BIOCHEMICAL FACTORS INFLUENCING LATEX FLOW DURING STRESS, TAPPING FREQUENCY AND STIMULATION IN *HEVEA BRASILIENSIS*

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By

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DECLARATION

I hereby declare that the thesis entitled **Biochemical factors influencing latex flow during stress, tapping frequency and stimulation in *Hevea brasiliensis*** is a bonafide record of the research work carried out by me at the School of Biosciences, M.G. University, Kottayam and Rubber Research Institute of India, Kottayam, under the joint supervision of Dr. G. Muraleedhara Kurup, Professor, School of Biosciences, M.G.University, Kottayam and Dr. K.R.Vijayakumar, Joint Director (Research), Rubber Research Institute of India, Kottayam. I further declare that the thesis has not been previously formed the basis for the award of any degree.

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CONTENTS

Symbols and Abbreviations

AOS	-	Active oxygen species
ATP	-	Adenosine 5' triphosphate
ADP	-	Adenosine 5' diphosphate
AMP	-	Adenosine 5' monophosphate
AEC	-	Adenylate energy charge
BI	-	Bursting index
Ca	-	Calcium
C	-	Celsius, centigrade
cm	-	centimetre
Cu	-	Copper
$CuSO_4$	-	Copper sulphate
CD	-	Critical difference
DTNB	-	5,5' Dithio-bis-2-Nitro benzoic acid
DRC	-	Dry rubber content
et al	-	*et alibi*, and others
etc	-	*et ceteri* and the other
EDTA	-	Ethylene diamine tetra acetic acid
$Fe\ Cl_3$	-	Ferric chloride
GSH	-	Glutathione (reduced)
GSSG	-	Glutathione (oxidized)
GR	-	Glutathione reductase
g	-	gram
HEPES	-	(N -[2-hydroxyethyl piperazine- N'-[2-ethane sulfonic acid)
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
IFR	-	Initial flow rate
kD	-	kilo Dalton
LDH	-	Lactate dehydrogenase
M	-	Molar
Mg	-	Magnesium
$Mg\ SO_4$	-	Magnesium sulphate
$Mg\ Cl_2$	-	Magnesium chloride
MES	-	(2-[N-Morpholino] ethane sulfonic acid

mg	-	milli gram
ml	-	milli litre
mM	-	milli molar
NR	-	Natural Rubber
NEM	-	N-ethyl maleimide
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced)
N	-	Normal
ns	-	not significant
NBT	-	nitro blue tetrazolium
OD	-	Optical density
Pi	-	Phosphorus (inorganic)
PI	-	Plugging index
PAGE	-	poly acrylamide gel electrophoresis
PK	-	Pyruvate kinase
rpm	-	Revolutions per minute
RSH	-	Reduced thiols
RRII	-	Rubber Research Institute of India
RRIM	-	Rubber Research Institute of Malaysia
SDS	-	Sodium dodecyl sulphate
SE	-	Standard Error
SOD	-	Superoxide dismutase
TPD	-	Tapping Panel Dryness
TEMED	-	N,N,N',N' Tetramethyl ethylene diamine
TSC	-	Total solid content
Tris	-	Tris hydroxymethyl amino methane
TCA	-	Trichloroacetic acid
TP	-	Turgor pressure
UV	-	Ultra violet
viz	-	videlicet, namely
%	-	percentage
μ	-	micro

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Hevea brasiliensis (Para rubber tree) is the major crop cultivated worldwide as a source of Natural Rubber (NR). The economic product of the tree is latex, which is the fluid cytoplasm of laticiferous cells. Generally a rubber tree is productive for a period of 25-30 years. Rubber plantations provide a regular source of income during its exploitation, which begins about 6-7 years after planting. Latex is obtained from the bark by tapping the trees at regular intervals. During tapping laticifers are severed and latex flows out of the tree, which upon coagulation and subsequent processing leads to Natural Rubber (cis- 1,4 polyisoprene).

Latex contains all the subcellular organelles of non-photosynthetic cells such as vacuoles, plastids, mitochondria, nuclei, endoplasmic reticulum and polysomes (D` Auzac and Jacob, 1989; de Fay *et al.*, 1989). Nuclei and mitochondria are not coming out with latex during tapping because of their parietal position (Dickenson, 1965).

Fresh latex is a polydisperse system, which can be separated into four main fractions by ultracentrifugation at 59,000g (Cook and Sekhar, 1953). These fractions include a white upper layer of rubber particles, below which an orange or yellow layer containing Frey Wyssling particles, an aqueous

serum called C-serum and a bottom fraction containing vacuoles called lutoids.

Rubber particles are spherical or pear shaped form with diameter ranging from 0.1-0.5 μm and are essential components in rubber synthesis. They are surrounded by a surface film of~10nm thick and composed of proteins and phospholipids (Cockbain and Philpott, 1963; Ho, *et al.*, 1976). This film carries a negative charge and it is responsible for the stability of rubber particles suspended in the latex serum. A few rubber particle proteins were studied such as Rubber Elongation Factor (REF), involved in rubber biosynthesis (Dennis *et al.*, 1989) and a small rubber particle protein (Oh *et al.*, 1999). The average molecular weight of rubber from fresh latex has been found to ranges from 0.7-40x 10^5 in a bimodal distribution (Westall, 1968).

The C- serum contains most of the soluble substances including aminoacids, proteins, polyols, glucids, organic acids, nucleotides, reducing agents and miscellaneous solutes (Archer, 1963b, 1969).

Quebrachitol (methyl inositol), sucrose and glucose are the major soluble carbohydrates in latex. The total protein content is ~1% of which 20% is adsorbed on rubber particles, an equal quantity in bottom fraction and the remainder in the C-serum. Lipids of fresh latex include fats, waxes, sterols, sterol esters and phospholipids (Ho, *et al*, 1976).

Lutoids and Frey Wyssling particles are the two major non-rubber particles in latex. Lutoids are single membrane bound microvacuoles (size 2-5μm) with lysosomal characteristics (Pujarniscle, 1968). They are fragile and

have a liquid content (B-serum) of pH ~5.5 and enclosed by a membrane consists of lipids and proteins. The membranes are negatively charged on its external surface. Lutoids accumulate and compartmentalize numerous ions in particular cations such as H^+, Mg^{2+}, Ca^{2+}, Cu^{2+} etc and a large amount of cationic proteins, Hevein (Van Parijis *et al.*, 1991), hydrolases, defence proteins such as chitinase, β, 1-3 glucanase (Subroto *et al.*, 1996) and stress proteins (D` Auzac, 1995). The release of these contents into the C-serum (cytosol) leads to aggregation of macromolecules, organelles and rubber particles leading to coagulation of latex after tapping. So the colloidal stability of the latex will depend upon the efficient compartmentation of all these coagulating factors and on the integrity of the membrane of lutoids.

Frey Wyssling particles are about 4-6μm in diameter and found much less than lutoids in latex (Dickenson, 1965). They are yellow or orange in colour due to the presence of carotenoids (Frey Wyssling, 1929) and possess a double membrane mainly composed of lipid material. They contain o-diphenol oxidase (Coupe *et al.*, 1972) indicates that they probably play a role in the coagulation processes associated with stopping of flow.

The actual rubber yield at each tapping depends on a number of factors including clone, age of the tree, tapping system, ecoclimatic conditions, stimulation of latex production using chemicals etc (Eschbach, 1984;Yeang and Paranjothy, 1982). Though latex-producing ability is a kind of genetic characteristic, it is also affected by external factors. Both the ecological

environment and climate directly affect the physiological/ biochemical reactions in the tree and thereby yield.

Latex production of *Hevea* is limited by factors related to the duration of latex flow, which determines the volume of latex collected at each tapping and the regeneration of latex between two tapping which is a function of frequency of tapping (Jacob *et al.*, 1989). The duration of flow is limited by the coagulation leading to plugging of the open extremities of the severed latex vessels (d` Auzac, 1989). The regenerative metabolism involves not only rubber biosynthesis but also the reconstitution of the lost sub cellular components during tapping.

1.1 Factors influencing latex flow

The main production-limiting factor in the tree was the duration of latex flow. Ecoclimatic conditions, tapping system, utilisation of stimulating agents, characteristics of the laticiferous system and its relation with the surrounding tissues in the bark influences the duration and mechanism of latex flow.

Rate and duration of latex flow is also dependent on external factors which directly affecting the flow such as relative air humidity and the amount of latex vessels opened by tapping and intrinsic factors such as anatomy of bark, latex composition and metabolic activity of latex vessels and surrounding tissues (Sethuraj, 1983).

In *Hevea*, the turgor pressure (TP) inside the laticiferous cells is directly responsible for latex flow at tapping (Buttery and Boatman, 1964;

Raghavendra *et al.*, 1984). Buttery and Boatman, (1966) developed methods for direct measurement of this pressure in latex vessels. The turgor of latex vessels is maximum during dawn, which ranges from 7.9-15 atmospheres, falls during day as a result of withdrawal of water under transpiration stress and rebuilt in the night (Buttery and Boatman, 1967). The poor latex yield when the trees are tapped much after sunrise is due to such diurnal variations in TP, which in turn could be due to the changes in water vapour deficit in the air (Paardekooper and Sookmark, 1969).

The initial flow of latex is due to elastic contraction of walls when the fluid cytoplasm is expelled after a sudden release in their turgor (Southorn, 1969; Boatman, 1970; Buttery and Boatman, 1976; Gomez, 1983). After a while capillary forces regulate flow until the flow ceases as the latex coagulates and plug the vessels (Boatman, 1966; Milford *et al.*, 1969).

It was shown that the availability of water for the laticiferous tissue is directly responsible for the TP, which is in turn responsible for latex flow. Availability of water is also involved in the dilution reaction and finally prolongs the latex flow. The origin of dilution reaction lies in the tapping cut, which brings the turgor pressure in the laticiferous tissue down to ~ 1 atm at the level of the cut itself. Dilution of latex favors latex flow in two ways i.e. it can reduce the viscosity and fluidity and helps to prevent the latex vessel collapse and prolonging latex flow (Pakianathan *et al.*, 1989).

There is a direct relationship between TP and flow during tapping. High TP is needed for latex expulsion to be more efficient. During tapping, it

involves water transfer from the apoplast of phloem tissues to the laticiferous tissue. This transfer is essential for flow. This phenomenon result in drop in Total solid content (TSC) during tapping indicating dilution of the rubber phase following an intake of water in the drained area. The easier is the hydric transfer, easier is the latex flow and conversely (Jacob *et al.*, 1997). This mechanism affects osmotic pressure within the laticifers and hence TP. Water transfer seems to be partly linked to biochemical energy availability in the laticifers.

The laticiferous system in *Hevea* consists of several rings which are mainly in the soft phloem, and the laticiferous vessels which form these rings are connected by anastomosis, a slightest wound can result in latex flow (Pakianathan *et al.*, 1989). The driving mechanism, dilution effect and kinetics of latex flow were studied much earlier (Arisz 1928; Gooding, 1952).

1.2 Limiting factors in latex flow

Studies by Milford *et al.*, (1969) revealed that the major limiting factor in the duration of flow is the plugging of the open extremities of the cut ends of the laticiferous vessels. Buttery and Boatman (1967) described the existence of a coagulum, which blocks the cut end of the vessels. Milford et *al.*, (1969) defined an index called Plugging Index (PI), which gives the extent of latex vessel plugging.

$$PI = \frac{\text{(Volume of latex in five minutes/5)}}{\text{Total volume of latex}} \times 100$$

Sethuraj *et al.*, (1978) and Yeang and Daud (1984) and measured the initial flow rate and the flow restriction index in relation to latex flow characteristics

and yield. Southorn and Gomez (1970) considered that shorter the tapping cut, greater the shear effect and consequently the plugging of latex tubes. For this they studied the intensity of plugging (IP).

