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Departments AMIS/Agronomy and CP/Rubber

HEVEA

Mission Report to RRIT-DOA in Thailand

Chachoengsao Rubber Research Center (CRRC)

from 4th to 15th December 2000

Anne CLEMENT

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Abstract
This mission was conducted in the frame of the SMOU 2 passed between the Rubber Programme of Cirad-cp (France) and RRIT-DOA (Thailand) devoted to the « Application of molecular biology in relation to plant breeding and plant physiology of the rubber tree ». The mission was coming after the previous missions of V. Pujade-Renaud and J.C. Prévôt and the recent training period of Mrs Pisamai Chantuma at Cirad Centre in Montpellier last June 2000.

The mission was aiming at giving training in some biochemistry techniques and at continuing a collaborative research project on oxidative stress in the laticiferous tissue. Training was delivered to Pisamai Chantuma, Somjintana Promson, Prasan Seubsuk and Kanlaya Prapan on electrophoresis and chromatography technics.

The project is currently focusing on dehydroascorbate reductase (DHAR) and the associated antioxudants (thiols and ascorbic acid). Analyses of thiols, ascorbic acid, proteinsand DHAR activity were performed on clones RRIM600, GT1 and PB235 at different stimulation frequencies. Latex sampling was made for further analyses.

DHAR activity may become a useful parameter to characterize clones (lowest for GT1, intermediate for RRIM600 and very high for PB235). But the levels are not yet related with the intensity of stimulation. This can be explained by the fact that, at the present stage of the experiment, the trees are not highly stressed by the exploitation. So, it is not yet possible to know whether this enzyme is a limiting factor affecting the protection against oxidative stress or not.

The levels of thiols and ascorbic acid are closely correlated.

Keywords
Rubber laticifer physiology and biochemistry, oxidative stress of the laticifer, enzymes, glutathion reductase (GR), dehydroascorbate reductase (DHAR), antioxidants, thiols, ascorbic acid, electrophoresis, SDS-PAGE, ELISA, western blotting, chromatography.

CONTENTS

I - Introduction and Objectives

This mission was conducted in the frame of the SMOU 2. It aims both to give training in some useful techniques and to continue a collaborative research project on oxidative stress.

Oxidative stress (uncompensated production of toxic oxygen species) is responsible for the degradation of a number of cellular compounds, and more specially the membranes. In rubber tree, oxidative stress generated by overexploitation may impair the yield of latex production by affecting both latex flow (the degradation of the lutoid membranes induces the release of coagulating factors responsible for earlier coagulation) and the regenerative metabolism. It may also affect the quality of the rubber product.

A number of enzymes involved in plant protection against oxidative stress have been previously described. Our aim is to identify among those some enzymes limiting for the yield of rubber production. The Yield Potential Trials set up by Dr Eric Gohet with and at CRRC provide a good experimental basis to try answering this question. These trials aim to compare several clones for their yield potential (evaluated by the production, the LD parameters, the observation of TPD symptoms...) in response to various intensities of exploitation (ie various numbers of stimulations per year), over several years, from the opening of the trees until the occurrence of physiological strain symptoms.

On this basis, some biochemical parameters (enzyme activity and antioxidant levels) can be regularly analyzed to check whether or not they are correlated to the clonal tolerance to exploitation stress. For a given enzyme identified as limiting, molecular analysis (of gene expression) should be performed to check whether the limitation in terms of activity can be accounted for a limitation in gene expression.

Previous missions of CIRAD agents at CRRC (V Pujade-Renaud/J-C Prévot in November 1999, J-C Prévot in Mai 2000) were launched to provide training both in molecular biology (gene expression analysis) and biochemistry (analysis of enzyme activities and antioxydants levels), and to initiate the collaboration around oxidative stress. From the analysis performed at CRRC, it appeared that the enzyme glutathion reductase was not limiting. On the other hand, preliminary results on dehydroascorbate reductase (DHAR) indicated clonal variations in terms of enzyme activity. Mrs Pisamai Chantuma together with Anne Clément-Vidal performed the purification of the enzyme at Montpellier (June 2000). From the purified DHAR enzyme, it will be possible to raise antibodies that will be useful for large scale screening of DHAR levels using SDS-PAGE/western blotting or ELISA techniques.

At that stage of the project, it was decided to focus on dehydroascorbate reductase and the associated antioxidants (thiols and ascorbic acid).

The mission of Anne Clement-Vidal at CRRC (Decembre 2000) had several objectives

1. To continue the biochemical analysis initiated at CRRC by J-C Prévôt and the CRRC researchers (Pisamai Chantuma, Somjintana Promson and Prasan Seubsuk) in order to confirm the preliminary results.

2. To provide theoretical and practical training on the separation and detection of proteins by electrophoresis (PAGE and SDS-PAGE) and on HPLC methods, as requested by CRRC.

3. To perform latex sampling to carry out gene expression analysis at Montpellier by V. Pujade-Renaud's team.

CRRC partipants: Pisamai Chantuma, Somjintana Promson, Prasan Seubsuk and Kanlaya Prapan.

II - Planning of activities

04/12/00: Explanation of mission objectives and main analysis carried out – Preparation of the reactives.

05/12/00: Latex sampling from the yield potential trial CH OE 02 (clone RRIM 600). Analysis of thiols, ascorbic acid, proteins and dehydroascorbate reductase activity.

06/12/00: Latex sampling from the yield potential trial CH OE 03 (clone GT 1). Analysis of thiols, ascorbic acid, proteins and dehydroascorbate reductase activity.

07/12/00: Latex sampling from the yield potential trial CH OE 01 (clonePB 235). Analysis of thiols, ascorbic acid, proteins and dehydroascorbate reductase activity.

08/12/00: Latex sampling from the yield potential trial CH OE 02 (clone RRIM 600) for biochemistry and molecular biology. Analysis of thiols, ascorbic acid, proteins and dehydroascorbate reductase activity.

11-12/12/00: training on electrophoresis technic

13/12/00: Latex sampling from the yield potential trial CH OE 03 (clone GT 1) for biochemistry and molecular biology. Analysis of thiols, ascorbic acid, proteins and dehydroascorbate reductase activity.

14/12/00: Latex sampling from the yield potential trial CH OE 01 (clonePB 235) for biochemistry and molecular biology. Analysis of thiols, ascorbic acid, proteins and dehydroascorbate reductase activity.

Meeting with CRRC team to present oxidative stress and to discuss about the objectives of the mission.

15/12/00 Training on chromatography technics

Uncountered persons:

Pisamai Chantuma: CRRC agrophysiologist.

Eric Gohet: CIRAD-CP/hevea, agrophysiologist.

Prawit Wongsukon: RRIT, former CRRC director (called to other responsibilities).

Chamnong Kongsing: RRIT, CRRC new director.

Arak Chantuma: RRIT, CRRC physiologist.

M. Suchin: RRIT, CRRC physiologist.

III - Experimental protocol of yield potential trial.

We analysed 3 clones: PB 235, RRIM 600 and GT 1 and 5 treatments with different stimulation frequencies. All trees were opened in may 1999 and they are tapped ½ S d/3 6d/7. The different treatments are :

B: no stimulation

- C: ET 2.5 % Pa 2/y
- D: ET 2.5 % Pa 5/y
- E: ET 2.5 % Pa 8/y
- F: ET 2.5 % Pa 12/y

Table 1: Operations schedule before and during analysis

PB 235 / *RRIM 600* / **GT 1**

➢ Latex sampling

Dates	Tappings	Stimulation	Analysis
01/11/00		DEF	
02/11/00	BCDEF	*DEF*	
03/11/00	*BCDEF*	**DEF**	
04/11/00	**BCDEF**		
05/11/00			
06/11/00	BCDEF		
07/11/00	*BCDEF*		
08/11/00	**BCDEF**		
09/11/00	BCDEF		
10/11/00	*BCDEF*		
11/11/00	**BCDEF**		
12/11/00			
13/11/00	BCDEF		
14/11/00	*BCDEF*		
15/11/00	**BCDEF**		
16/11/00	BCDEF		
17/11/00	*BCDEF*		
18/11/00	**BCDEF**		
19/11/00			
20/11/00	BCDEF		
21/11/00	*BCDEF*		
22/11/00	**BCDEF**	F	
23/11/00	BCDEF	*F*	
24/11/00	*BCDEF*	**F**	
25/11/00	**BCDEF**		
26/11/00			
27/11/00	BCDEF		
28/11/00	*BCDEF*		
29/11/00	**BCDEF**	E	

30/11/00	BCDEF	*E*	
01/12/00	*BCDEF*	**E**	
02/12/00	**BCDEF**		
03/12/00			
04/12/00	BCDEF		
➢ 05/12/00	*BCDEF*		*RRIM 600*
➢ 06/12/00	**BCDEF**		**GT 1**
➢ 07/12/00	BCDEF		PB 235
➢ 08/12/00	*BCDEF*		*RRIM 600*
09/12/00	**BCDEF**		
10/12/00			
11/12/00	BCDEF		
12/12/00	*BCDEF*		
➢ 13/12/00	**BCDEF**	F	**GT 1**
➢ 14/12/00	BCDEF	*F*	PB 235
15/12/00	*BCDEF*	**F**	

Trees selection

We analysed the same trees than previously analysed by J. C. Prevot. We used 5 trees by treatment with homogeneous production and girth.

B			C			D			E			F		
PB 235	RRIM600	GT 1	PB 235	RRIM600	GT 1	PB 235	RRIM600	GT 1	PB 235	RRIM600	GT 1	PB 235	RRIM600	GT 1
2	1	2	4	1	1	5	3	4	3	1	2	1	2	3
3	3	5	6	3	4	6	9	5	6	2	4	3	5	5
4	7	6	8	8	6	10	10	6	8	6	7	5	8	9
7	11	7	9	9	10	12	12	7	9	7	10	6	10	10
8	15	13	10	14	12	13	15	11	12	11	12	12	11	12
9	4	4	11	2	3	8	11	1	1	9	5	2	15	2
10	14	8	13	7	11		13	10	2	12	13	13	12	4

All trees were sampled at the minimum 5 days after stimulation (Table &), to the exceptionof sample F from RRIM 600 wich was collected 1 day only after stimulation.

Latex sampling

- For biochemical analysis the latex was harvested between the 5 th and the 30 th minutes after tapping in a Whirlpack bag. For each clone, 10 ml of latex from 5 trees was collected and pooled in another bag. From this stock 1 ml of each clone was sampled and added to 9 ml of TCA 2.5 % + 0.01 % EDTA to carry out latex diagnosis and 1 ml was collected in a task to make Total Solid Contents. All samples were transfered in ice-box to the laboratory. Samples were centrifuge at 9000 rpm for 30 min, then white fractions were

centrifuged for 1h at 50 000 rpm. Clear C serum was used to carry out biochemical analysis.

