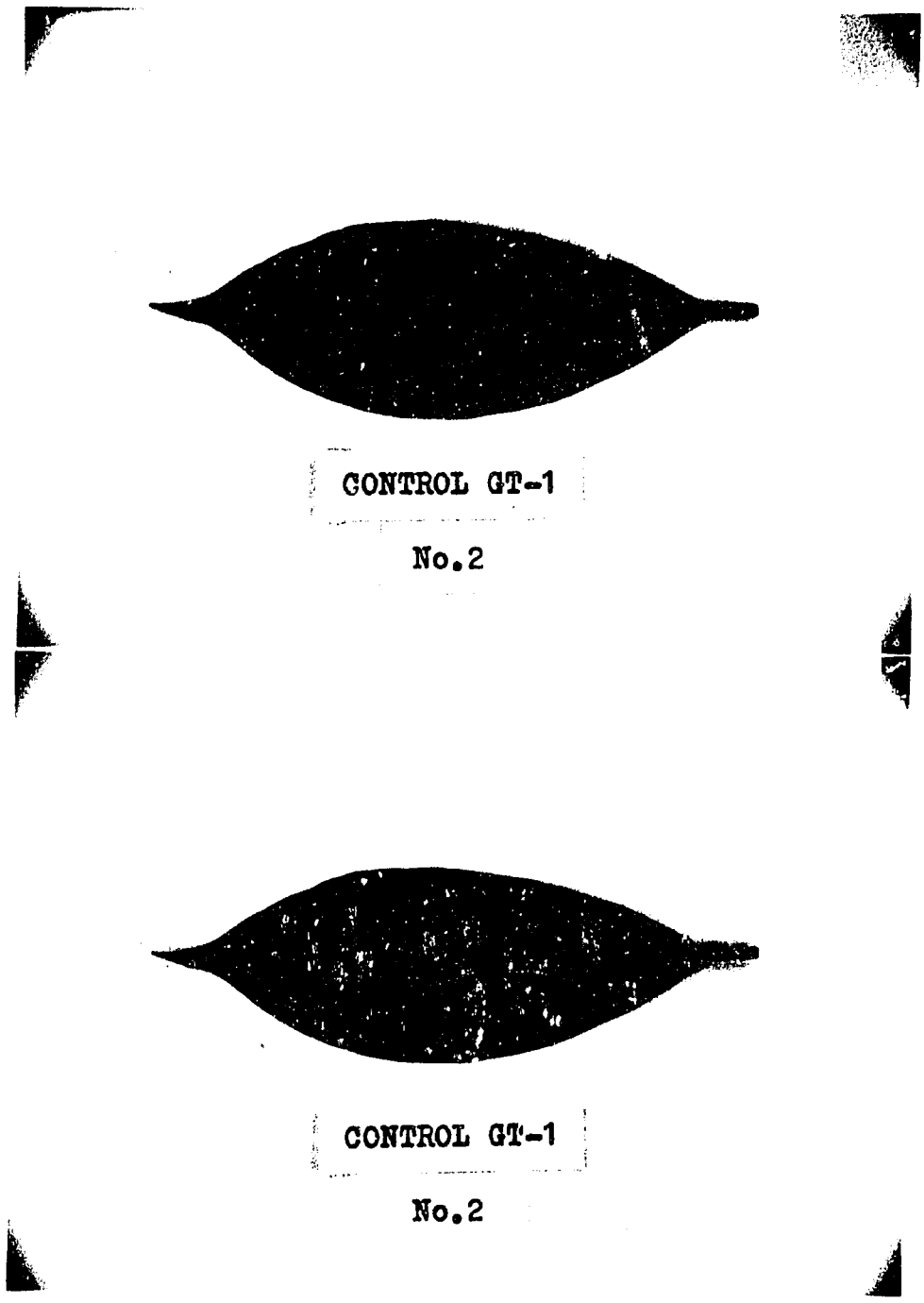


Fig. 13. Control GT-1 leaf after 72 hours of inoculations with contaminated and uncontaminated agar discs containing non pathogenic fungus of unifentified species before (upper picture) and after (lower picture) the removal of agar discs.