IP= 100 (b-a)/a where 'a' is the flow during the minute preceding retapping and b is the flow during the minute following retapping. The IP values indicated the extent to which flow had been impeded just before retapping and found that very short tapping cut (1-2 mm long) gave much higher IP in two clones studied i.e. RRIM 501 and PR 107.

PI was shown to be a clonal character (Paardekooper and Somosorn, 1969: Milford *et al.*, 1969) but it is affected by the exploitation technique as well as the environmental stress. Correlation between PI and yield, and clonal variation in latex flow patterns were studied by Raghavendra *et al.*, (1984); Muji Lasminisingh and Thomas (1995) and Dey *et al.*, (1995).

Latex flow characteristics have been shown to affect rubber yield (Narayanan *et al.*, 1974). The volume of latex and the percentage of rubber it contains determine the yield of rubber from a tree on tapping. Sethuraj (1981) derived a formula for calculating the production of rubber by any tree.

Y=F.l.Cr / P where Y is the yield of rubber obtained from a tree each time it is tapped

F- average initial flow rate/ cm of tapping cut after tapping l- length of the tapping cut, Cr- dry rubber content of latex and P- plugging index. These components are influenced by internal and external factors. The rubber content is determined by the extent of assimilate partitioning into rubber. l, is a direct

proportion of the girth of the tree. The exploitation systems and clonal characters also influences F, Cr and P. The important sub components of these major yield components were the stability of rubber and lutoid particles, flocculation potential of lutoid serum, antogonising effect of C-serum on lutoid serum activity, dilution reaction after tapping etc. Dey *et al.*, (1995) studied the seasonal variations in some of the physiological and biochemical parameters, which regulate yield in different clones.

Tapping systems and use of stimulants also governs the mechanism of flow and regeneration of latex (Jacob *et al.*, 1989) and the results can be measured as physiological parameters (Eschbach *et al.*, (1984). Commercial scale experiments proved that tapping patterns and stimulation intensity could regulate the dynamic balance between latex flow and regeneration. Occurrence of Tapping Panel Dryness (TPD) – a major syndrome encounterd in rubber plantations is also controlled (Xu Wenxian *et al.*, 1994). Jacob *et al.*, (1989) has established the physiological parameters linked with flow and their correlation with yield.

1.3 Parameters linked with flow

1.3.1 Total solids content (TSC)

High TSC values can limit production, mainly latex flow because of the resulting high viscosity of latex. Vangils (1951) showed a negative correlation between TSC and production. This phenomenon was more marked when the water factor is limiting (Yeang and Paranjothy, 1982) and when

there is inadequate transfer of water from parenchymatous tissue to the laticifers.

1.3.2 Bursting Index (BI)

The BI of latex is a measure of the degree of intactness of lutoids (Ribaillier, 1971). It is the ratio of free acid phosphatase activities in the cytosol (derived from the destruction of part of lutoids) to total acid phosphatase activities determined after bursting the lutoids with a detergent (0.1% Triton X-100). This ratio represents the ~% of degraded lutoids. The BI is generally negatively correlated with yield (Eschbach *et al.*, 1984; Prevot *et al.*, 1984; Jacob *et al.*, 1986). Low BI indicates high stability of latex and hence easy flow and higher yield. A high BI reflects the extensive release of lutoid serum into the cytosol and indicates considerable destabilization of latex and consequently early plugging and rapid stopping of flow. Damaged lutoids are more in early flow fractions but later when the flow became very slow, damaged lutoids and rubber-lutoid microflocs slowly began to plug the open extremities of latex tubes causing the flow to stop (Yeang and Hashim, 1996). The relationship between bursting index and enzymes involved in latex flow and its cessation has not been reported.

1.3.3 Thiols

Latex thiols consist of cysteine, methionine and glutathione (Mc Mullen, 1960). Glutathione (RSSG-RSH) forms the largest proportion of thiols in latex. Reduced thiols (RSH) play an important role in the mechanism governing lutoid stability and hence latex flow (Cretin and Bangratz, 1983;

Chrestin, 1984). They inhibit peroxidases in the medium by preventing the production of toxic oxygen or catalyzing their transformation into non-toxic substances. They trap various forms of toxic oxygen, which are the classical byproducts of any cell metabolism. The formation of toxic oxygen is less when the metabolism is normal but it can attain considerable importance when cells are subjected to any stress. Toxic oxygen causes damage by degrading genes and moreover may cause cellular decompartmentation as a result of peroxidative degradation of membrane phospholipids. In latex it effectively cause the destruction of lutoids with harmful results with regards to flow. Thiols trap these toxic forms by protecting the cell compartmentation of latex and hence the functioning of laticifers and in particular flow at tapping.

Reduced thiols are also activators of the two major enzymes in the glycolytic pathway such as invertase and pyruvate kinase and thus have a positive effect on latex regeneration (Jacob *et al.*, 1982 & 1981). The RSH are thus capable of favoring latex flow and regeneration of latex. A highly significant positive correlation between RSH content, bursting Index and yield was reported by Chrestin and Bangratz, (1983); Eschbach *et al.*, (1984) and Prevot *et al.*, (1984). Hence the process which regenerate reduced thiols from its oxidised form has a certain importance. Jacob *et al.*, (1984) showed the presence of an enzyme glutathione reductase (GR) (E C.1.6.4.2) in the latex cytosol capable of carrying out this reduction. The variations in reduced thiols in latex and C-serum and GR activity in C- serum during stress, under different tapping frequency and stimulation has not been reported.

1.3.4 Magnesium (Mg^{2+})

Magnesium is present in the cytosol but accumulated in lutoids. When Mg^{2+} is released from the lutoids, its positive charge has a destabilising effect on the negative charges of the colloidal suspension formed by latex, making it to coagulate and thus limiting flow. In certain clones whose latex has a high Mg^{2+} content showed frequent latex coagulation at the tapping cut. Mg^{2+} acts as an inhibitor of invertase in latex (Tupy and Primot, 1976) and acid phosphatase (Jacob *et al.*, 1989). It is also an activator of enzymes such as transferase, PEP carboxylase (Jacob *et al.*, 1981) and ATPase (Chrestin *et al.*, 1985).

1.4 Carbohydrate metabolism and latex flow

High invertase activity and sucrose catabolism increase the dilution of latex during its flow after tapping. Yeang *et al* (1984) reported a negative relationship between invertase activity in latex and plugging index. Latex pH and actual invertase activity in successive latex fractions after tapping exhibit a decline and are very low at cessation of flow (Chong, 1981; Tupy, 1973).

1.5 Adenylates and latex flow

The quantity and turn over of adenylate pool (ATP, ADP and AMP) also have a fundamental importance to latex flow and regeneration and consequently on rubber yield (Jacob *et al.*, 1997). The variations in the ATP level in latex during different seasons; effect of high and low frequency-tapping systems and immediate effect of stimulation has not been reported earlier.

1.6 Factors involved in the cessation of flow after tapping

Flow of latex stops after a few hours after tapping by a process of coagulation at the cut surface. Cessation of latex flow is caused by the formation of latex coagulum at the cut ends of the latex vessels. The coagulum act as physical barriers to latex flow and the phenomenon is called latex vessel plugging.

Several mode of action have been proposed to explain the mechanism of latex vessel plugging. The first hypothesis was the collapse of latex vessel after tapping put forward much earlier by Arisz, (1920) and Frey Wyssling, (1932).

The progressive plugging of latex vessels during the course of latex flow following tapping restricts the quantity of latex and hence it is an important determinant of yield output. The influence of lutoids on stability and flow of latex were studied by Yip and Southorn (1968) and Southorn and Edwin, 1968). They have shown that latex had an inherent clotting mechanism, which could give rise to an internal plug formation . In this mechanism lutoids are ruptured during latex flow. This breaking is due to osmotic shock (Pakianathan *et al.*, 1966), shear (Yip and Southorn, 1968 & 1973) and other factors and resulting in the release of their contents. This leads to flocculation of latex particles (Southorn and Edwin, 1968; Southorn and Yip, 1968) and consequently initiating latex vessel plugging. All these studies revealed the importance of the damage of lutoids in the formation of plugs.

Southorn and Edwin (1968) have demonstrated the coagulant role of intra lutoid serum (B-serum). The high protein content of B-serum (~3.5%) and a major part of this was positively charged led to suspicion of their role in the destabilization of latex. When the proteins of the B-serum were discarded by precipitation after boiling, most of the destabilizing activity was removed with the precipitate.

Further experiments showed that a cationic protein such as cytochrome c which has a high isoelectric point (10.5) causes immediate flocculation when added at normal lutoid protein concentration (3%) to a suspension of 2% rubber particles. This is same as the process observed with natural B-serum protein.

Like wise Southorn and Yip, (1968) reported that the addition of a cationic surfactant Cetyl Pyridinium Chloride (CPC) to latex causes immediate flocculation.On the other hand, an anionic surfactant like sodium dioctyl sulfosuccinate (Aerosol OT), was found to be suitable for titrating the coagulating power of lutoid serum in the same way as the protective power of C-serum was titrated by CPC (Southorn and Yip, 1968).

The lutoid serum can destabilize the latex was confirmed by the determination of the surface potential (zeta potential) of the rubber particles and by lowering and neutralizing it by adding B-serum or CPC to a suspension of rubber particles (Southorn and Yip, 1968b). C-serum contains negatively charged proteins at biological pH values imply charge neutralization reactions when proteins of B and C sera are put into contact.

From all these studies it was concluded that the breakage of lutoids within latex vessels leads to the formation of microflocs inside and this might play an important role in the stopping of latex flow.

The breakdown of lutoids during or after tapping liberates some hydrolytic enzymes. They attack the phospholipoprotein films around the rubber particles. Among the lutoid enzymes (Pujarniscle, 1968) only a protease displaying very acid pH $\leq$ 3.5 was suspected in this process. Lysozyme (hydrolytic lutoid enzyme) is not suited to attack the protective film of rubber particles (Woo, 1973). The role of enzymes such as phospholipase, coagulase and phenol oxidase was studied by Smith, (1954); Woo (1973); Brozozowska- Hanower *et al.*, (1978). Yip and Gomez (1984) studied the characterization of cell sap of *Hevea* and its influence on cessation of latex flow.

Thus the duration of latex flow and production depend on the greater or lesser wholeness of lutoids when they flow out of the laticiferous tubes. Two types of analysis were reported to study the stability of lutoids. Bursting index (BI) of lutoids by Ribaillier (1968) which is a measure of the integrity of lutoids and the Plugging Index (PI) by Milford *et al.*, (1969) which is a measure of the more or less rapid blocking of the open extremity of the laticiferous tubes by internal plugs and the thick external cap of coagulum (Milford, 1969).

Ribaillier (1968) who introduced the concept of BI was not able to establish a correlation between BI and PI. But Sherief and Sethuraj (1978)

established a correlation between BI of lutoids and PI in a group of seedling trees. Yeang and Paranjothy (1982) have also observed a relationship between PI and BI.

The fragility of lutoid membrane may be one of the primary factors in the destabilization of these particles when they are subjected to substantial stresses. Fall in turgor pressure, which occurs when latex vessels are opened at tapping, is in fact a considerable mechanical stress. Early fractions of latex flow were rich in damaged lutoids (Pakianathan, 1966, Yeang and Hashim, 1996).