- For RNA extraction for each treatment (B, C, D, E, F),1 ml of latex was collected from 6 trees and pooled in a tube containing 6 ml of buffer. Latex and buffer were carefully mixed and immediately frozen in liquid nitrogen. It was necessary to use gloves and steril gutters (sterilisation with pasteur oven), to prevent RNA degradation.

IV - Biochemical analysis methods

DHA reductaseactivity measurement

50 mM pH 7,0 phosphate buffer	1 ml	
40 mM GSH	25 µl	Final concentration:1 mM
40 mM DHA	15 µl	Final concentration: 0.6mM

Wavelenght = 265 nm.

The reaction was started by addition of 7µl of enzyme. It was necessary to subtract the blanck value. Blanck consisted of the chemical reaction between glutathione and dehydroascorbate.

Proteins assay

Protein contents were determined by the Bradford method using Coomassie brillant blue reactive and bovin serum albumin as standard (0,5 mg/ml). The staining solution contained 100 mg of brillant blue diluted in 50 ml of ethanol. 100 ml 85 % w/v phosphoric acid were added to this solution and diluted to a final volume of 1 liter. This solution was filtered on filter paper.

Standard curve

Volume SM µl	0	10	15	20	25	30	40	50
Quantity µg	0	5	7.5	10	12.5	15	20	25
Reactive ml	1	1	1	1	1	1	1	1

For C serum sample 10 and 20 µl were used after 10 times dilution.

C serum deproteinisation for thiols and ascorbate measurement

500 µl of C serum sample were diluted in 500 µl of TCA 5 %. After centrifugation analysis was carried out on the supernatant.

Thiols measurement

Thiols concentration was determined using the method of Ellman.

DTNB reactive: 99,125 mg of DTNB (10 mM) + 186 mg EDTA (20 mM) in 6 ml of 0,5 M Tris, the volume was adjusted to 25 ml with distilled water.

Standard solution: 1 mM GSH in 2.5 % TCA.

1 mM GSH µl	0	20	40	60	80	100
2,5 % TCA µl	1000	980	960	940	920	900
GSH mM	0	0.02	0.04	0.06	0.08	0.1

50µl of DTNB were added and 1 ml 0.5M Tris in every solution. For sample, 100 and 200 µl were added to 900 and 800 µl of TCA 2.5 % + 50µl of DTNB + 1 ml 0.5M Tris.

Reading: Wavelenght = 412 nm between 2 and 30 minutes.

Ascorbic acid measurement

Ascorbic acid (AsA) concentration was measured by the method of Okamura.

Reactive preparation:

Reactive A:

0.15 M Potassium phosphate buffer pH 7.4	10 ml
10 % TCA	25 ml
DW (distilled water)	15 ml
Phosphoric acid ½ in DW	20 ml
4 % 2,2' dipyridyl in ethanol	20 ml (daily preparation)

Reactive B: 3 % $FeCl_3$ in distilled water.

Standard curve: 0.5 mM AsA prepared in 2.5 % TCA

2 mM AsA µl	0	10	20	30	40	50
2.5 % TCA µl	50	40	30	20	10	0
AsA Mm	0	0.4	0.8	1.2	1.6	2
Réactive A ml	1.8	1.8	1.8	1.8	1.8	1.8
Réactive B ml	0.2	0.2	0.2	0.2	0.2	0.2

Wavelenght = 525 nm, reading after 45 minutes.

25 and 50 µl of sample were used for analysis.

Methods for SDS-PAGE

Gels preparation:

Solutions: 1.5 M Tris-HCl buffer pH 8.8

0.5 M Tris-HCl buffer pH 6.8

10 % SDS: 100 mg/ml

10 % ammonium persulfate: 100 mg/ml

	10 % Acrylamide gel	**12 % Acrylamide gel**
Distilled water	4.85 ml	4.35 ml
1.5 M pH 8.8 Tris	2.5 ml	2.5 ml
SDS	100 µl	100 µl
Acrylamide 40%	2.5 ml	3 ml
Air was removed		
Ammonium persulfate	50 µl	50 µl
TEMED	5 µl	5 µl

Stacking gels preparation

	Stacking gels 4 % Acrylamide
Distilled water	6.4 ml
Tris 0.5 M pH 6.8	2.5 ml
SDS	100 µl
Acrylamide 40%	1 ml
Air was removed	
Ammonium persulfate	50 µl
TEMED	10 µl

Polymerisation time was about 1 hour

Sample preparation:

50 µl of mercaptoethanol were added to 950 µl of Laemmli solution, 50µl of this solution was added to 25 µl of C serum.

BioRad standard 97, 66, 45, 31, 21.5, 14 kDa : 2 µl was added to 40 µl of Laemmli solution .

Samples and standard were boiled at 100° C in a waterbath for 5 minutes.

10 µl of each sample were distributed in wells after cooling.

Preparation of running buffer 5X

Tris : 15g/l

Glycine : 72g/l

SDS : 5g/l.

Buffer dilution: 150 ml 5X buffer +750 ml distilled water.

Electrophoresis conditions : first electrophoresis was carried out at 75 Volts for 15 minutes, then conditions changed to 125 V. Current was stopped when bromophenol bluereached the edge of the gel.

Coomassie staining

Staining solution preparation: 0.1 % Coomassie R250 was diluted in a solution prepared with 40% methanol, 10% acetic acid, 50 % distilled water. After about 30 minutes gel was destained in a bath with 40% methanol, 10% acetic acid, 50 % distilled water.

V - Results-Discussion

Parameters linked to oxidative stress (appendix 1)

Dehydroascorbate reductase activity and thiols and ascorbic acid levels were measured on the 3 clones (RRIM600, GT1, PB235) of the first yield potential trial opened in May 99. The analysis was repeated on new samples collected 3 to 7 days later.

The steady state level of dehydroascorbate reductase activity was significantly different depending on the clone. It was the lowest for GT1 (0.03 to 0.14 u/mg), intermediate for RRIM600 (0.09 to 0.18 u/mg) and very high for PB235 (0.24 to 0.52 u/mg). This confirms results observed by J-C Prévôt from experiments in Ivory Coast and in CRRC. DHAR may therefore become a useful parameter to characterize clones.
However, for all clone the level of DHAR activity remained rather stable whatever the number of stimulation per year. At that stage of the experiment, the trees are not highly stressed yet by the exploitation. We cannot conclude yet whether DHAR can be a marker of the tolerance to oxidative stress. The experiment must be conducted further so that the level of DHAR can be evaluated in the situation of high oxidative stress.

The levels of thiols and ascorbic acid had similar evolutions depending on the number of stimulation per year. However, the results obtained on the second set of samples differed significantly from the first one.
On the first set of samples, RRIM 600 showed a very slight increase in both Thiols and ascorbic acid for the stimulated trees. This was confirmed on the second set of samples.
GT1 and PB235 presented a parabolic evolution on the first set of samples, with a significant decrease for most of the stimulated samples. However, on the second set of samples, variations were not as marked and rather showed a slight increase in case of GT1.
The reason for the differences between the 2 measurements is not known. Results are difficult to interpret as the time lag between stimulation and latex collection is not the same for all samples. It may also be a technical artifact. It is therefore important to repeat such measurements.

An other surprising observation is that thiols values were very high for PB235 whereas they are usually weaker than in other clones.

Latex diagnosis parameters (appendix 2)

For these parameters, the results measured on the first set of samples were confirmed on the second set (which tends to indicate that the discrepancy observed previously for thiols and ascorbic acid may be an artifact).

RSH : For all clones, this parameter showed very little variation according to the stimulation treatments (slight increase for GT1 and RRIM 600, slight decrease for PB235). The RSH values in PB235 were very high, as previously thiols parameters.

Pi : phosphorus content increased with stimulation. The highest values were obtained for E treatment (8 stimulations per year). However, this sample shows the shortest time lag after stimulation which may explains why the Pi value, which reflects the intensity of the metabolism, was higher in E than in F. PB235 clone had the highest values and GT1 the lowest, which is in agreement with previous results

Sucrose : For GT1 clone, sucrose content was high in non stimulated trees and decreased strongly when stimulation frequencies increased. RRIM600 showed low sucrose values, without variations related to stimulation frequency. PB235 had very low sucrose values, as usually described, which did not vary with the stimulation frequency.

TSC : This parameters did not vary significantly with the treatment nor with the clones.

Inter-parameters correlation (appendix3)

The parameters of stress (thiols, ascorbic acid, dehydroascorbate reductase) and latex diagnosis were correlated 2 per 2, without clonal distinction. All clones and treatments merged. The results are shown (appendix 3) only when the correlation coefficients (R^2) were below 1%, indicating a significant correlation.

The inverse Pi/sucrose correlation has been already observed in monoclonal analysis. It is very evident here, although it is a pluriclonal analysis. It may indicate that the more active the metabolism, the more sucrose is consumed.

The acid ascorbic/thiols correlation was very strong whereas the acid ascorbic/RSH (from latex diagnosis) correlation was not clear (not shown in appendix 3). As a matter of fact, thiols and RSH were not clearly correlated. This surprising phenomenon can be explained perhaps by differences in methodology because LD parameters were measured on complete latex and the "stress parameters" on C serum only.

DHAR showed a good positive correlation with Pi and RSH, and a negative correlation with sucrose. As the shape of the curve is not linear for DHAR/Pi or DHAR/sucrose, it will be interesting to perform this analysis clone by clone, to see the contribution can be attributed to different clones.

SDS-PAGE experiments

SDS PAGE analysis was carried out on each sample of the 3 clones. The first electrophoresis pattern did not show differences, neither when comparing the treatments nor the clones. But the main goal of this experimentation was to train CRRC researchers on this technique which is a step towards western blot analysis and bidimentional protein profile comparison, using their own equipment (mighty small 2 Hoefer apparatus SE250/SE260).

VI - Perspectives /Recommendations

The interesting data on the dehydroascorbate reductase clonal differences allow to consider a publication. However, as the trees from the Yield Potential Trial have not yet reached a real stress state, whatever the clone, it is not yet possible to know whether or not this enzyme is a limiting factor for the protection of rubber tree against oxidative stress. It is important to continue the experiment during 1 or 2 cycles of production, with measurements of the same parameters (DHAR activity, ascorbic acid, thiols, and LD parameters) once every 2 month, in order to answer this question.

It may also be interesting to carry out the same analysis on gas stimulation trials and low intensity tapping systems (CHOE10), once per month.

More DHAR enzyme will be purified at our laboratory in Montpellier and antibodies directed against the enzyme will be made. They will be useful to perform large scale screening of the DHAR level in the various field trials, by ELISA or Western blotting. As SDS-PAGE is required to perform this last technique we recommend that CRRC research continue practicing SDS-PAGE analysis to master the technique routinely.

The sequencing of the purified protein and/or the use of the antibodies may give the necessary tools to clone a DHAR probe, in order to perform gene expression analysis.