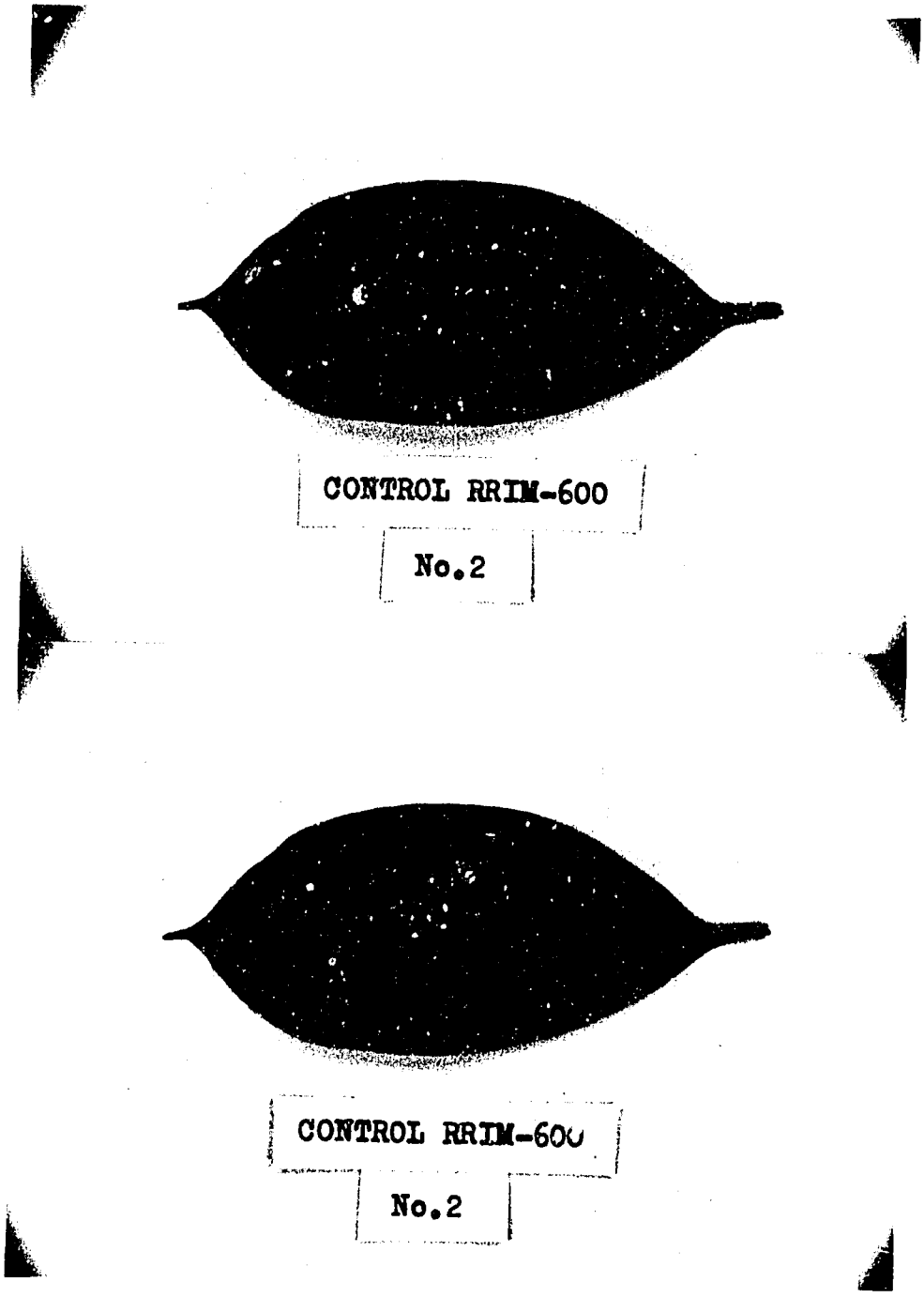


Fig. 14. Control RRIM-600 leaf after 72 hours of inoculation with contaminated agar discs containing non-pathogenic fungus of unidentified species before (upper picture) and after (lower picture) the removal of agar discs.

Table I

Effect of C-serum on HMGR activity obtained from the bottom fraction suspension of latex from high, medium and low yielding rubber trees.

	HMGR activity (nmole MVA formed/min/mg.protein)						
Source of bottom fraction \ Source of C-serum added	Control	^{H}RRIM 600	^{M}RRIM 600	^{L}RRIM 600	^{H}KRS 208	^{M}KRS 208	^{L}KRS 208
^{H}RRIM 600	2.19	4.06 (85%)	2.71 (23%)	2.43 (10%)	3.66 (67%)	2.84 (30%)	2.20 (0.46%)
^{M}RRIM 600	1.10	1.99 (80%)	1.73 (57%)	1.30 (18%)	2.34 (112%)	2.12 (92%)	1.56 (14.8%)
^{L}RRIM 600	1.28	2.19 (41%)	1.59 (24%)	1.33 (4%)	2.07 (67%)	1.49 (1.6%)	1.47 (14.8%)

H, M and L represent latex obtained from high, medium and low yielding trees accordingly.

( ) Percent of activation is shown in parenthesis.

Table II

Purification protocol of HMGR activator (calmodulin)

Purificaiton 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
bioled 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 III

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 IV

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 V

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+}	2.0	168

Table VI

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	0.80	3

Table VII

Effect of *Hevea* calmodulin on NAD kinase activity

treatment	% activation	Specific activation (unit*)
Hevea NAD kinase	0	0
Hevea NAD kinase + *Hevea* calmodulin	134	187
Liver NAD kinase	0	0
Liver NAD kinase + *Hevea* calmodulin	48	158
Liver NAD kinase + brain calmodulin	91	161

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

Table VIII

Effect of HMGR activator, phosphatases 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