The stability of lutoid membrane, which is a clonal character, must be very important among the factors governing the duration of flow. Dupont *et al.*, (1976) studied the phospholipid and fatty acid composition of lutoid membrane and characterized by a high content of phosphatidic acid. Usha Nair *et al.*, (1995) showed that two characters related to lutoid membrane i.e. phospholipid content of bottom fraction and protein content of lutoid membrane have a high correlation with BI and PI and thereby yield. Sreelatha *et al.*, (1995) have studied the lutoid membrane composition and polypeptide profile of high and low yielding clones and found that high yielding clones have a high content of protein in the lutoid membrane and an enhanced expression of two polypeptides with molecular weight 63.1 and 79.4 kD.

A completely different mechanism i.e. the peroxidation pathway that degrades lutoid membrane was proposed by (Chrestin, 1984b). Evidence of an NADP (H) oxidase activity in the lutoid tonoplast, which generate toxic forms

of oxygen, was reported by (Chrestin *et al.*, 1986). It was shown that there is a strong positive correlation between activity of lutoid NADH oxidase and BI of lutoids (D'Auzac *et al*, 1986). The various forms of toxic oxygen released (O_2^- H_2O_2, OH^-) during NAD (P) H dependent consumption of oxygen leads to a rapid peroxidative degradation of the lutoid membrane as well as exogenous unsaturated lipids and the damage was evidenced by the appearance of malondialdehyde (a product of peroxidation of polyunsaturated lipids).

Lutoids from healthy high yielding and medium yielding trees exhibit only traces of NAD (P) H oxidase activity. The lutoids from very low yielding trees with abnormally high plugging index and bursting index showed a higher activity which generate toxic oxygen and able to induce the peroxidative degradation of the unsaturated lipids of the membrane leading to lysis of the lutoids. But if the scavenging chemicals and enzyme activities were fully active, this peroxidative degradation would not result in any harmful effects. The factors likely to protect the membrane from the deleterious effects of toxic oxygen are superoxide dismutase, catalase, reduced thiols and ascorbic acid.

It is well known that two basic proteins of *Hevea* latex (hevein and hevamine) were involved in the plugging phenomenon of latex vessels after tapping (Tata, 1976; Archer, 1976). This involvement is partly due to their high isoelectric point and their consequent ability to flocculate the negatively charged rubber particles in latex.

The acidic protein hevein is the major protein in the lutoid body fraction of rubber tree latex (Archer, 1969). Hevein is a single chain protein of

43 aminoacids rich in glycine and cysteine (Walujono *et al.*, 1975) and an apparent molecular weight of 5000 daltons (Tata, 1976). The three dimensional structure has been determined by X-ray diffraction and NMR spectroscopy (Rodriguez- Romero *et al.*, 1991; Anderson *et al.*, 1993). Hevein is formed from a precursor protein pro-hevein. Crude preparations of hevein contain a small amount of pseudo-hevein, which is a minor hevein component. The difference between hevein and pseudo-hevein is the replacement of a tryptophan residue by a tyrosine at position 21 of the sequence in the carbohydrate-binding site (Soedjanaatmadja *et al.*, 1994). Pseudo- hevein elutes earlier than hevein when fractionated using affinity chromatography indicating the lower binding strength to this polypeptide. It was shown by Gidrol *et al.*, (1994) that hevein is a lectin like protein involved in the coagulation of latex by bringing together rubber particles. This bridging is mediated by N-acetyl glucosamine and involved a 22 KD glycoprotein localized on the rubber particle surface, which act as receptor for hevein. The binding of N-acetyl glucosamine to hevein is Ca2+ dependent. Lutoids are known to have an acidic pH of 5.5 and highly buffered content and very rich in Ca^{2+} (1.5 mM) as compared with C-serum (0.2 mM). The bursting of lutoids leads to the release of divalent cations along with hevein and provokes the acidification of the immediate vicinity where the process of coagulation starts.

Van Parijis *et al.*, (1991) have suggested that hevein play a role in the protection of wound site from fungal attack. Broekaert *et al.*, (1990) studied

the accumulation of mRNA containing a hevein sequence in laticifers of rubber tree. Sivasubramanium, (1995) presented evidence that hevein is a novel stress induced gene.

Breton *et al.*, (1995) reported the presence of a β,1-3 glucanase (E.C.3.2.1.39) in the lutoid body fraction of *Hevea*. The presence of chitinase (3.2.1.14) in latex (Subroto *et al*, 1996) inhibits latex coagulation by removing N-acetyl glucosamine moiety from the 22 KD receptor proteins. It also increases the concentration of free N-acetyl glucosamine in the latex and blocking hevein-binding sites. The level of hevein and chitinase expression in latex is a clonal characteristic linked to latex flow (Chrestin *et al.*, 1997).

N-acetyl glucosaminidase (E.C.3.2.1.36) which catalyses the hydrolysis of the β-N-acetyl glucosaminyl linkages exists in carbohydrates and glycolipids (Giordani *et al.*, (1992). It is a 92kD protein consists of two 46kD subunits and has a glycoside content of 16%. This enzyme inhibits latex coagulation (Chrestin *et al.*, 1997).

In contrast to the above findings on destabilizing effect of hevein, Ukun *et al.*, (1999) presented evidence that hevein has a stabilizing effect on suspension of rubber particles. The results showed that hevein content of lutoids in high yielding clones was higher compared to low yielding clones.

From the above studies it is evident that the processes involved in latex coagulation are antagonistic. Hevein and glucanase induce coagulation (Chy and Cheung, 1995) and N-acetyl glucosaminidase and chitinase slow it down (Martin, 1991).

1.7 Implications of stress on latex flow and yield

Latex harvesting is performed over most of the year and therefore a study of the effect of climatic conditions on yield would strengthen the knowledge and help to improve the effectiveness of exploitation. Yield is influenced by climatic factors. Climate is one of the ecological factors that affect the growth and yield of crops. There was a dual effect of rain on latex yield. On the one hand, soil humidity is increased and water status in the tree was improved, led to an increased yield. But on the other hand, it prevented tapping thus reducing the number of tapping days leading to reduce harvest yield.

Yield is influenced by the effect of different environmental parameters. In India, the period of peak yield is from September – January (non-stress period) and dry period is from February- April (stress period). The latex yields are generally reduced at low soil moisture levels prevalent in summer months. The soil moisture remarkably altered the pattern of latex flow. The duration of flow as well as the amount of latex was reduced during water stress conditions (Sethuraj *et al.*, 1984). According to Sethuraj and George (1976a), the drop in latex yield under soil moisture stress was due to enhanced plugging and restricted drainage area.

Clonal variations in the kinetics of latex flow at two-soil moisture levels were studied by Raghavendra *et al.*, (1984). The existence of two distinct phases such as an initial curvilinear rapid expulsion and a later

exponential slow phase were demonstrated. At low soil moisture levels duration of latex flow was shortened particularly in the later phase.

Sethuraj (1983) reported that the major genetic variance possibly existed in some physiological processes related to yield. Some physiological characteristics are very susceptible to environment. Annual yield depends upon the average values of initial flow rate, plugging index and rubber content through different seasons of a year. Clones of *Hevea* vary in their sensitivity to water stress (Saraswathyamma and Sethuraj, 1975)

Information on the seasonal variation of the limiting factors i.e. flow and regeneration of latex will be useful. The two main determinants of yield variations are the initial flow rate and the latex vessel plugging during latex flow (Yeang and Paranjothy, 1982). The extent of damage to lutoids in the latex is an important factor in determining seasonal changes in latex vessel plugging.

Initial flow rate of latex was influenced by panel turgor. Panel turgor might be influenced by the moisture status of the tree and could be diminished by water stress (Yeang and Paranjothy, 1982). Water status of the bark tissue is reflected in the panel turgor. So a high turgor is the resultant of high water content of the latex with a consequent depression in dry rubber content (DRC)

In the regulation of the final yield output from the tree on a seasonal basis, the duration of flow is relatively more important than the severity of the initial decrease in flow rate.

Prolonged flow of latex depends partly on the influx of water from the surrounding cells. Any condition contributing to good supply of water to tissue or reductions in the loss of water by evapotranspiration are favorable for latex flow and high production. The main reason for seasonal variation in production should therefore be sought in rainfall pattern, which is different in the various rubber producing regions.

PI displayed a very marked seasonal variation which is probably connected with seasonal variation in rainfall i.e. availability of water to the tree (Paardekooper and Somosorn 1969). Premakumari *et al.*, (1980) reported that the increase in PI caused by drought was related to a fall in the phospholipid and neutral lipid content of latex. A negative correlation between neutral lipids in rubber particles, phospholipid contents of luoid fraction and PI during drought were observed. There was decrease in production by decrease in lipid content which would lead to increased fragility of lutoid membrane and hence a high PI.

Sethuraj, (1995) studied the clonal variation in the percentage reduction of rubber yield in summer and showed that high yielding clones showed relatively greater % reduction in the summer compared to low yielding trees.

Seasonal variation in yield, yield components and components of water relations were studied in some *Hevea* clones by Devakumar *et al.*, (1988). It was seen that summer yield drops were low in clones like RRII 105 and Gl-1. High latex vessel turgor and low solute potentials in clone RRII 105 in the dry

season indicate the presence of osmotic adjustment. Higher plant water status and lowered transpiration in this clone might help in better turgor. In another study by Satheesan *et al.,* (1982), the drought tolerant clones have shown to maintain high solute potential in their C- serum (to keep the lutoids intact) even in summer months. Calcium and magnesium do not seem to contribute to the seasonal trend in latex vessel plugging (Yeang and Paranjothy, 1982).

1.8 Implications of tapping frequency on latex flow and yield

Tapping is a direct mechanical injury to the laticiferous system. The economic life of a rubber tree depends to a great extent on the frequency of tapping and the tapping system adopted. Frequency of tapping is a factor, which would influence the physiology of the tree as well as the yield from the tree. It would affect the physiological balance between extraction of latex and its regeneration in the latex vessels.

Various tapping patterns resulted in different physiological mechanism. In addition to the tapping method itself, tapping frequency and use of hormonal stimulation using an ethylene generator such as 2-chloroethyl-phosphonic acid was also considered. Too low a tapping frequency would result in reduced yield. A reduction in tapping frequency requires an increase in metabolic activity by hormonal treatments to obtain similar production. Increase in tapping frequency also leads to the incidence of tapping panel dryness (TPD) (Vijayakumar *et al.*, 1991). The tapping system chosen for a clone should take into account the tapping frequency, the

stimulation intensity and also the panel management (Gener and du Plessis, 1975).

Several researchers have established that the yield variability of low frequency system is very much dependent upon the levels of stimulation (Eschbach and Banchi, 1984; Sivakumaran *et al.*, 1982; Sivakumaran and Ismail Hashim, 1985; Vijayakumar, 2001). Over stimulation or excessive latex flow could produce drastic physiological damages in the tree. These include excessive loss of active substances of latex vessel system on the flow area, including various organelles related to latex regeneration or disintegration of nuclear membranes. This give rise to functional exhaustion of latex production and finally *in situ* self destruction of laticifers and leads to TPD (Pakianathan *et al.*, 1982). Do Kim Thanh *et al.*, (1996) studied some physiological parameters in relation to tapping frequency in clone RRIM 600 and found that trees tapped on d/2 frequency recorded lower readings of PI, IFR, TSC and DRC but higher bottom fraction, thiols and inorganic phosphorus in latex compared to d/4 tapped trees. The role of enzymes related to latex flow and cessation of flow under high and low frequency-tapping systems has not been studied.

1.9 Effect of Stimulation on latex flow and yield

Yield stimulation of *Hevea* with ethephon, a proprietary formulation containing 10% (ai) 2-chloro-ethyl phosphonic acid is now a common practice in most of the NR producing countries. External application of ethephon in the bark is known to transiently overcome the two intrinsic limiting factors by delaying coagulation and stimulating latex regeneration and resulting in

increased latex yield (Ho and Paardekooper, 1965). All these characteristics like duration of latex flow, latex regeneration, response to yield stimulation and sensitivity to tapping panel dryness syndrome etc depend on clones.