Acknowledgement

I wish to thank the RRIT for having allowed me to carry out this mission in such good conditions, as well as the CRRC team for its cordial greeting and its availability. May Pisamai Chantuma and all her team, as well as Somjintana Promson, Kanlaya Prapan and Prasan Seubsuk receive my congratulation for their dynamism and motivation during these two weeks.

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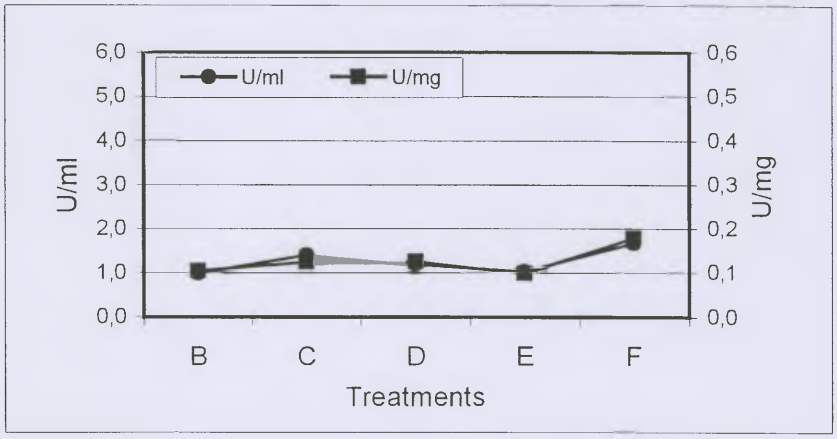
APPENDIX 1

Parameters linked to oxidative stress

Dehydroascorbate reductase activity measurements

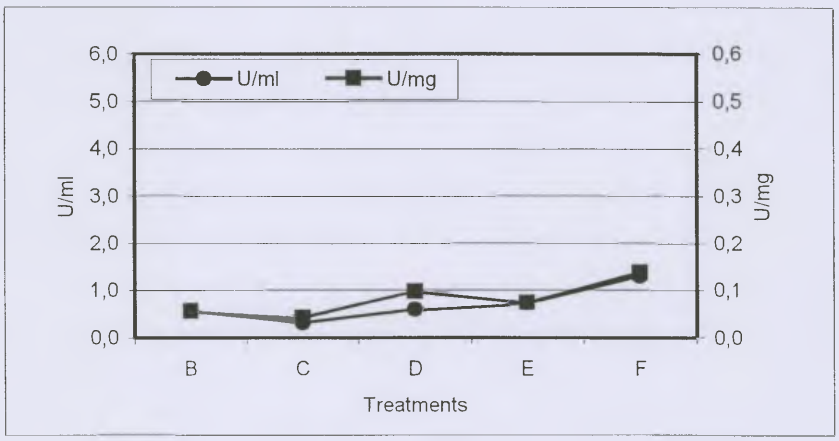
RRIM 600 05/12/2000

Treatments	U/ml	U/mg
B	0,960	0,102
C	1,389	0,122
D	1,187	0,125
E	1,010	0,096
F	1,659	0,177



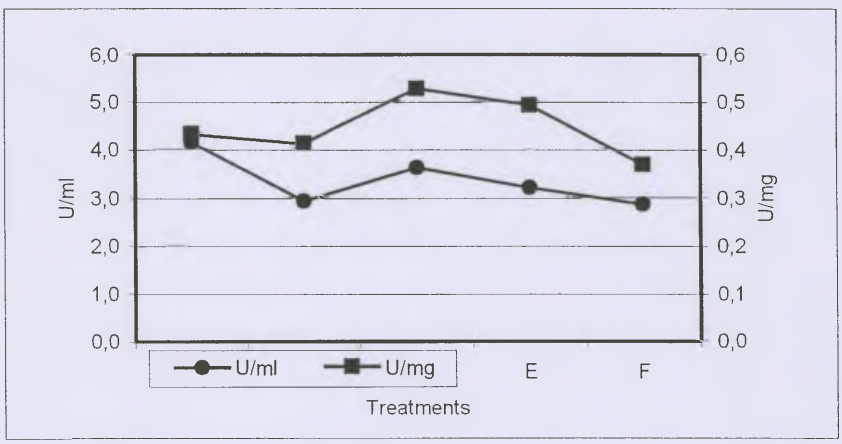
GT1 06/12/2000

Treatments	U/ml	U/mg
B	0,552	0,053
C	0,297	0,040
D	0,581	0,097
E	0,716	0,073
F	1,289	0,136



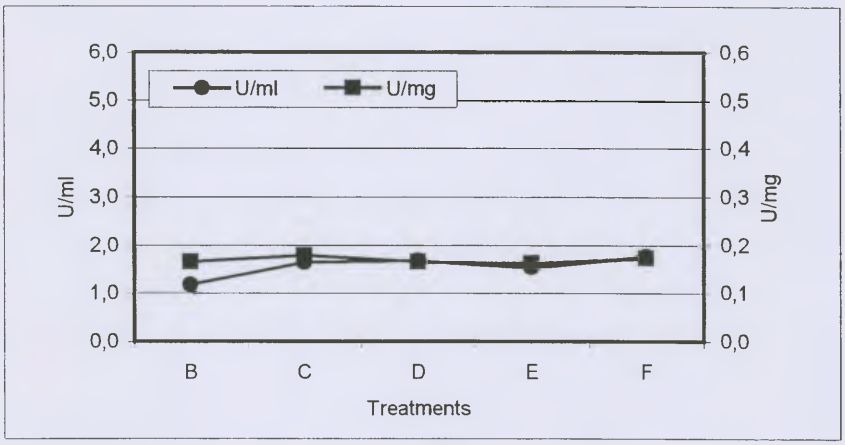
PB 235 07/12/2000

Treatments	U/ml	U/mg
B	4,140	0,432
C	2,909	0,413
D	3,604	0,527
E	3,190	0,492
F	2,847	0,368



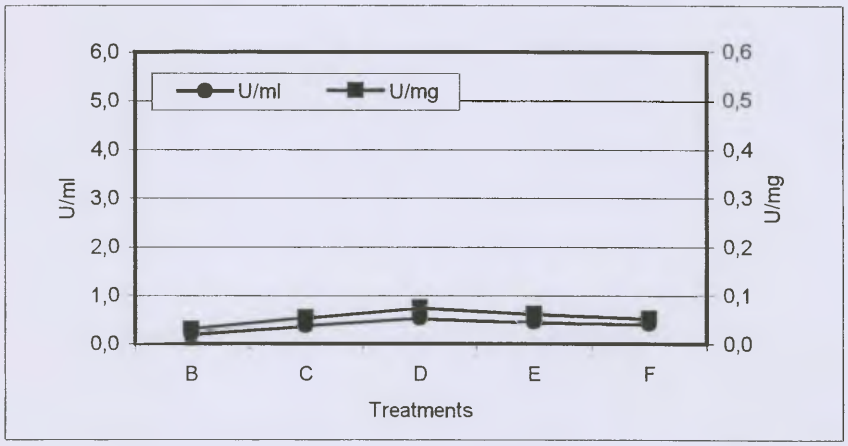
RRIM 600 08/12/2000

Treatments	U/ml	U/mg
B	1,164	0,165
C	1,626	0,177
D	1,671	0,164
E	1,513	0,161
F	1,756	0,172



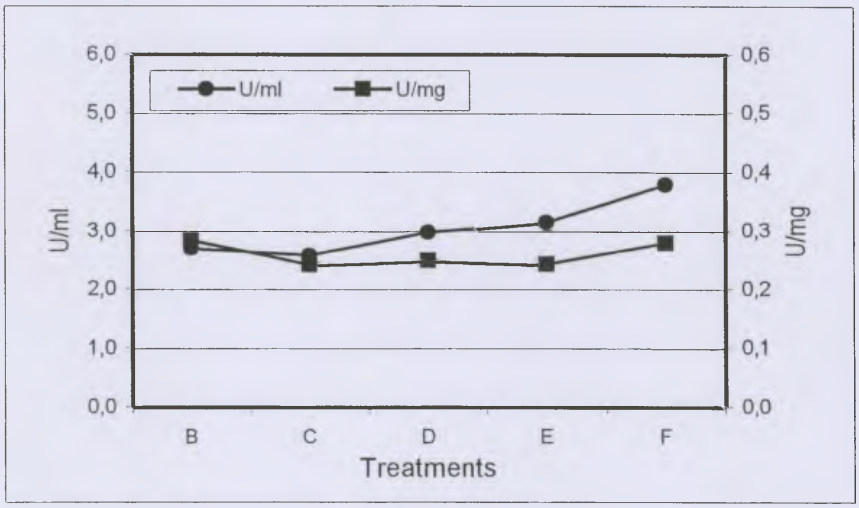
GT 1 13/12/2000

Treatments	U/ml	U/mg
B	0,185	0,029
C	0,356	0,053
D	0,510	0,073
E	0,435	0,060
F	0,395	0,051



PB 235 14/12/2000

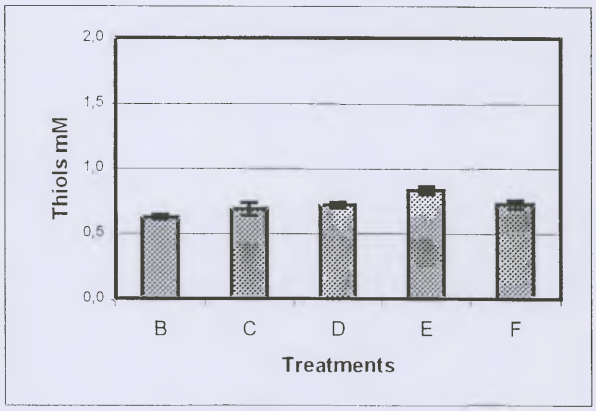
Treatments	U/ml	U/mg
B	2,679	0,282
C	2,565	0,240
D	2,965	0,248
E	3,136	0,241
F	3,774	0,277



Thiols determination

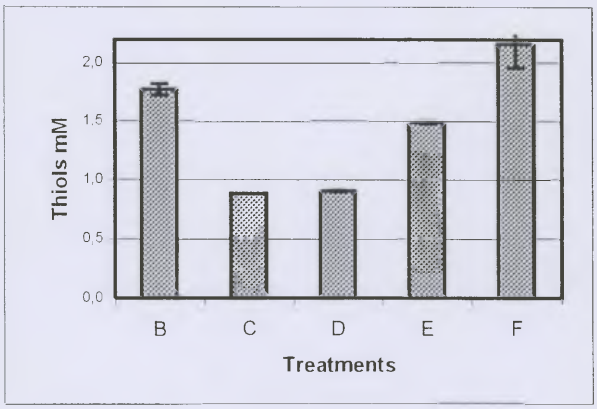
RRIM 600 05/12/2000

Treatments	Thiols mM	STD
B	0,622	0,019
C	0,682	0,050
D	0,716	0,022
E	0,829	0,028
F	0,720	0,030



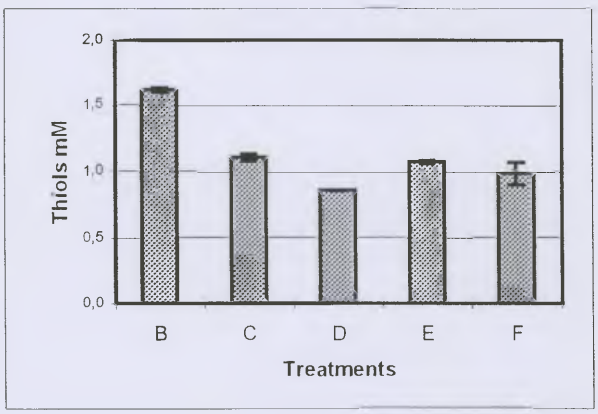
GT1 06/12/2000

Treatments	Thiols mM	STD
B	1,767	0,048
C	0,883	0,002
D	0,901	0,004
E	1,486	0,010
F	2,157	0,204



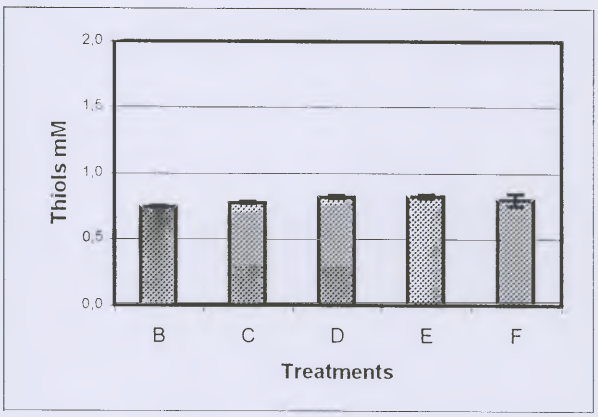
PB 235 07/12/2000

Treatments	Thiols mM	STD
B	1,611	0,019
C	1,098	0,028
D	0,850	0,004
E	1,073	0,011
F	0,978	0,085



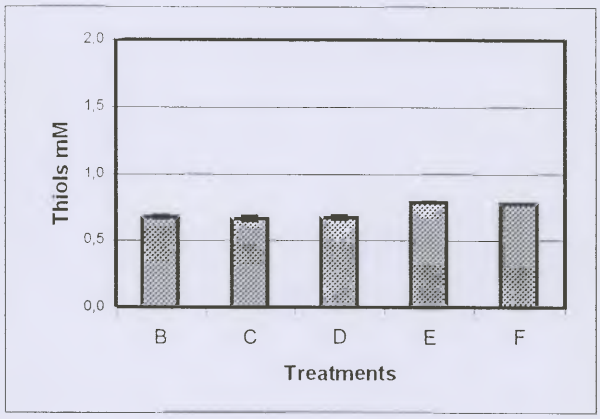
RRIM 600 08/12/2000

Treatments	Thiols mM	STD
B	0,743	0,007
C	0,778	0,008
D	0,820	0,011
E	0,825	0,014
F	0,798	0,046



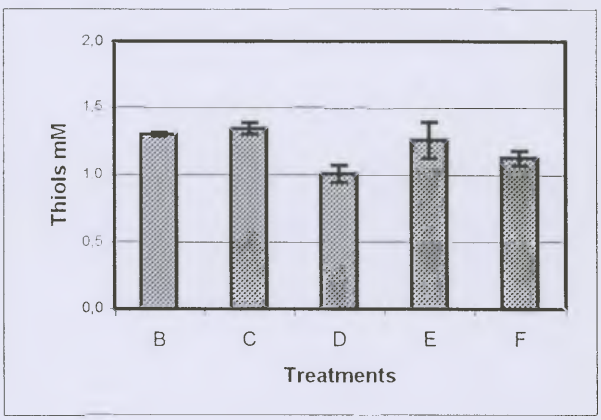
GT 1 13/08/2000

Treatments	Thiols mM	STD
B	0,673	0,009
C	0,665	0,017
D	0,673	0,012
E	0,789	0,003
F	0,773	0,002



PB 235 14/12/2000

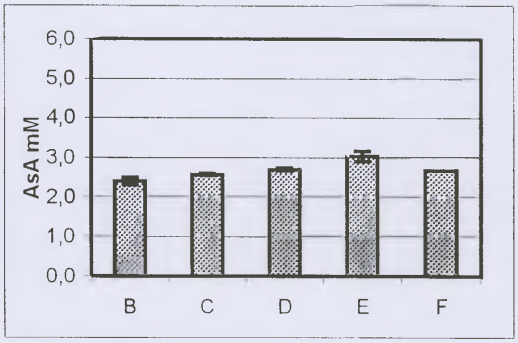
Treatments	Thiols mM	STD
B	1,300	0,015
C	1,339	0,043
D	1,001	0,064
E	1,252	0,135
F	1,120	0,054



Ascorbic acid (AsA) determination

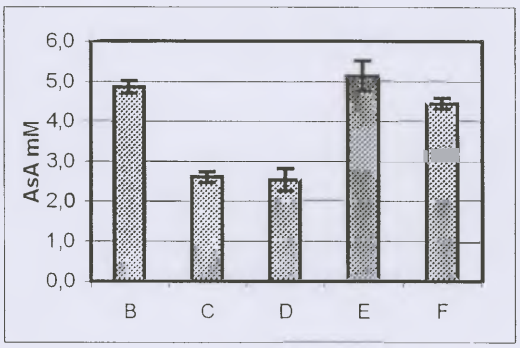
RRIM 600 05/12/2000

Treatments	AsA mM	STD
B	2,396	0,092
C	2,563	0,026
D	2,693	0,041
E	3,027	0,129
F	2,672	0,004



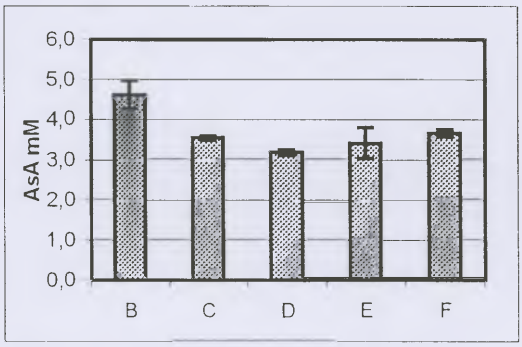
GT1 06/12/2000

Treatments	AsA mM	STD
B	4,849	0,162
C	2,594	0,136
D	2,529	0,273
E	5,112	0,380
F	4,442	0,133



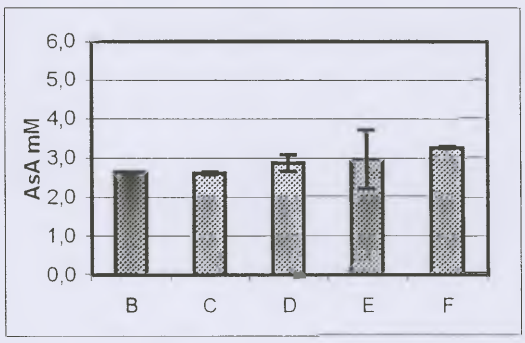
PB 235 07/12/2000

Treatments	AsA mM	STD
B	4,598	0,350
C	3,542	0,056
D	3,176	0,067
E	3,411	0,384
F	3,658	0,101



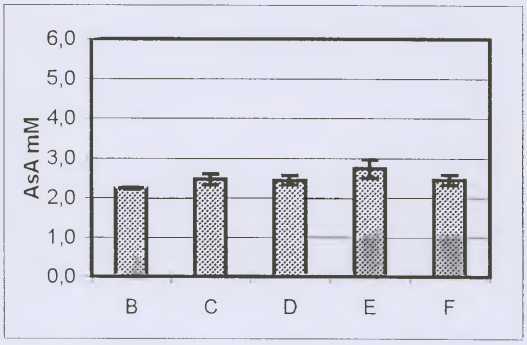
RRIM 600 08/12/2000

Treatments	AsA mM	STD
B	2,597	0,010
C	2,597	0,038
D	2,855	0,212
E	2,925	0,755
F	3,258	0,024



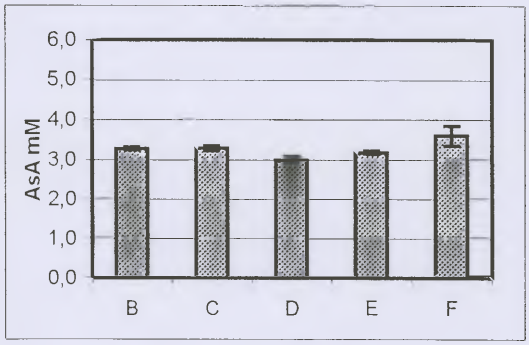
GT 1 13/12/00

Treatments	AsA mM	STD
B	2,234	0,025
C	2,455	0,138
D	2,432	0,121
E	2,733	0,234
F	2,450	0,131



PB 235 14/12/2000

Treatments	AsA mM	STD
B	3,267	0,040
C	3,286	0,067
D	2,981	0,067
E	3,176	0,047
F	3,595	0,249



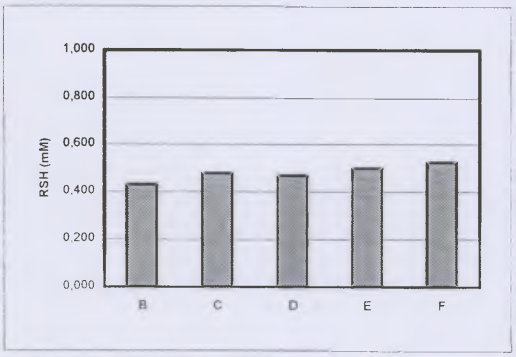
APPENDIX 2

Latex diagnosis parameters

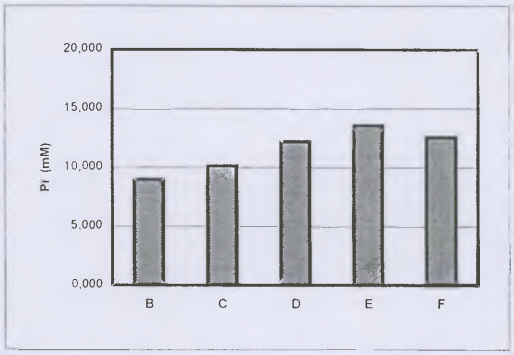
Latex diagnosis

RRIM 600 05/12/2000

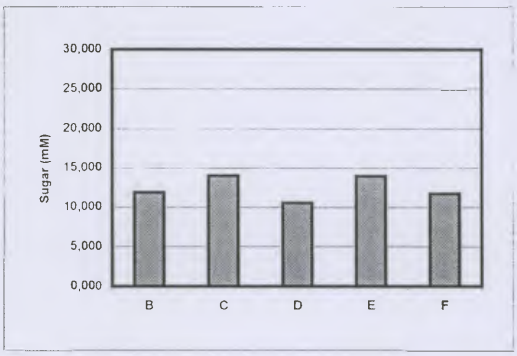
Treatments	RSH mM
B	0,429
C	0,476
D	0,467
E	0,497
F	0,523