Yield response to ethephon greatly depends on sucrose availability in latex vessels and an enhancement of invertase activity, which is the rate-limiting enzyme in carbohydrate metabolism (Tupy, 1973). Tupy and Primot, (1976) reported that stimulation was not effective and leads to very low increase in latex yield in trees with very low latex sucrose.

Stimulation is an indirect chemical damage to the system. Abraham *et al.*, (1968) and d` Auzac and Ribaillier (1969) were the first to report the yield enhancing properties of this substance. First response in yield increase was noted 24 hrs after application of the stimulant. De Jonge (1955) showed that duration of flow can be even doubled or more, but Boatman (1966) pointed out that, this enhancement did not persists from one tapping to the next eventhough total latex yield continued to increase. Stimulation lowered the dry rubber content of latex. Latex viscosity is related to DRC, which may account for the increase in flow rate at tapping. Boatman (1966) eliminated the role of latex viscosity because there was no correlation between viscosity and production.

Buttery and Boatman (1964 &1966) showed that stimulation could not be ascribed to an increase in turgor pressure but stimulation prolongs flow under low pressure and delays plugging of the latex vessels.

Osborne and Sargent (1974) observed that under stimulation, *Hevea* should develop wider vessels with thicker and rigid walls. After tapping these vessels would lead to reduce shearing forces and prolonged latex flow.

De Jonge (1953) proved that after stimulation there was an extension of the drainage area. Pakianathan *et al.*, (1976) showed that the displacement area was enhanced by stimulation.

Stimulation with ethrel promotes transmembraneous water exchange resulting in a decrease of TSC and partly account for the increased ease of flow observed after treatment and the resulting increase in production (Jacob *et al.*, 1992).

In a normally tapped tree, the flow stops progressively by a process of coagulation at the cut surface a few hours after incision of the bark. Boatman (1966) found that stimulation enhances production by improving latex stability and thus lengthening duration of flow.

Ribaillier (1971&1972) showed that lutoids became more stable after stimulation. Milford *et al.*, (1969) found a direct correlation between the Plugging Index and the response to stimulation. The bursting of lutoids can cause slowing of flow. For the first ten tapping after stimulation there was a striking increase of the stability of the lutoids as measured by the BI. The lutoids from stimulated trees gained new properties such as more resistance to various destabilizing treatments like osmotic lysis, shearing and reduced destabilizing acivity of lutoid serum. Latex from stimulated trees coagulated less rapidly than the latex from unstimulated controls.

Coupe (1977) proved that ethylene stimulation did not affect the osmotic pressure of either C or B serum. Physiological and biochemical evidence showed that ethylene act on membrane permeability leading to prolonged latex flow as well as general regenerative metabolism (Coupe and Chrestin, 1989).

1.10 Stimulation induced metabolic changes

Treatment of the bark with ethephon induces an acceleration of sugar loading (Lacrotte *et al.*, 1985), and its catabolism (Tupy and Primot, 1976) in addition to the general increase in protein turn over within the latex cells (Coupe and Chrestin, 1989). Stimulation with ethrel also induces a cytosolic alkalinisation (Coupe *et al.*, 1976) coupled with an acidification of lutoids (Chrestin *et al.*, 1984). Thomas *et al.*, (1999) studied the mechanism of ethylene-induced stability of lutoids in clone RRII 105 under d/2 system of tapping. They presented evidence that ethephon application increases the rubber yield by altering the water relations of latex. Because of the loss of osmotic gradient, continued flux of water into lutoid particles was avoided and thus they remained intact for a long time.

Ethylene activates ATPase and pyrophosphatase activities of lutoid tonoplast, which are involved in the proton transport between lutoid and the cytosol and thus maintaining the pH of latex (Chrestin *et al*, 1984; Gidrol *et al.*, 1988).

Modifications of the cytosol and intra lutoid serum (Jacob *et al*, 1992); protein biosynthesis (Prevot *et al*, 1992); and hevein transcripts (Broekaert *et*

al., 1990), increase in glutamine synthetase activity (Pujade Renaud, 1994) by ethrel stimulation were also reported.

Ethylene stimulation activates cell metabolism particularly in the adenylate pool and also substantially increases the penetration speed of water from phloem to laticiferous tissue even before tapping. This is probably one of the major positive effects of ethylene treatment on flow and hence on yield stimulation (Gidrol *et al.*, 1988; Amalou *et al.*, 1992 and Jacob *et al.*, 1997).

While ethephon stimulation can stress the tree physiologically by increasing the yield output, it also induce other stress effects such as increasing the production of toxic oxygen from lutoidic NAD (P) H oxidase activity (Chrestin, 1984b) which also causes a fall in RSH content (Prevot *et al.*, 1986). Actually, the intensity of stimulation and hence an increase in laticifer activity, leads to a drop in reduced glutathione content (Gohet *et al.*, 1997).

Apart from physiological and biochemical studies on yield stimulation, one aspect of practical importance is the optimum use of stimulation in conjunction with tapping systems. Studies on regulation of enzymes which influencing latex flow and cessation of flow under low frequency tapping system with stimulation has not been reported earlier.

1.11 Latex flow characteristics and Tapping panel dryness (TPD)

TPD, which is generally regarded as a physiological disorder of *Hevea*, is of great economic importance since it leads to the loss of yield particularly in high yielding clones. In India, the TPD incidence is 15-30% among high

yielders and 5-10% among seedlings. The high yielding clone RRII 105 is very susceptible. No *Hevea* cultivar is completely resistant to TPD. However different clones are known to possess varying degree of relative resistance to dryness.

Most of the physiological/ biochemical studies including histological observations indicate that it is a disorder that originates in the laticiferous system of the tree (Chua, 1966; Paranjothy *et al.*, 1976; Gomez, 1990).

It has been shown that excessive flow of latex due to over exploitation or over stimulation is a primary cause for the onset of tree dryness of *Hevea.* These treatments would change the physical balance between latex regeneration and flow, resulting in metabolic disorders.

The onset of TPD has often been associated with latex instability within the latex vessels. This leads to blockage of the latex vessels and the cessation of flow when the tree is tapped.

Cellular metabolism normally produces a certain quantity of toxic oxygen species (AOS) during electron transfers occuring in mitochondria, peroxisomes, plasmalemma, chloroplasts etc. Various detoxification reactions occur to neutralise these AOS. These are enzymes such as super oxide dismutase (SOD) (E.C.1.15.1.1), catalase (E.C 1.11.1.6), peroxidase (E.C.1.11.1.7) or antioxidants such as glutathione, ascorbic acid etc. All these enzyme systems have been detected in latex (Chrestin, 1984b; Jacob *et* al., 1997).

Krishnakumar *et al.*, (1999) studied the biochemical composition of soft bark tissues affected by TPD and showed that TPD affected tissues contained comparatively higher levels of sugars, phenols and soluble proteins than healthy tissues. Also there was significantly higher activity of peroxidase and lower activity of polyphenol oxidase in the affected tissues than healthy tissues. Thomas *et al.*, (1998) studied the biochemical composition of latex (sucrose, thiols and inorganic phosphorous) in clone RRII 105 at biweekly intervals with the objective of relating any changes in these parameters with the onset of TPD. Six trees out of fifty trees showed symptoms of TPD during the 20 months period. The concentration of sucrose and Pi in the latex was higher in the affected trees than healthy trees 15 to 45 days before the onset of TPD. These results showed that lack of availability of sucrose was not the cause for TPD.

Yang Shaoquiong *et al.*, (1997) reported the physiological changes in the onset and development process of whole cut dryness which include a short time increase in IFR and latex yield prior to dryness and an increase in inorganic phosphorus in the course of onset and development of dryness. Gitali Das *et al.*, (2002) reported the over exploitation associated free radical changes and its scavengers during the occurrence of TPD.

The accumulation of proline in plant tissue is a common reaction to various forms of physiological stress, such as that induced by drought, cold or salt (Aspinall and Paleg, 1981). As water is the main constituent of latex,

excessive withdrawal of latex may not lead to a localized water deficit in the region of the tapping panel.

Faridah Yusof *et al.*, (1995) studied the effects of various stress inducing treatments on latex proline and showed an increase in proline content of about 60% compared to control at the 7th pweek of the experiment. By the 25th week the proline content of latex did not showed any significant difference between control and stress treatments. Their results suggest that an increase in latex proline might serve as a warning indicator of tapping panel dryness.

As it is evident from this review, no definite information is available on the biochemical factors influencing latex flow under various conditions such as stress, tapping frequency and low frequency tapping system with stimulation and in high and low yielding clones. Hence, experiments were carried out in newly opened trees of clone RRII 105 (a high yielding Indian clone) to relate the biochemical factors influencing latex flow characteristics, cessation of flow and production. The specific objectives are

1. To study the biochemical factors influencing latex flow, its cessation and reduction in yield during [illegible]-stress season.
2. To study the variations in latex flow characteristics associated with high and low frequency- tapping systems.
3. To study the immediate effect of stimulation on parameters associated with latex flow, cessation of flow and yield in trees tapped under low frequency tapping system.

4. Observations on the natural development of tapping panel dryness syndrome (TPD) in trees tapped under high and low frequency tapping systems (at the time of opening the trees for tapping onwards) and their biochemical components before and after the incidence.
5. To study the clonal variations in biochemical parameters associated with latex flow and its cessation.

CHAPTER 2

MATERIALS AND METHODS

2.1. Location

All the experiments were carried out at Rubber Research Institute of India, Kottayam (9 ^{0}32′ N, 76^{0}36′ E, 73 M).

The study consists of four experiments viz.

1. Seasonal variations in latex flow characteristics and yield
2. Effect of tapping frequency on latex flow characteristics and yield
3. Immediate effect of stimulation on latex flow characteristics and yield
4. Clonal variations in latex flow characteristics and yield

2.2. Plant materials for Experiment 1&2

Hevea trees of clone RRII 105, which were planted in 1988 at field V of Rubber Research Institute of India, Kottayam, were selected for these experiments. 48 trees of uniform girth (50 cm) were selected and opened for tapping in February 1997. These trees were then divided into two equal groups. ½ S d/2 6d/7 (alternate daily) tapping system was adopted for one group and the other group was tapped under ½ S d/4 6d/7 (fourth daily) tapping system. Individual tree yield in terms of latex volume were recorded monthly up to November 1998. Then 16

trees with uniform yield were selected from each group for physiological and biochemical measurements. Monthly data on yield and physiological parameters were recorded for two years from November 1998- April 2000.

For comparison of seasonal variation, the values of 16 trees, which were under ½ S d/2 6d/7 tapping system, were used. Mean values of September – November 1999 were taken as peak yielding season and February- April 2000 as stress season.

To study the effect of tapping frequency, 16 trees from both groups were selected. Mean values for the peak yielding period (September- November 1999) were used for comparison.

2.3. Plant materials for Experiment-3

36 *Hevea* trees of clone RRII 105 with uniform girth and comparable yield were selected for this study. The trees were under ½ S d/4 6d/7 tapping system. The following treatments were imposed with six replications.

T1 - ½ S d/4 6d/7 (un stimulated control)

T2 - ½ S d/4 6d/7 + 2.5 % Ethephon

T3 -1/2 S d/4 6d/7 + 2.5% Ethephon and the tapping system changed to ½ S d/2 6d/7

T4 -1/2S d/4 6d/7 + 2.5% Ethephon and tapping rest for 2 days

T5 -1/2S d/4 6d/7 + 2.5% Ethephon and tapping rest for 6 days

T6 -1/2S d/4 6d/7 + 2.5% Ethephon and tapping rest for 10 days

T7 -1/2S d/4 6d/7 + 2.5% Ethephon and tapping rest for 14 days

The study was conducted during October 2000

2.3 .1. Method of stimulation

Commercially available 10% solution of ethephon (2-chloro ethyl phosphonic acid, Bethel products Pvt. Ltd. Bangalore) was diluted to 2.5 % with coconut oil. The solution was applied at the rate of 1.5 ml per tree by panel application (ethephon was applied with a brush just above the tapping cut to a width of 1 cm).

Pre treatment yield as well as physiological and biochemical parameters of latex were recorded from all trees from the tapping done before the application of ethephon. Three types of comparisons were made

1. T1 and T2 (control and stimulated). For this the measurements were made on four tappings after stimulation.
2. Before stimulation and T3: Latex samples were collected 2-day interval after stimulation (intensive tapping).
3. Before stimulation and T4, T5, T6 and T7: Latex samples were collected from trees, which were given tapping rest for 2,6,10 and 14 days after stimulation

2.4. Plant materials for Experiment-4

The study was carried out in four clones viz. RRII 105, RRIM 600 (representing high yielding clones) and RRII 38 and HP-20 (representing low yielding clones). Six trees from each clone were used. The tapping system adopted was ½ S d/2 6d/7. The experiment was carried out during the peak-yielding season of 1999.

2.5. Physiological parameters

2.5.1. Latex yield

Total latex yield of the tree was determined by measuring the whole latex collected from the tree after tapping and represented in terms of volume of latex in ml $tree^{-1}$ tap^{-1}.

2.5.2. Dry rubber yield

Dry rubber yield was calculated using the formula

$$\text{Dry rubber yield} = \frac{\text{DRC} \times \text{Total volume of latex (ml)}}{100}$$

It was represented in g $tree^{-1}$ tap^{-1}

2.5.3. Dry rubber content (DRC)

Dry rubber content was determined by gravimetric method. About 10 g latex was coagulated with 3% acetic acid. The coagulum was washed in running water and oven dried at 80^0 C for 72 hrs. Dry weight of the rubber was determined after cooling. DRC of latex was calculated using the formula

$$\text{DRC} = \frac{\text{Weight of oven dry rubber}}{\text{Weight of fresh latex}} \times 100$$ and was expressed as percentage.

2.5.4. Total solid content (TSC)

TSC was determined by drying 1g of latex at 80^0 C to constant weight and its dry weight determined after cooling to room temperature. TSC was calculated using the formula

$$\text{TSC} = \frac{\text{Weight of oven dry latex}}{\text{Weight of fresh latex}} \times 100$$ and was represented as percentage.

2.5.5. Turgor pressure (TP)

Pre tapping latex vessel turgor was measured according to the method described by (Raghavendra *et al.*, 1984) using disposable mini manometers comprising of polythene surgical tubing sealed at one end and fitted with a 21-gauge hypodermic syringe needle at the other. The total length of the manometer was 20 cm. The needle was inserted gently into the bark at 5 cm below the tapping cut and latex would be entering the sealed surgical tube. The length of the air column trapped at the sealed end of the surgical tube was measured and the turgor pressure was computed using a calibration curve prepared against known pressures and length of air column in a similar tube and expressed as bars.

2.5.6. Initial flow rate / unit length of tapping cut

IFR per unit length of tapping cut (ml/min/cm) was determined by dividing the volume of latex collected during the first 5-min by 5 and length of tapping cut (cm).

2.5.7. Plugging index (PI)

Plugging index was determined by measuring the initial flow rate IFR and total volume of latex and calculated using the formula described by Milford *et al.*, (1969).

PI = IFR × 100/ Total volume (ml)

2.6. Collection and separation of different fractions of latex for biochemical analysis

After tapping the tree, the first 5ml of exuded latex was discarded and about 50 ml of subsequent flow was collected from individual trees into ice-chilled

containers. Latex centrifugation was carried out immediately at 23,000 rpm for 45 minutes at 4°C in a Sorvall OTD 55 B Ultracentrifuge. The upper rubber phase was removed and the middle serum (C-serum) was collected using a syringe and used for various biochemical estimations.

2.6.1 Preparation of B-serum

The fraction at the bottom (lutoid) after centrifugation of latex was removed and washed 3-4 times with 0.4 M mannitol and subjected to repeated freezing and thawing to rupture the lutoids and to liberate the serum inside the lutoids. This was centrifuged at 20,000 for 30 minutes to remove the lutoid membrane fragments and the supernatant (B-serum) was collected and used for biochemical analysis.

2.7 Biochemical parameters

2.7.1. Bursting index (BI) of lutoids

Bursting index of lutoids was determined by the method of (Rebaillier, 1968)

Reagents

(1) 0.8M Acetate buffer pH 5.0

27.128 g of anhydrous sodium acetate was dissolved in 250 ml distilled water. 9.2 ml glacial acetic acid was diluted to 200 ml with distilled water. The two solutions were mixed in the ratio 2:1 and the pH adjusted to 5.0.

(2) 0.8M sodium paranitrophenyl phosphate

2.11 g of sodium paranitrophenyl phosphate was dissolved in 100 ml acetate buffer.

(3) 0.5% Triton X100

0.5 ml Triton X100 was mixed with 0.5 ml 1N NaoH and made up to 100 ml with distilled water.

(4) 0.6M Mannitol

21.84g mannitol was dissolved in 200 ml distilled water.

(5) 2N Trichloroacetic acid

32.678 g trichloroacetic acid was dissolved in 100 ml distilled water.

(6) 1N NaOH

4g NaOH dissolved in 100 ml distilled water.

(7). PAT solution (for total acid phosphatase)

50 ml p-nitrophenyl phosphate was added to 100 ml Triton and 245 ml water. Kept at 20 ^{0}C.

(8) . PAL solution (for liberated acid phosphatase)

50 ml p-nitrophenyl phosphate was added to 200 ml mannitol and 145 ml water. Kept at 20^{0} C

(9) P-nitrophenol standard: 10 mg p-nitro phenol was dissolved in 25 ml distilled water. From this 1ml was diluted to 25 ml for working standard.

Procedure

Liberated total (LT)

2.9 ml PAL solution was pipetted in test tubes and 100µl latex was added. The mixture was incubated for 10 minutes and the reaction was stopped by adding 1 ml 2N TCA and filtered using whatman No.1 filter paper.

Liberated control (LC)

2.9 ml PAL solution and 1ml 2N TCA were taken in test tubes and added 100µl latex. Mixed well and the resultant mixture was filtered using whatman No.1 filter paper.

Test total (TT)

2.9 ml PAT solution was pipetted in test tubes and 100µl latex was added. The mixture was incubated for 10 minutes and the reaction was stopped by adding 1 ml 2N TCA and filtered using whatman No.1 filter paper.

Test Control (TC)

2.9 ml PAT solution and 1ml 2N TCA were taken in test tubes and added 100µl latex. Mixed well and the resultant mixture was filtered using whatman No.1 filter paper.

Estimation

0.5 ml of the supernatant was added to 0.5 ml 1N NaOH and 4.0 ml water. The p-nitrophenol liberated was measured spectrophotometrically at 410 nm. Bursting Index was calculated using the formula

BI = LT-LC / TT-TC x 100 (expressed as %)

2.7.2. Extraction of thiols and inorganic phosphorus from latex

About 1 gm of fresh latex was extracted with 2.5 % trichloro acetic acid and made up to 10.0ml with 2.5% TCA. Filtered the solution using Whatman No.1 filter paper and the filtrate was used for the estimation of thiols and inorganic phosphorus.

2.7.2.1. Estimation of thiols in latex

Estimation of thiols was done using the method of (Boyne and Ellman, 1972)

The thiol group of RSH reacts with DTNB (5,5' Dithiobis 2-nitro benzoic acid) to form TNB, which strongly absorbs at 412 nm. The R-SH groups become oxidized quickly and the analysis was done within 24 hours of latex collection.

Reagents

1. 0.5 M Tris(hydroxymethyl aminomethane) solution

(6.06 gm Tris hydroxy methyl aminomethane was dissolved in 100 ml of distilled water)

2. 10 mM DTNB (Dithio-bis-2-nitro benzoic acid)

In a small beaker added 79.4 mg DTNB and 140.3 mg EDTA (Ethylene diamine tetraacetic acid disodium salt). Add ~10.0ml water and adjusted the pH to 6:5 with 0.5 M Tris and made up to 20.0 ml in a volumetric flask and stored in the refrigerator.

Glutathione standard

25.0mg reduced glutathione (GSH) was made up to 25 ml distilled water in a volumetric flask. 1.0 ml of the above solution was diluted to 25ml with 2.5% TCA and used as working standard.

Estimation

For estimation, 2.0ml of latex TCA extract was pipetted in test tubes and 0.1ml DTNB and 2ml tris were added. Standards were also made up to 2.0ml and treated in the same way. Mixed well and optical density was measured at 412nm.

2.7.2.2. Estimation of thiols in C - serum

To 25 µl of C- serum, added 5.0 ml 2.5 % Trichloroacetic acid to precipitate the proteins and centrifuged at 5000 rpm for 15 minutes. To 2.0 ml of the supernatant added 0.1 ml DTNB and 2.0 ml 0.5 M Tris. Mixed well and read the optical density at 412nm along with the glutathione standard.

2.7.2.3. Estimation of oxidised glutathione in C-serum

Oxidised glutathione was measured using the method of (Smith and Kendal, 1984).

Reagents

1. 2.5 %TCA
2. 0.04M N-ethyl maleimide
3. 0.1M Sodium phosphate buffer pH 7.6
4. 5 mM EDTA
5. 6 mM DTNB
6. 1mM NADPH
7. Glutathione reductase (0.09 units/mg solid)
8. Oxidised glutathione std

Procedure

Serum samples were deproteinised with 2.5%TCA. The precipitate was removed by centrifugation at 4000 × g for 10 min. The deproteinised supernatant was directly used for assaying oxidised glutathione.

Oxidised glutathione was determined after removing the reduced glutathione with N-ethyl maleimide (NEM). For removing reduced glutathione, 1 ml of the TCA

free extract was incubated with 1 ml of 0.04 M NEM in 0.1M sodium phosphate buffer pH 7.6 for 1 hour at 25^0 C. The NEM was removed by extracting 10 times with two volumes of diethyl ether and the residual ether was evaporated. The resultant solution contained GSSG.

The standard incubation mixture for estimation of oxidized glutathione contained 0.4 ml 0.1 M sodium phosphate buffer pH 7.6 containing 5 mM EDTA, 0.2 ml 6 mM DTNB, 0.2 ml 1mM NADPH and 2ml extract. The components were equilibrated in a cuvette for 100 seconds and the reaction initiated by the addition of 0.1 ml of yeast glutathione reductase. The change in optical density at 412nm was measured and was corrected for the reaction of DTNB with glutathione reductase in the absence of sample. 1 mol of GSSG is equivalent to 2 moles of GSH.

2.7.3 Estimation of inorganic phosphorous in latex

Inorganic phosphorus content of latex was estimated according to (Tausky and Shore, 1953)

Reagents

1. Sulphomolybdic acid reagent: In a 1000ml standard flask added 700ml-distilled water, 278-ml sulphuric acid, 100 gm ammonium molybdate. Made up the volume to 1000ml with distilled water. Kept in fridge.
2. Ferrous sulphate solution: At the moment of experiment 5.0 gm ferrous sulphate was dissolved in 50.0ml-distilled water. To this added 10.0ml of the above sulphomolybdic acid reagent and made up the volume to 100ml with water

3. Standard: KH2PO4 (~ 200 microgram/ml).

Estimation

Pipette out 0.5 ml of the TCA extract and made up to 2.0ml with 2.5 % TCA. Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0 ml standards and made up to 2.0ml with 2.5 %TCA. Added 2.0ml ferrous sulphate reagent to all the tubes and read the standards and tests at 740nm after 10 minutes.