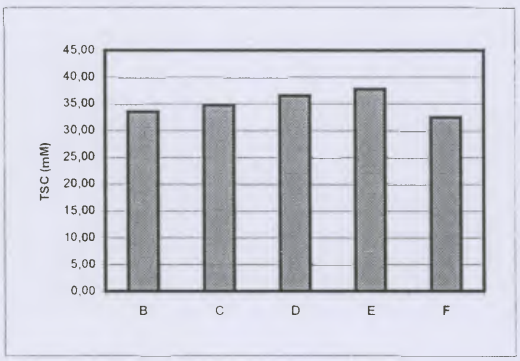
Treatments	Pi mM
B	8,948
C	10,123
D	12,151
E	13,527
F	12,537



Treatments	sugar mM
B	11,915
C	14,018
D	10,594
E	13,956
F	11,733

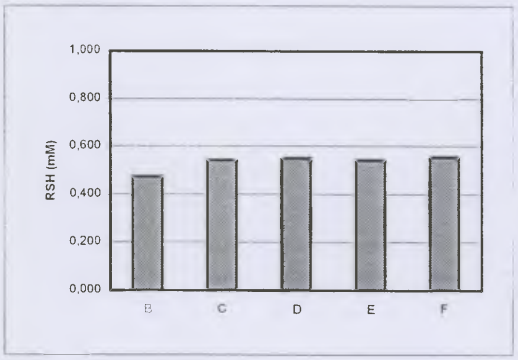


Treatments	TSC %
B	33,51
C	34,72
D	36,50
E	37,69
F	32,50

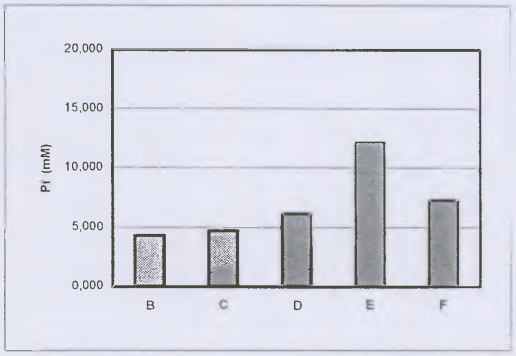


GT 1 06/12/2000

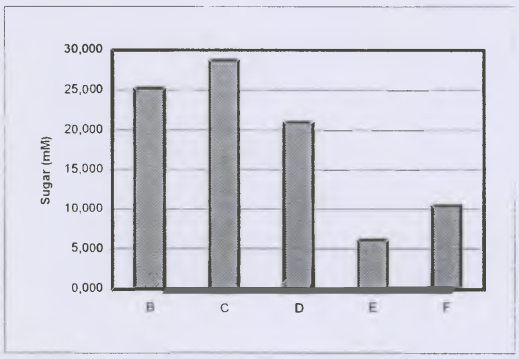
Treatments	RSH mM
B	0,472
C	0,540
D	0,549
E	0,541
F	0,554



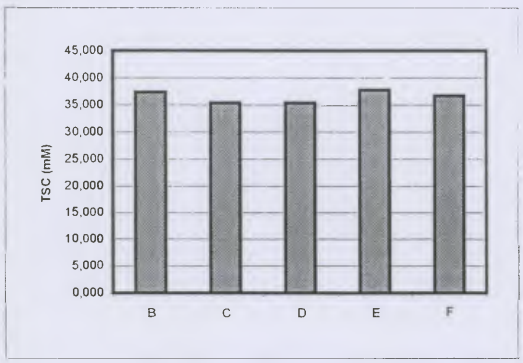
Treatments	Pi mM
B	4,328
C	4,719
D	6,103
E	12,195
F	7,252



Treatments	Sugar mM
B	25,209
C	28,642
D	20,960
E	6,065
F	10,400

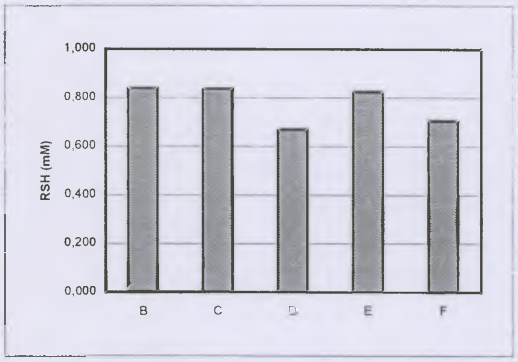


Treatments	TSC %
B	37,374
C	35,354
D	35,354
E	37,755
F	36,735

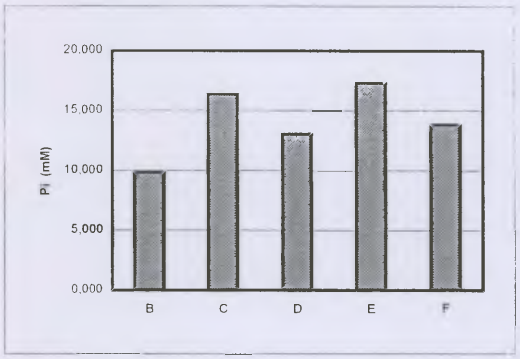


PB 235 07/12/2000

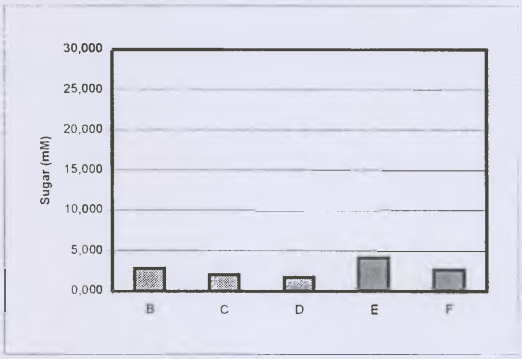
Treatments	RSH mM
B	0,840
C	0,838
D	0,669
E	0,825
F	0,704



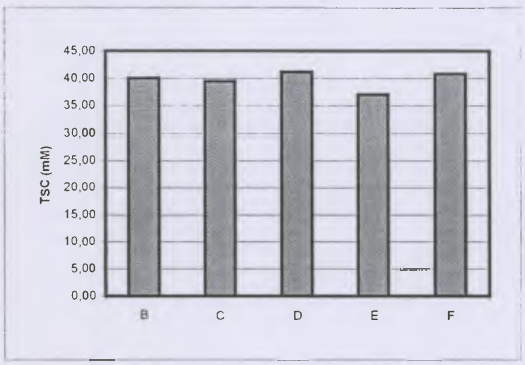
Treatments	Pi mM
B	9,830
C	16,370
D	13,034
E	17,301
F	13,797



Treatments	Sugar mM
B	2,837
C	2,052
D	1,756
E	4,047
F	2,515

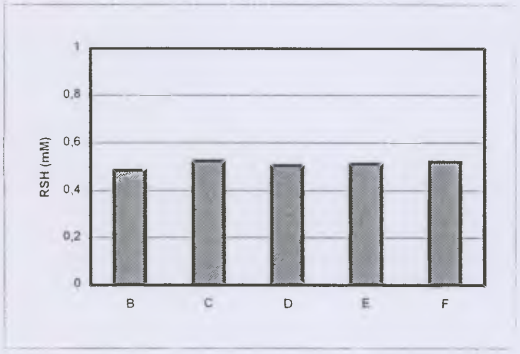


Treatments	TSC %
B	40,00
C	39,39
D	41,18
E	37,00
F	40,82

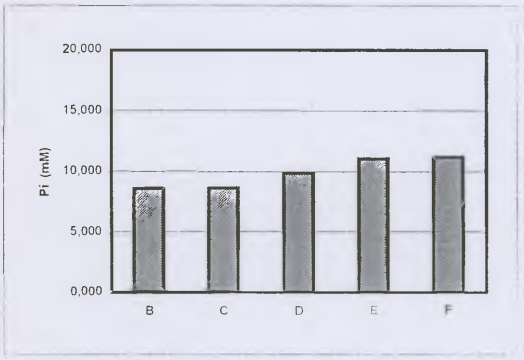


RRIM 600 08/12/2000

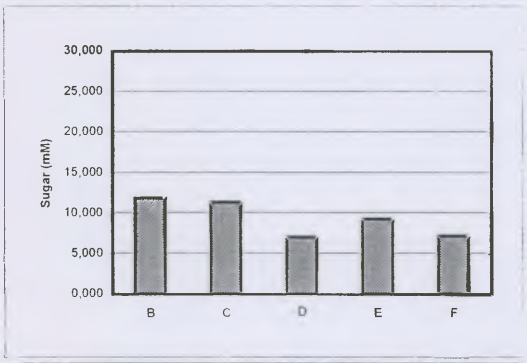
Treatments	RSH mM
B	0,485
C	0,523
D	0,504
E	0,511
F	0,522



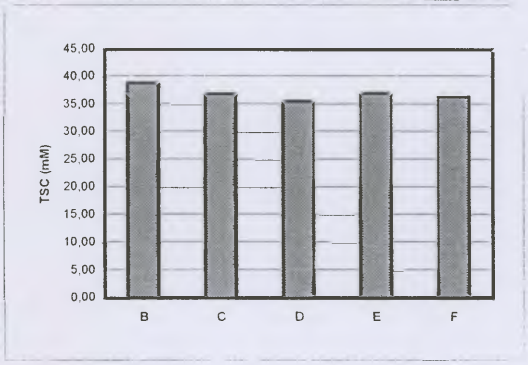
Treatments	Pi mM
B	8,605
C	8,630
D	9,856
E	11,061
F	11,127



Treatments	Sugar mM
B	11,877
C	11,205
D	6,908
E	9,139
F	7,069

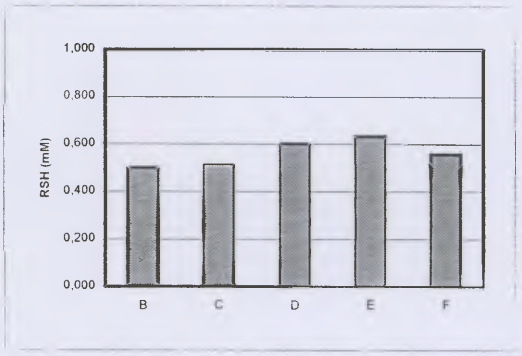


Treatments	TSC
B	38,71
C	36,73
D	35,42
E	36,84
F	36,46

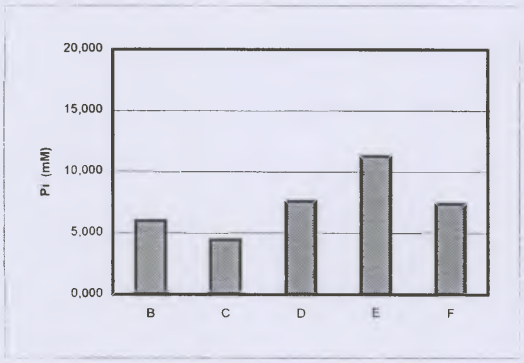


GT 1 13/12/2000

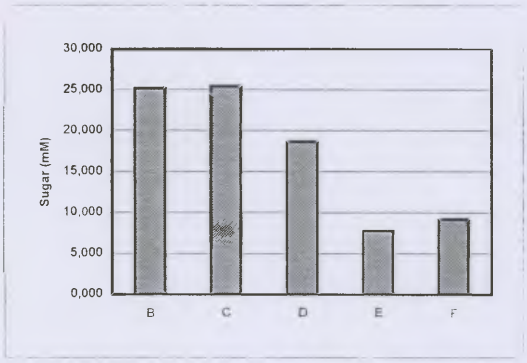
Treatments	RSH mM
B	0,499
C	0,513
D	0,600
E	0,631
F	0,556



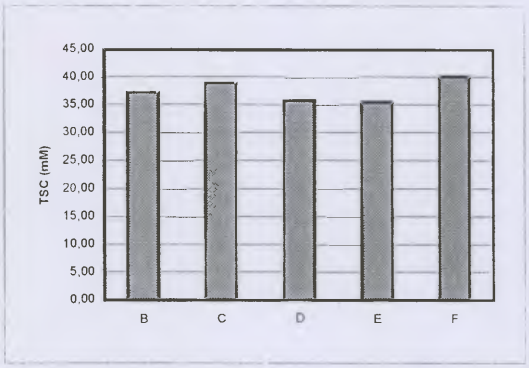
Treatments	Pi %
B	5,960
C	4,409
D	7,557
E	11,244
F	7,359



Treatments	Sugar %
B	25,168
C	25,388
D	18,602
E	7,775
F	9,177

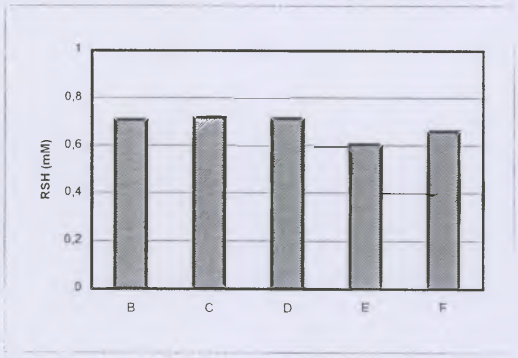


Treatments	TSC %
B	37,23
C	38,95
D	35,79
E	35,42
F	40,00

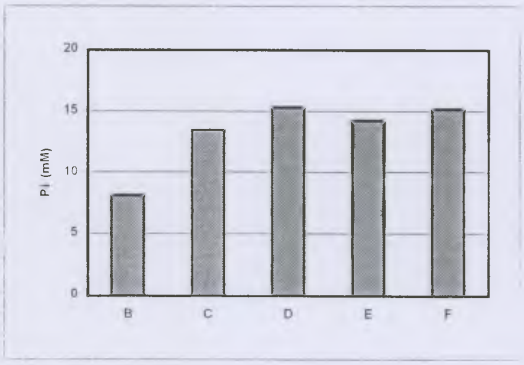


PB 235 14/12/2000

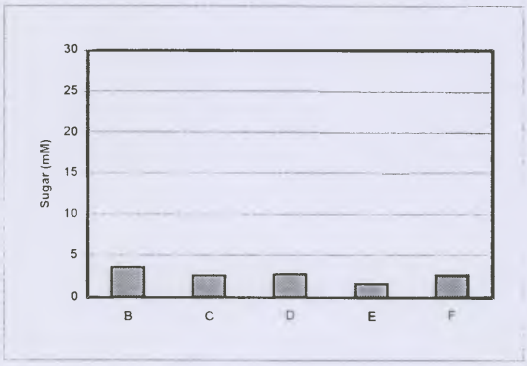
Treatments	RSH %
B	0,708
C	0,719
D	0,713
E	0,602
F	0,658



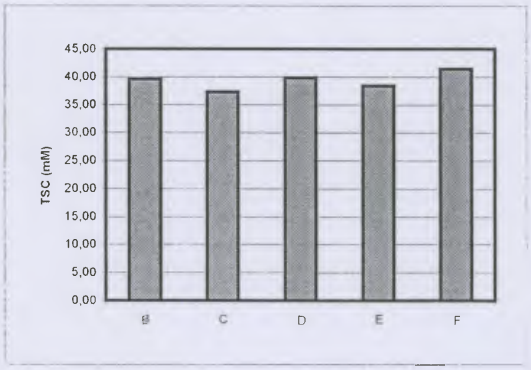
Treatments	Pi %
B	8,066
C	13,482
D	15,277
E	14,219
F	15,16



Treatments	Sugar %
B	3,632
C	2,621
D	2,76
E	1,662
F	2,647

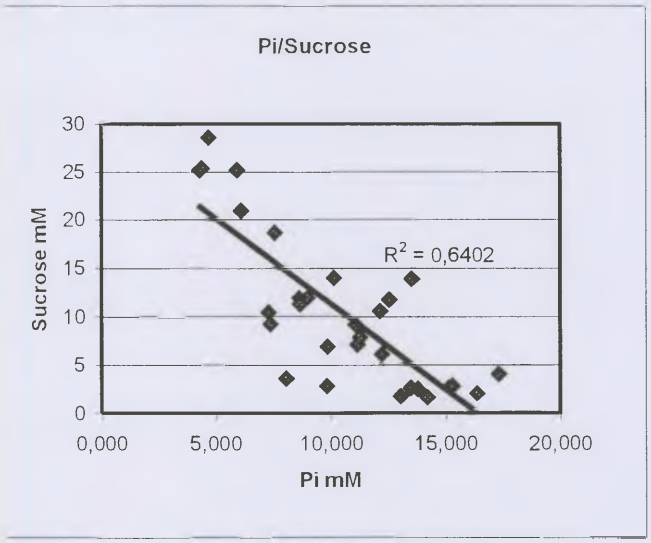


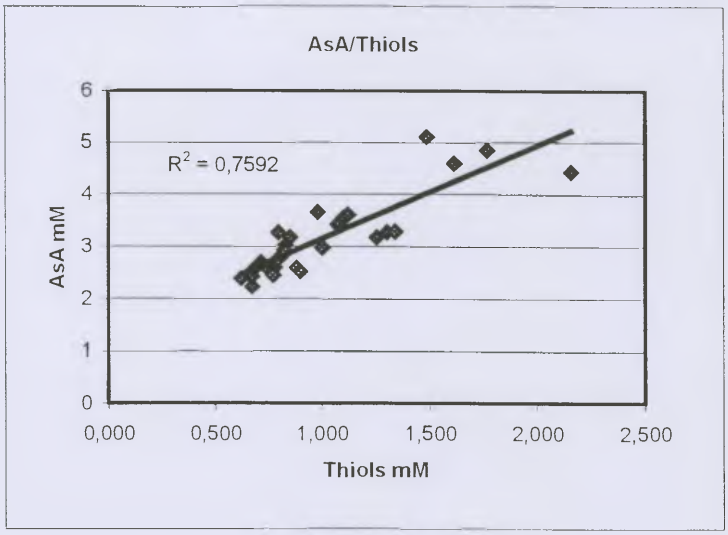
Treatments	TSC %
B	39,60
C	37,25
D	39,80
E	38,38
F	41,41

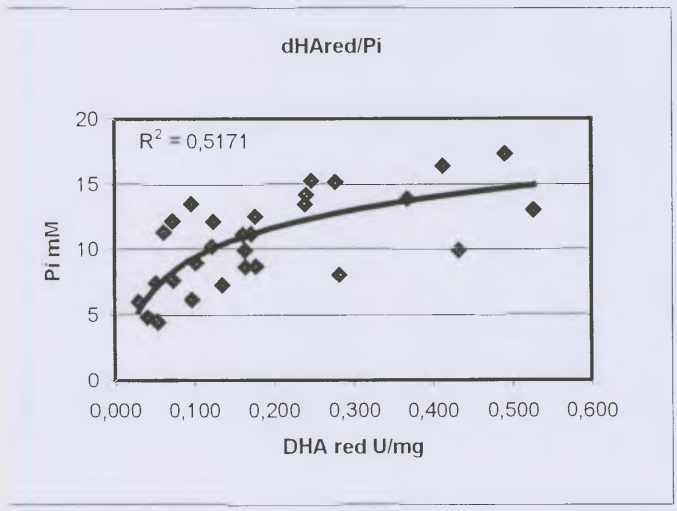


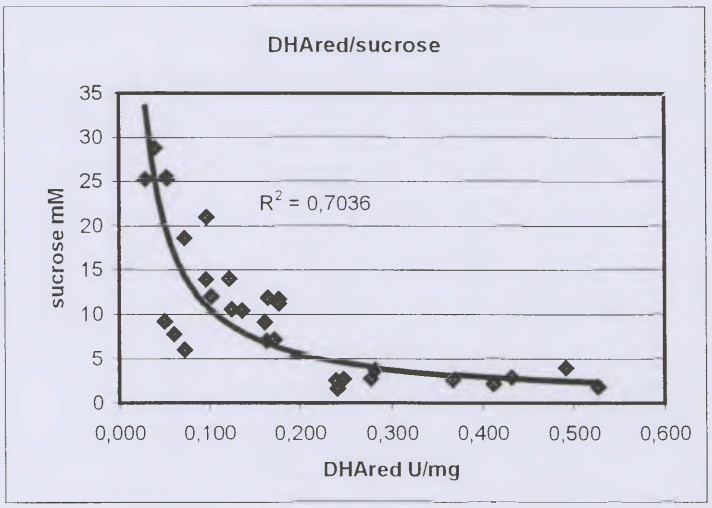
APPENDIX 3

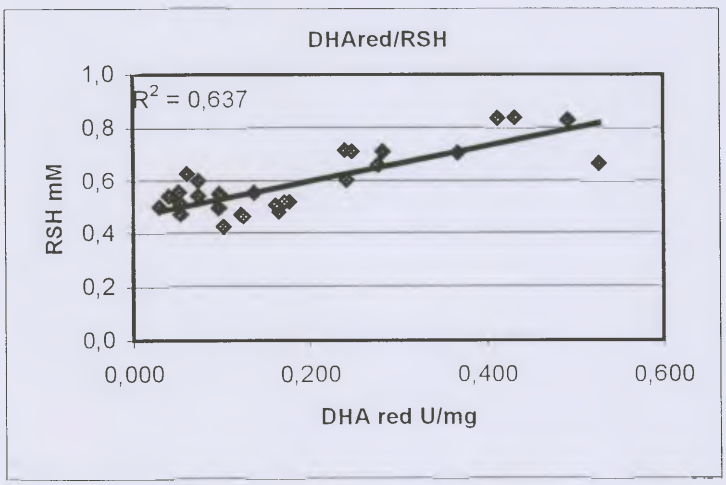
Interparameter correlations


Pi/Sucrose
Sucrose mM
Pi mM
R^2 = 0,6402
0
5
10
15
20
25
30
0,000
5,000
10,000
15,000
20,000