2.7.4. Extraction and estimation of phenols in latex

Extraction: ~ 1 gm latex was extracted with 80% alcohol and kept at 80^0C for 30 minutes. Repeat the extraction 3-4 times and made up to a known volume and filtered. The filtrate was used for the estimation of phenols.

Reagents

1. Saturated sodium carbonate

2. Folins reagent (1N)

3. Catechol standard (10 mg catechol in 80% alcohol)

Estimation

Phenol content in latex was estimated by the method of (Swain and Hillis, 1959) 0.5 ml extracts were evaporated to dryness and added 0.5 ml distilled water and 0.5-ml folins reagent (1N) and 1.0 ml saturated sodium carbonate. The mixture was incubated for an hour after making final volume up to 10.0 ml with distilled water. Standards were also treated in the same way as tests. The optical density of standards and tests was measured at 725 nm against a blank with water and reagents

2.7.5. Extraction and estimation of proline in latex

Extraction

0.5 g latex was extracted with 5ml of 3% sulfosalysilic acid. The mixture was stirred to coagulate the rubber and the extracts were made up to a known volume and filtered. The filtrate was used for the estimation of proline.

Estimation

Latex proline was estimated by the method of (Bates *et al*, 1973)

Reagents

1.Glacial acetic acid

2. 6 M Orthophosphoric acid

3. Ninhydrin reagent

Acid ninhydrin was prepared by warming 1.25-g ninhydrin in 30.0-ml glacial acetic acid and 20.0 ml 6M ortho phosphoric acid. Kept cool (store at 4^0C).

4. Toluene

5. L–Proline standard (10mg L-proline was dissolved in 50 ml sulphosalicylic acid).

Estimation

2.0 ml filtrate was added to 2.0-ml ninhydrin reagent and 2.0 ml glacial acetic acid. Heated in boiling water bath for 1 hour and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4.0-ml toluene, mixed vigorously in a stirrer for 15-20 seconds. The chromophore containing toluene was aspirated from the aqueous phase, cooled to room temperature and the optical density read

at 520 nm using toluene as blank. The proline concentration was determined from a standard curve using L- proline.

2.7.6. Extraction and estimation of adenine nucleotides in latex

Adenine nucleotides in latex was extracted and estimated by the method of Amalou *et al.*, (1992)

Extraction

Reagents

1. 2.5% Trichloro acetic acid
2. Diethyl ether
3. 0.1 N KOH
4. 30mM HEPES buffer pH 7.4
5. Pyruvate kinase-PK; ATP: Pyruvate 2-O-phospho transferase (EC.2.7.1.40)

   One unit will convert 1.0 micromole of phospho enol pyruvate/min at pH 7.6 at 37°C.

6. Myokinase: (Adenylate kinase); ATP: AMP phosphotransferase (EC 2.7.4.3)

   One unit will convert 2 micromoles of ADP to ATP+AMP/min at pH 7.6 at 37^0C.

6. PK/LDH enzymes: solution in 50% glycerol containing 10mM Hepes pH 7.0,100 mM KCl, 0.1mM EDTA.

~1.0 gm latex was coagulated with 2.5%TCA. Coagulated rubber was rinsed twice with TCA and then dried (24 hr at 85^0C) to determine the sample dry rubber content (SDRC) of each latex sample. The TCA extracts were centrifuged at 7000

g for 10 min at 4^0C and TCA was removed by three successive extractions with cold ether. The residual ether was evaporated. The aqueous samples were neutralized with 0.1N KOH and the volume was adjusted to 10ml with 30mM Hepes buffer pH 7.4.

Estimation

ATP was quantified by a bioluminescence method using the ATP bioluminescent kit (FL-AA 89 H 9803, Sigma Chemical Company, USA) which is luciferin luciferase complex. To 100µl extract add 100µl assay mix from the kit and ATP was measured using a Luminometer (Stratec electronic GmbH, Brikenfeld, Germany). ADP and AMP were assayed by the same method after being phosphorylated to ATP using commercial pyruvate kinase and adenylate kinase respectively. Adenine nucleotide concentrations were expressed in µM in the whole latex based on the calculation

$$\text{Latex [AN]} = \text{ANs} \times \text{LDRC} \times \text{SDRC}^{-1}$$

Where latex [AN] is the concentration of a given adenine nucleotide in the whole latex expressed in µM in latex; ANs is the total amount of a given adenine nucleotide in the 10.0 ml latex sample extract (weight of latex) as expressed in µM; LDRC is the latex dry rubber content expressed in g L^{-1} and SDRC is the sample dry rubber content expressed in gram. The AEC is expressed according to the method of Hansen and Karl (1978).

$$\text{AEC} = \frac{[\text{ATP}] + 1/2\,[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]}$$

2.7.7 ATPase ACTIVITY OF LUTOIDS

ATPase activity of lutoids were measured according to Gidrol *et al*, (1988).

Reagents

1. 50mM Hepes-Mes Tris (pH 7.0)
2. 300mM mannitol
3. 5mM Mg SO_4
4. 0.1mM ammonium molybdate
5. 5mM ATP

Preparation of lutoid fraction and assay of ATPase.

The fresh latex was collected in glass vessels held in melting ice. The first 20.0ml were discarded in order to avoid possible bacterial contamination and damaged particle content. The fresh latex was then immediately centrifuged at 35000 g for 20 min at 4^0C. The supernatent serum (cytosol) and the poly isoprenoid particle fractions were discarded. The pellet resuspended in 5 volumes of a 50 mM Hepes-Mes-Tris (pH 7.0), 300mM mannitol buffer form the crude lutoid fraction. The crude lutoid fraction was washed three times with the same buffer. The sediment obtained by the centrifugation of the lutoid suspension at 35000g for 10minutes at 4^0c was resuspended in the ATPase buffer (50mM Hepes –Mes-Tris pH 7.0, 300mM mannitol, 5mM MgSO4, 0.1mM ammonium molybdate) in a final volume of 10 ml of 250mg fresh lutoids . Assay was performed in 2.5ml assay buffer with 10% lutoid. The reaction was started by the addition of 5mM ATP at pH 7.0. The incubation time was 10 min at 26^0C under continuous stirring. Then enzymatic

hydrolysis of ATP was stopped by adding ice cooled TCA at a final concentration of 0.5 mM. The Pi released was measured spectrophotometrically.

2.7.8. Estimation of total protein in C-and B-serum

Total soluble proteins in C- and B- serum were estimated by the method of (Lowry *et al*, 1951).

Reagents

1. A: 2% Sodium carbonate in 0.1 N NaOH
2. B: 0.5% Copper sulphate in 1% sodium potassium tartarate
3. Alkaline copper reagent C: 50.0 ml A + 1.0 ml B
4. Diluted folins reagent (1: 2)
5. 10.0% Trichloroacetic acid
6. Bovine Serum Albumin standard

Estimation

Proteins were precipitated from the serum (25µl each) by adding 5.0ml 10.0% TCA and centrifuged. The precipitate was dissolved in a known volume of 0.1 N NaOH. 0.5 ml sample and standards were then made upto 1.0 ml with 0.1N NaOH. A blank containing 1.0 ml 0.1N NaOH was taken. To all the tubes added 5.0-ml alkaline copper reagent. Kept for 10 min. Added 0.5ml diluted folins reagent. Mixed well and the tubes were kept for 30 min. The optical density was measured at 660 nm in a UV visible spectrophotometer. Bovine serum albumin was used as standard.

2.7.9 Electrophoresis of C-serum proteins using phast system (Pharmacia LKB Biotechnology)

Details of gels, separation and staining methods (Phast System Separation technique file. No. 111)

Phast Gel Homogeneous 12.5 % Gel:

These gels have a 13-mm stacking gel zone and a 32-mm separation gel zone. The concentration of polyacrylamide in the stacking and in the separation gel zone is

Stacking gel zone: T 6% C 3%

Separation gel zone: T 12.5% C 2%

The gels are approximately 0.45 mm thick and the buffer system in the gels is of 0.112-M acetate, 0.112 M Tris hydroxymethyl aminomethane, at pH 6.5.

For SDS- PAGE, gels were run with Phast gel SDS buffer strips, which contain 0.2 M Tris, 0.55 M SDS, pH 8.1. The buffer strips are made of 2% Agarose.

Separation method for SDS- PAGE with Phast gel homogeneous 12.5 % gel is as follows.

Sample application down at		0.0 Vh			
Sample application up at		0.0 Vh			
Separation	step 1. 250 V	10.0 mA	3.0 W	15^0 C	1 Vh
	step 2. 250 V	1.0 mA	3.0 W	15^0C	1 Vh
	step 3. 250 V	10.0 mA	3.0 W	15^0C	70 Vh

Fast Coomassie Staining of the Gel

Stock solution (0.2%): Dissolved one tablet of Phast gel blue R in 80.0 ml of

distilled water and stirred for 5-10 minutes. Added 120 ml of methanol and stirred for 2-3 min.

Stain: 0.1 % Phast gel blue R solution in 30 % methanol and 10 % acetic acid in distilled water.

Final solution: Mix 1 part of filtered stock solution with 1 part of 20 % acetic acid in distilled water.

Destaining solution: 30% methanol, 10% acetic acid in distilled water (3:1:6)

Preserving solution: 10.0 % glycerol and 10.0 % acetic acid in distilled water.

Coomassie blue staining method for SDS- PAGE

The gels were stained for 8 min in staining solution at 50^0C. Destained the gels using destaining solution as follows

Step 1. 5 min at 50^0C

Step 2. 8 min at 50^0C

Step 3. 10 min at 50^0C

Then preserved the gels in preserving solution. The relative intensities of the bands were quantified using the Image Master VDS system (Pharmacia Biotech)

Markers used (Sigma-marker wide range MW 6.5 –205 Kd)

Markers	MW (KD)
1. Aprotinin (bovine lung)	6.5
2. α-Lactalbumin	14.2
3. Trypsin Inhibitor (soybean)	2 0.0
4. Trypsinogen (bovine pancreas)	24.0

5. Carbonic anhydrase	29.0
6. Glyceraldehyde –3- phosphate dehydrogenase	36.0
7. Ovalbumin	45.0
8. Glutamic dehydrogenase	55.0
9. Albumin (bovine serum)	66.0
10. Fructose-6- phosphate kinase	84.0
11. Phosphorylase b	97.0
12. β- Galactosidase	116.0
13. Myosin	205.0

2.7.10 Eectrophoresis of B-serum proteins using Phast System (Pharmacia LKB Biotechnology)

Details of gel, separation and staining methods (Separation technique file. No.130)

Phast gel gradient (4-15 %)

The gel has a 13 mm stacking gel zone (4.5 % T, 3 % C) and a 32mm continuous 5-15 % gradient gel zone with a continuous 1-2 % gradient cross linker. The gel is 0.45 mm thick.

The buffer system in the gel is of 0.112 M acetate (leading ion), 0.112 M Tris pH 6.4 . The gel is run with Phast Gel SDS buffer strips, which contain 0.2 M tricine, 0.20 M Tris, 0.55 % SDS pH 8.1. The buffer strips are made of 3 % agarose.

Optimised method for SDS- PAGE with Phast Gel gradient (4 – 15) to programme into the separation file of Phast system.