AsA/Thiols
AsA mM
Thiols mM
R^2 = 0,7592
0
1
2
3
4
5
6
0,000
0,500
1,000
1,500
2,000
2,500


dHAred/Pi
Pi mM
DHA red U/mg
R^2 = 0,5171
0
5
10
15
20
0,000
0,100
0,200
0,300
0,400
0,500
0,600


DHAred/sucrose
sucrose mM
DHAred U/mg
R^2 = 0,7036
0
5
10
15
20
25
30
35
0,000
0,100
0,200
0,300
0,400
0,500
0,600


DHAred/RSH
RSH mM
DHA red U/mg
R^2 = 0,637
0,0
0,2
0,4
0,6
0,8
1,0
0,000
0,200
0,400
0,600

APPENDIX 4

Pisamai Chantuma training report (June 2000)

Preliminary study of dehydroascorbate reductase: purification and characterization

Biochemistry training of Pisamai Chantuma (RRIT)

Persons in charge of the training course: A. Clement (CIRAD-amis) and J. C. Prevot (CIRAD-CP)

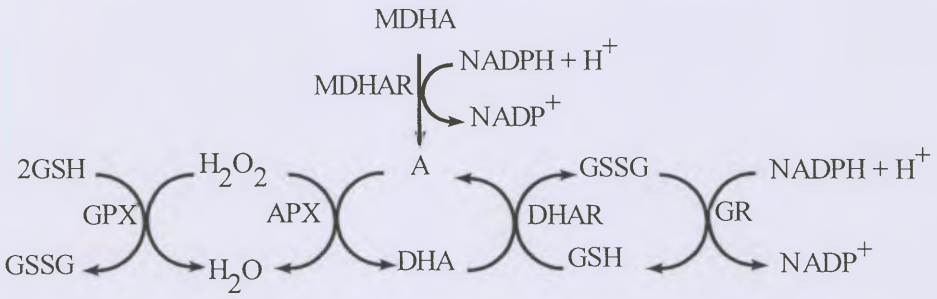
CIRAD Montpellier 29 mai 2000-26 juin 2000

OBJECTIVE

Ascorbate acts as an important antioxidant in both enzymatic and non enzymatic reactions in plant cells. Oxidation of ascorbate produces dehydroascorbate (DHA) which must be regenerated to ascorbate to keep the scavenging cycle (figure 1) operative.

Dehydroascorbate reductase (EC 1.8.5.1.) is abble to realize this reaction. This enzyme catalyses the reduction of dehydroascorbate (DHA) using reduced glutathione (GSH) as electron donor.

Of the antioxidants required for the scavenging cycle mainly ascorbate (2-5 mM) and GSH (0.5-1.5 mM) can be measured in latex. Oxidized glutathione (GSSG) and DHA are in traces. It appears that the latex dehydroascorbate reductase can play an important role in reactive oxygen detoxification and its study is very relevant.



MDHA: monodehydroascorbate

MDHAR: monodehydroascorbate reductase

GPX: glutathione peroxidase

APX: ascorbate peroxidase

GR: glutathione reductase

Figure 1: Scavenging cycle

MATERIALS AND METHODS

Plant material

For all experiments the latex used was pooled from trees of the same genotype harvested in Ivory Coast at IDEFOR (Bimbresso). The latex was collected on ice and was centrifuged at 8000 x g for 15 min. at 4°C. The pellet composed of lutoïds and Frey-Wyssling particles was separated and the supernatant was centrifuged 45000 x g, 90 min, to remove rubber particles from the cytosol. The cytosol was freeze dried and stored at 30°C.

Enzyme assays

Dehydroascorbate reductase activity was determined by spectrophotometry by following ascorbate formation at 265 nm. The reaction mixture contained: phosphate buffer 50 mM pH 7.4, GSH 1 mM and DHA 0.5 mM. A blank was realised with only ascorbate and GSH, then it was substracted from the measure of dehydroascorbate reductase activity.

Proteins assay

Protein contents were determined by the Bradford method using albumin bovin as standard

Table Preparation of standard protein curve

Tube no.	1	2	3	4	5	6	7	8	9
protein (µg)	0	2	4	6	8	10	12	15	20
Coomassie Brilliant Blue G-250 (ml)	1	1	1	1	1	1	1	1	1

The solution was mixed and the absorbance at 595 nm was measured after 10 min and before 1 hour in 1 ml cuvettes against a reagent blank.

Beer – Lambert law

$$ABS = \varepsilon lc$$

$\varepsilon = 1/k$: extinction coefficient (M^{-1} cm^{-1})

l = lenght and c = protein concentation

pH influence

For the pH curve two buffers were used: 50 mM phosphate buffer (pH between 5.8-8) and tris/HCl buffer 50 mM (pH between 7.5-9).

Enzyme purification

All purification steps were conducted at 4°C. Crude extract: 1g of freeze dried cytosol was resuspended in buffer (25 mM MOPS/KOH pH 7.4 : buffer A). The suspension was stirred for 20 min and then centrifuged at 11000 x g for 15 min (Prolabo SR2000).

Ammonium sulfate precipitation: proteins precipitated between 50-75% ammonium sulfate were collected by centrifugation 11000 x g for 15 min and resuspended in buffer A.

Sephacryl chromatography: the protein extract was applied on a 100 x 1.6 cm column (Sephacryl S200 Pharmacia) equilibrated and eluted with buffer A at 1 ml/min. The fractions (5 ml) containing dehydroascorbate reductase activity were pooled.

Ion exchange chromatography: the extract was applied to 1.5 x 10 cm column of dimethyl aminoethyl gel (Fractogel EMD DMAE, Merck) pre-equilibrated with buffer A. After washing the column with buffer A, dehydroascorbate reductase elution was carried out by KCl linear gradient (0-0.3 M for 180 ml). The fractions (5 ml) were collected with a flow rate of 0.8 ml/mn. Those containing activity were pooled.

RESULTS

Purification of dehydroascorbate reductase

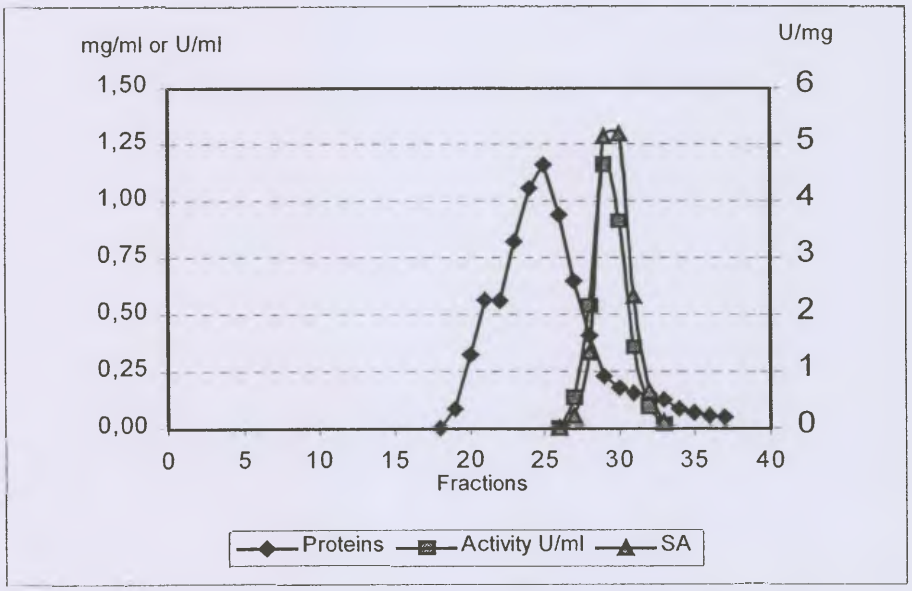


Figure 2: Elution profile from Sephacryl chromatography

(SA: specific activity)

Sephacryl chromatography: one activity peak was found which had the highest specific activity (figure 2). The fractions containing dehydroascorbate reductase activity were pooled.

Ion exchange chromatography

The column was first washed with the equilibration buffer and then the enzyme was eluted with the KCl gradient.The elution profile is shown in figure 3 There was 3 activity peaks. At first the more important activity peak was analysed. It constituted our enzyme-enriched extract and at the end it was purified 136 fold with 15 % of initial activity. Purification of the enzyme is summarized in Table 1.

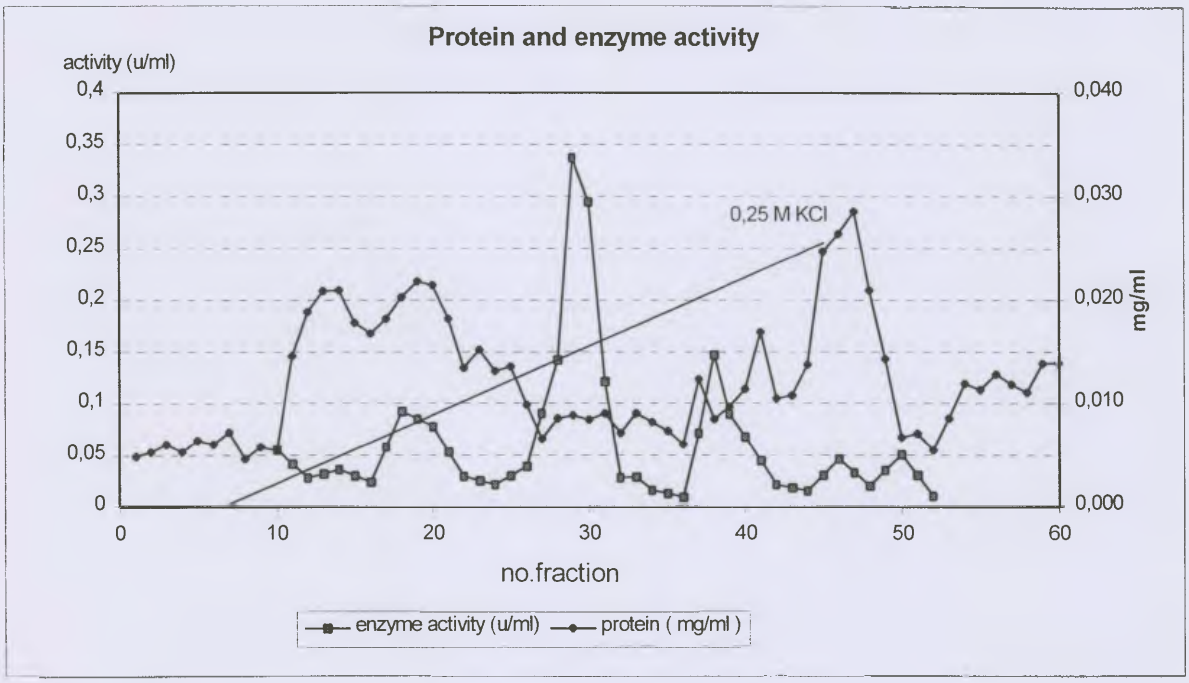


Figure3: Elution profile from DMAE

Table 1: The different purification steps of DHA reductase

Step	Protein (mg)	U	S.A.	Purification factor	% recovery
1. crude extract	128.2	26.8	0.21	1	100
2. $(NH_4)_2SO_4$	8.2	14.0	1.7	8	52
3. Sephacryl	1.4	3.4	2.5	12	13
4. DMAE	0.2	4.1	28.4	136	15

Enzyme properties of DHA reductase

Influence of pH

The optimum pH of DHA reductase was about 7.8-8.0 . At alkaline pH, the non-enzymatic reduction of DHA by GSH increased with increasing pH and measuring the enzyme activity above pH 8.3 was difficult.