Sample application down at	1.1				0.0 Vh
Sample application up at	1.2				1.0 Vh
SEP 1.1	250V	10.0 mA	3.0W	15°C	1.0 Vh
SEP 1.2	250V	1.0 mA	3.0 W	15°C	1.0 Vh
SEP 1.3	250V	10.0 mA	3.0 W	15°C	63.0 Vh

Staining of the gels was made using coomassie blue for 8 min at 50°C. Then destained the gel using destaining solution and preserved in preserving solution.

2.7.11. Glutamine synthatase activity in C-serum

Glutamine synthetase activity in C- serum was estimated by the method of (Shapiro *et al*, 1970)

Reagents

1. 0.1M Tris-HCl pH 7.7
2. 20mM glutamate
3. 10mM magnesium chloride
4. 2mM ATP
5. 2mM $(NH4)_2SO_4$
6. 100μM ammonium molybdate
7. Ferric chloride solution: (5g $FeCl_3$, 0.402 ml concentrated HCl and 1.634g TCA in 50ml water. The solution was filtered and used fresh.

8. Assay

Glutamine synthatase activity in C- serum (~50µl) was assayed at 30^0C in a medium containing 2.5 ml 0.1M Tris-HCl buffer pH 7.7 containing 20mM glutamate, 10mM $MgCl_2$, 2mM ATP, 2mM $(NH4)_2$ SO_4 and 100µM ammonium molybdate to inhibit latex phosphatase activity. Assays without glutamate and without enzyme solution were run as controls. Incubated test and controls at 37^0C for 15 min. 0.75ml $FeCl_3$ solution was added and OD read at 535nm against a blank without enzyme and without glutamate and corrections were made using blank. The enzyme activity was expressed as units/min/mg protein.

2.7.12 Glutathione reductase activity in C- serum

Glutathione reductase activity in C- serum was measured according to (Akerboon and Sies, 1981)

Reagents

1. 3.6mM NADPH
2. 7.6mM DTNB
3. 15mM oxidised glutathione
4. 0.1M Sodium phosphate buffer pH 7.5
5. Glutathione reductase standard (sigma)

Assay

Glutathione reductase activity was determined spectrophotometrically at 412nm by measuring the rate of reaction between glutathione and DTNB. Each analysis contained 50 µl serum, 0.1ml 7.6mM DTNB, 0.1ml 15mM oxidised glutathione

and 2.0 ml sodium phosphate buffer pH 7.5. The reaction was started by the addition of 0.1ml 3.6mM NADPH and the rate of reaction was monitored for 4 minutes. Purified glutathione reductase was used to generate standard calibration.

2.7.13. Superoxide dismutase activity in C- and B- serum

Superoxide dismutase activity in C- and B- serum was measured according to (Giannopolitis and Ries, 1977)

Reagents

1.Potassium phosphate buffer pH 7.8

2. Sodium carbonate (1.5M)

3. Methionine (0.13M)

4. Riboflavin (13 μM)

5. Nitroblue Tetrazolium (NBT) (0.63 mM)

6. Ethylene diamine tetra acetic acid (10.0 mM)

Assay

Test: 1.6 ml buffer was pipetted in test tubes and added 0.1 ml sodium carbonate, 0.3ml methionine, 0.3ml EDTA, 0.3ml NBT, 0.3ml riboflavin and 0.1 ml enzyme.

Control: 1.6 ml buffer was pipetted in test tubes and added 0.1 ml sodium carbonate, 0.3ml methionine, 0.3ml EDTA, 0.3ml NBT, 0.3ml riboflavin and 0.1 ml enzyme (without light).

Light control: 1.7 ml buffer was pipetted in test tubes and added 0.1 ml sodium carbonate, 0.3ml methionine, 0.3ml EDTA, 0.3ml NBT, 0.3ml riboflavin (without enzyme)

Test & light control kept under fluorescent light for 30 min. The OD of test, control and light control were read at 560 nm. (First read test, light control and then control)

Calculation

$$\text{SOD (units/mg protein)} = \frac{\text{100-OD sample x100/OD light control /mg protein}}{50}$$

OD sample= OD test-OD control

2.7.14. Peroxidase activity of C and B serum

Peroxidase activity of C and B serum was measured according to Guilbaut, (1976)

Reagents

1. 50 mM Phosphate buffer pH 7.0
2. 0.1mM H_2O_2
3. 20mM pyrogallol

Assay

The reaction mixture contained 2.5 ml phosphate buffer, 200µl pyrogallol and 100µl C serum. (For B- serum 50µl was used). The reaction was started by the addition of 50µl H_2O_2. The oxidation of pyrogallol was followed by monitoring the increase in absorbance at 430nm for four minutes. A blank without enzyme was taken as control and corrections were made. The activities were expressed in enzyme units/ mg protein where one enzyme unit was defined as the change in absorbance/min caused by the enzyme.

2.7.15 Catalase activity in C and B serum

Catalase activity in C and B serum was measured according to (Chance and Maehly, 1955)

Reagents

1.50mM phosphate buffer pH 7.0

2. 0.1mM H_2O_2

Assay

The reaction mixture contained 3.0 ml phosphate buffer and 100µl c or B- serum. The reaction was started by the addition of 50µl H_2O_2. The decrease in absorbance at 240 nm was recorded for four minutes. A blank without enzyme was taken as control and corrections were made. The activities were expressed as units/mg protein where one enzyme unit was defined as the change in absorbance/min caused by the enzyme.

2.7.16 β- 1,3 Glucanase activity in B- serum

β- 1,3 Glucanase activity in B- serum was measured acoording to the method of **Subroto** *et al.*, 1996)

Reagents

1. 50mM sodium acetate buffer pH 4.5
2. 0.5ml laminarin (1mg/ml in acetate buffer)
3. Copper reagent A

Dissolve 25g of anhydrous sodium carbonate, 25g sodium potassium tartarate (Rochelle salt), 20g sodium bicarbonate, 200g anhydrous sodium sulphate in

800ml water and diluted to 1 litre. Stored the reagent at room temperature. Filtered the solution before use.

4. Copper reagent B- 15% $CuSO_4.7H_2O$ containing one or two drops of concentrated H_2SO_4 in 100ml distilled water.

5. Arsenomolybdate colour reagent.

Dissolve 25g ammonium molybdate in 450ml distilled water, added 21ml of concentrated H_2SO_4 and mixed. 3.0g $Na_2HAsO_4.7H_2O$ was dissolved in 25ml water, both the solutions were mixed and placed in an incubator at 37^0 C for 24-48 hours or heated the solution to 55^0C for 25 min with continuous stirring. The solution was stored in brown bottle and kept at room temperature. 5ml

6. Glucose standard

Assay

β-1,3 Glucanase activity was measured in a reaction mixture contained 1.0ml 50mM sodium acetate buffer pH 4.5, 100µl b-serum and 0.5ml laminarin (1mg/ml in acetate buffer) as substrate. 0.5ml copper reagent was added and the mixture was incubated in a waterbath for 10 min at 37^0C. After cooling 0.5ml arsenomolybdate reagent was added and made up the solution to 12.5 ml with distilled water. The OD was measured at 540nm using glucose as standard. Enzyme activity was expressed as mg glucose liberated /min/mg protein.

2.7.17. N- acetyl glucosaminidase activity in B-serum

N- acetyl glucosaminidase activity in B-serum was measured according to Roger

Giordani, *et al.*, (1992).

Reagents

1. 0.1 M Succinate buffer pH 6.0
2. 0.02%Sodium azide
3. 2.5 mM p-nitrophenyl β-D- N acetyl glucosaminide (pNP-β-D-GlcNAc)
4. 0.2 M Sodium carbonate (Na_2CO_3)

Assay

The incubation medium used to study the enzyme activity contained 0.1M succinate buffer pH 6.0 (2.5 ml), 0.05ml sodium azide, 0.2 ml pNP-β-D-GlcNAc and 50 µl B-serum at 30^{0}C. After incubation 0.25 ml of 0.2 M Na_2 CO_3 was added to stop the reaction and the absorbance of p-nitrophenol formed was read at 400 nm and compared with a control sample containing only substrate. The specific activity of enzyme was defined as the amount of p-nitrophenol liberated/min/mg protein.

2.7.18 Chitinase activity in B-serum

Chitinase activity in B-serum was measured according to Pedraza-Reyas and Lopez-Romero (1991).

Reagents

1. 50 mM phosphate buffer
2. 1mM Magnesium chloride
3. Chitin azure

Procedure

100µL B-serum was mixed with 1.0 mg chitin azure in 0.5 ml 50 mM phosphate

buffer pH 6.5 with 1mM $MgCl_2$. The reaction mixture was incubated at 30^0C with gentle shaking for 48 hours followed by centrifugation at 14000 g for 4 min. The absorbance at 575 nm of the supernatant fluid was then measured. Chitinase activity was defined as the amount of protein that results in an increase of 0.01 A_{575} units under the above conditions.

2.7.19. Hevein content of B-serum (Ukun et al., 1999)

Lyophilised B-serum was suspended in water to which 0.5g/l sodium thionite was added. After centrifugation, solutions were 100% saturated with ammonium sulphate and centrifuged again. Precipitates were dissolved in small volumes of 0.2 M acetic acid and submitted to gel-filtration on a column of Sephadex G-25. 5mg protein was applied to the column and eluted with 1.2 M acetic acid. The fractions were collected at the rate of 3.0 ml/minute and optical density was measured at 280 nm.

2.7.20. Extraction of bark samples for biochemical analysis

Soft bark tissues were extracted in 80 % alcohol for phenol, in 2.5 % TCA for thiols and in 0.1 M sodium phosphate buffer pH 7.6 for proteins. Then the estimations were made.

2.7.21. Statistical Analysis

1. Analysis of variance
2. Covariance analysis
3. Regression analysis

CHAPTER-3

RESULTS

3.1 Seasonal variations in physiological and biochemical parameters associated with latex flow, cessation of flow and production.

Results of the study conducted in clone RRII 105 under ½ S d/2 6d/7 tapping system are presented in this chapter. Analysis of variance was carried out to study the seasonal differences in dry rubber yield and physiological parameters in latex. September- November-1999 was taken as the peak yielding season and February – April as stress season. Regression analysis was carried out to study the relationship of these parameters with yield using the pooled data of one year. Independent t test was used to study the seasonal variations in latex flow characteristics and biochemical parameters

3.1.1 Monthly variations in yield and physiological parameters

Monthly variations in dry rubber yield and physiological parameters of these trees are presented in figures 1-6. Yielding pattern showed a high yielding trend during August- November and a low yielding trend during February- April. High yield was also observed during December and January, which was the defoliation-refoliation period (Fig-1). Phenol content in latex showed a wide range of variation with maximum values at the end of December every year and minimum values during February (Fig-5). Maximum proline content was observed during January every year (Fig-6).