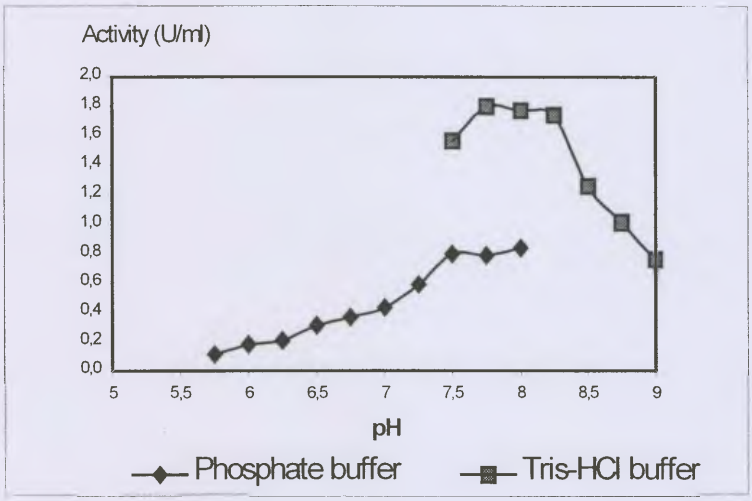


Figure 4: Effect of ph on the activity of DHA reductase

The activity was more efficiency in Tris buffer but the chemical reaction was higher in this buffer.

Dehydroascorbate reductase affinity for substrates

Affinity of enzyme for DHA was measured by keeping GSH at various fixed concentrations and varying the DHA concentration. The apparent Km values (Michaelis Menten constant) was determined. Km values for DHA was 0.22 and 0.83 mM for 0.5 and 2.5 mM GSH (figure 6). For high GSH concentration (5 mM) the blank increased in the same way as DHA, then it was impossible to evaluate Vmax. Km values for GSH were 1.49 and 1.38 mM respectivelyfor 0.25 and 0.5 mM DHA.

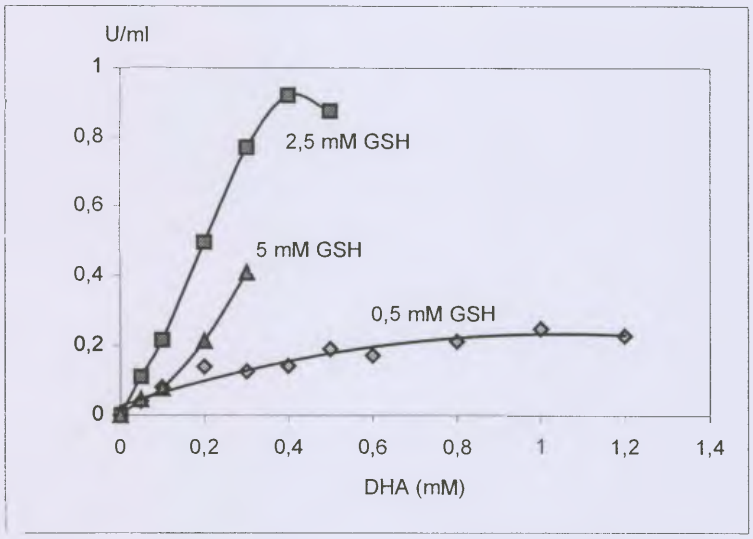


Figure 5: Enzyme activity as related to variable DHA concentrations with 3 different GSH concentrations.

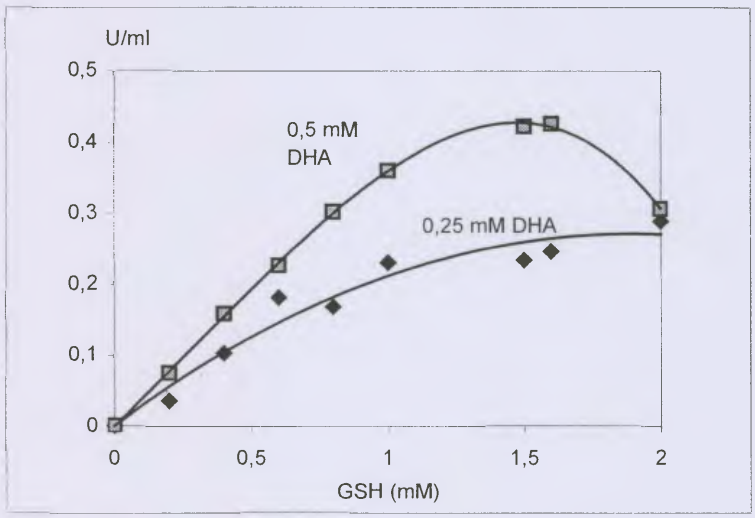


Fig 6: Enzyme activity as related to variable GSH concentrations with 2 different DHA concentrations.

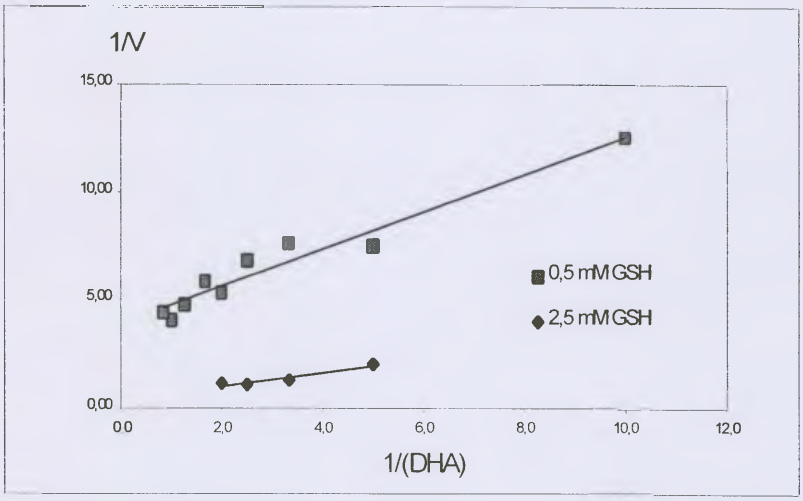


Fig 7: Km of DHA as related to variable GSH concentrations, Lineweaver – Burk representation[1/V = f (1/S)]

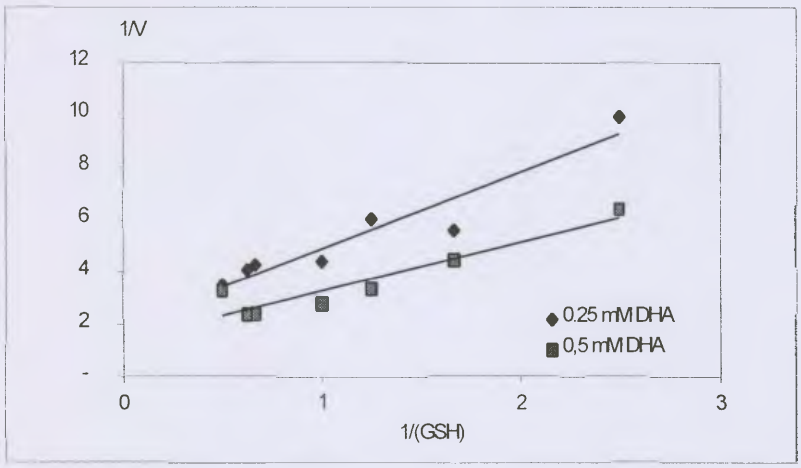


Fig 8: Km of GSH as related to variable DHA concentrations, Lineweaver-Burk representation [1/V = f (1/S)]

$$V = Vmax.[S]/(Km + [S])$$

$$1/V = \{(Km+[S])/[S]\,Vmax\}$$

$$1/V = \{Km/(Vmax \,.\, [S])\} + 1/Vmax$$

When $1/V = 0$ then $1/S = -1/Km$

Influence of different effectors

The effect of inhibitors on the enzyme activity was tested by incubating the enzyme with the inhibitors. The enzyme activity was determined after adding the standard assay mixture. The enzyme was inhibited by glutamate and a little by Mg^{2+}, citrate and malate and should be activated by potassium (Table 2). This compounds are present in latex and certainly control the activity of enzyme in physiological conditions. It will be necessary to confirm and study this phenomenom.

Table 2: Effect of inhibitors on dehydroascorbate reductase activity

Compound present during preincubation	Relative DHA reductase activity
None	(100)
MgCl 10 mM	80
KCl 10 mM	137
Glucose 1 mM	107
Fructose 1 mM	106
Sucrose 10 mM	ND*
Citrate 5 mM	81
Malate 10 mM	81
Alanine 10 mM	88
Aspartate 10 mM	104
Glutamate 10 mM	69
Glutamine 10 mM	98

* The effects of sucrose were difficult to measure and will be neccesary to realize new study.

CONCLUSION

The DHA reductase from latex rubber, was purified 136 – fold. The optimum pH of DHA reductase was measured between 7.8 – 8 while physiological pH was 6.8 – 7.3 . Therefore, dehydroascorbate reductase in physiological conditions is fonctionning, but under its optimum potential. The Km for GSH was high (1.45 mM) comparatively with the physiological concentration (0.5 – 1 mM). GSH concentration seems to control functioning of enzyme. The Km for DHA decreases when GSH concentration decreases too. At

physiological GSH concentration, it is near 200 μM. It will be interesting to complete this study and to evaluate the purity of the enzyme by electrophoresis.