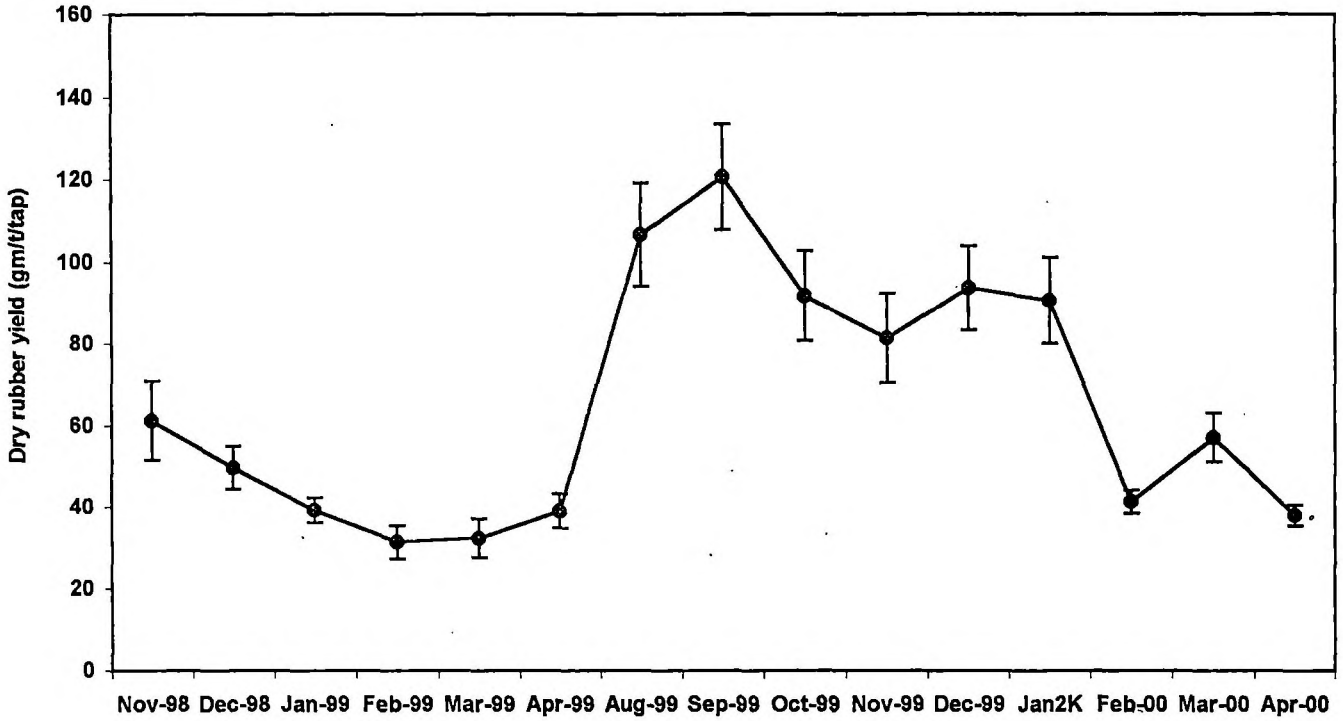


Figure-1. Monthly variations in dry rubber yield of newly opened trees of clone RRII 105 under1/2 S d/2 6d/7 tapping system

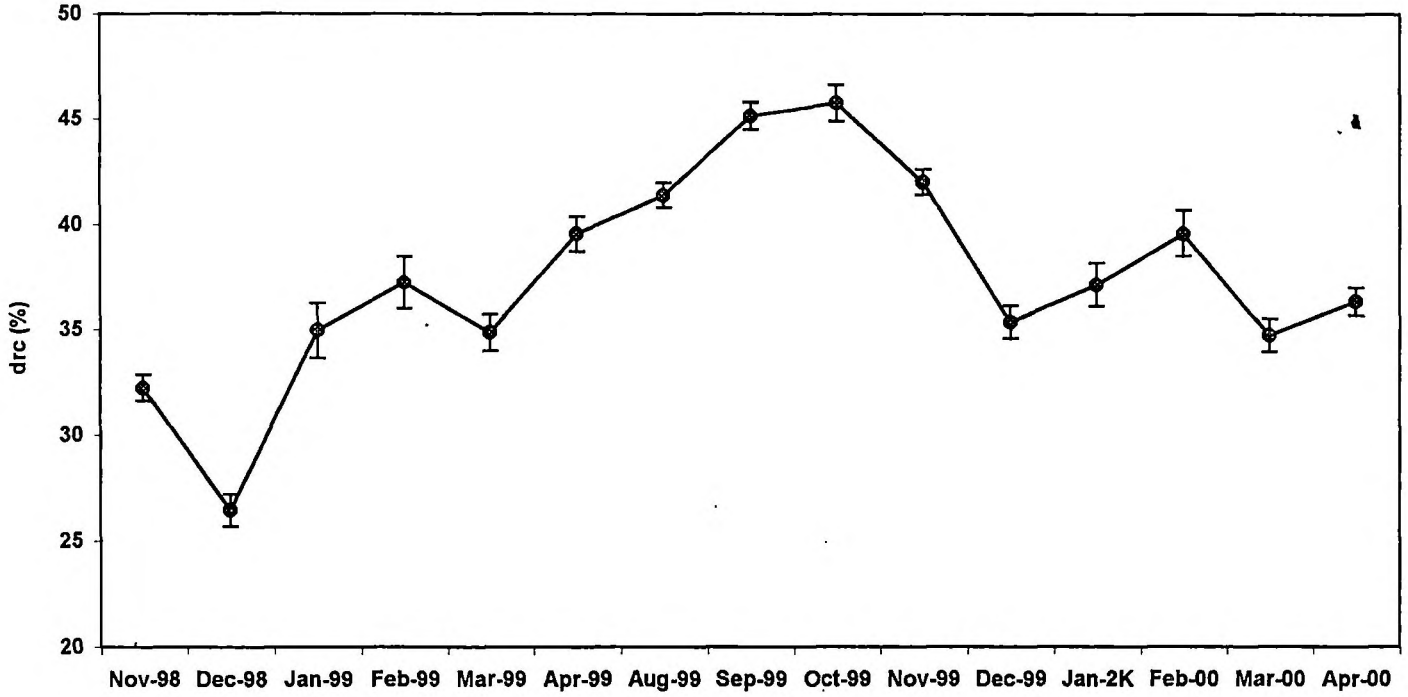


Figure-2 Monthly variations in dry rubber content in newly opened trees of clone RRII 105 under 1/2 S d/2 6 d/7 tapping system

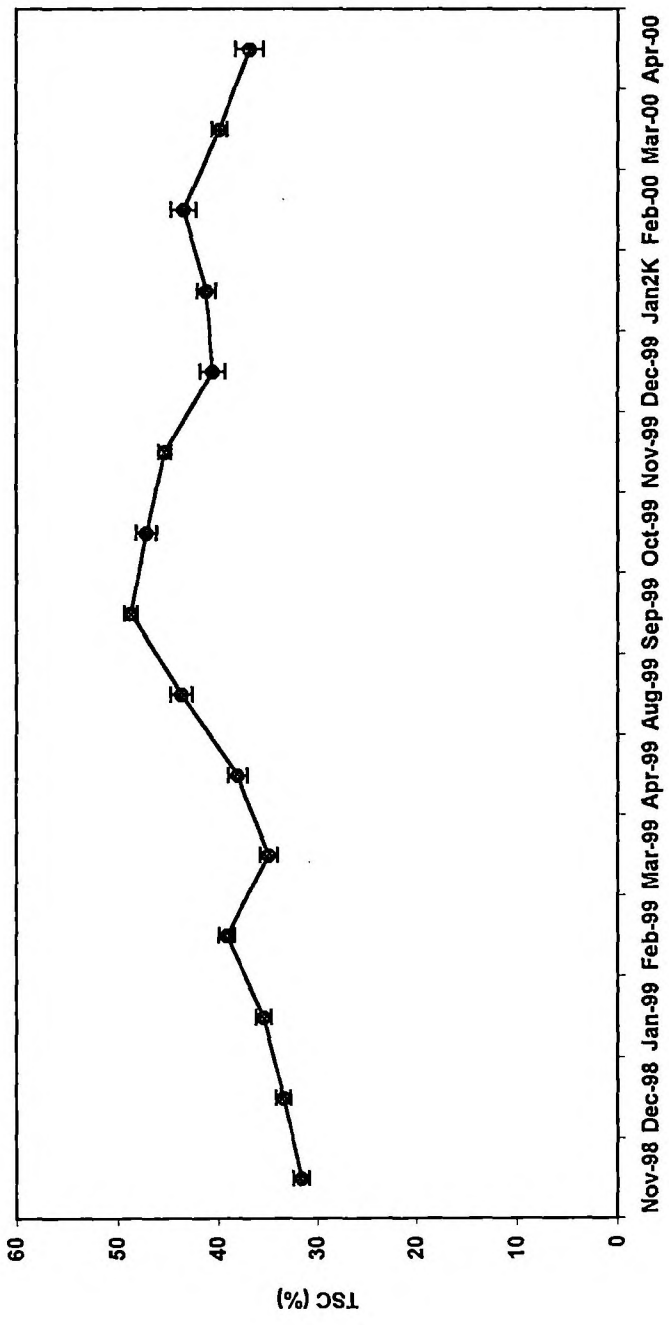


Figure-3: Monthly variations in total solid content in newly opened trees of clone RRII 105 under 1/2 S d/2 6 d/7 tapping system

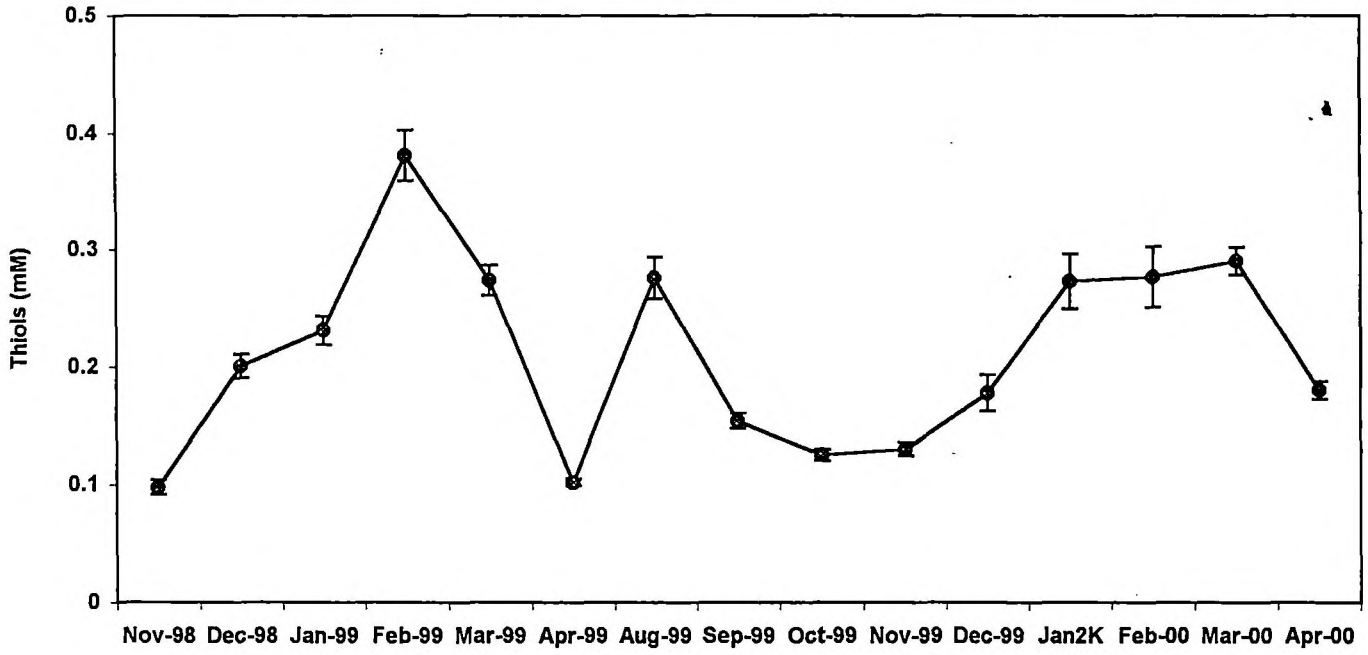


Figure-4 Monthly variations in latex thiols in newly opened trees of clone RRII 105 under 1/2 S d/2 6d/7 tapping system

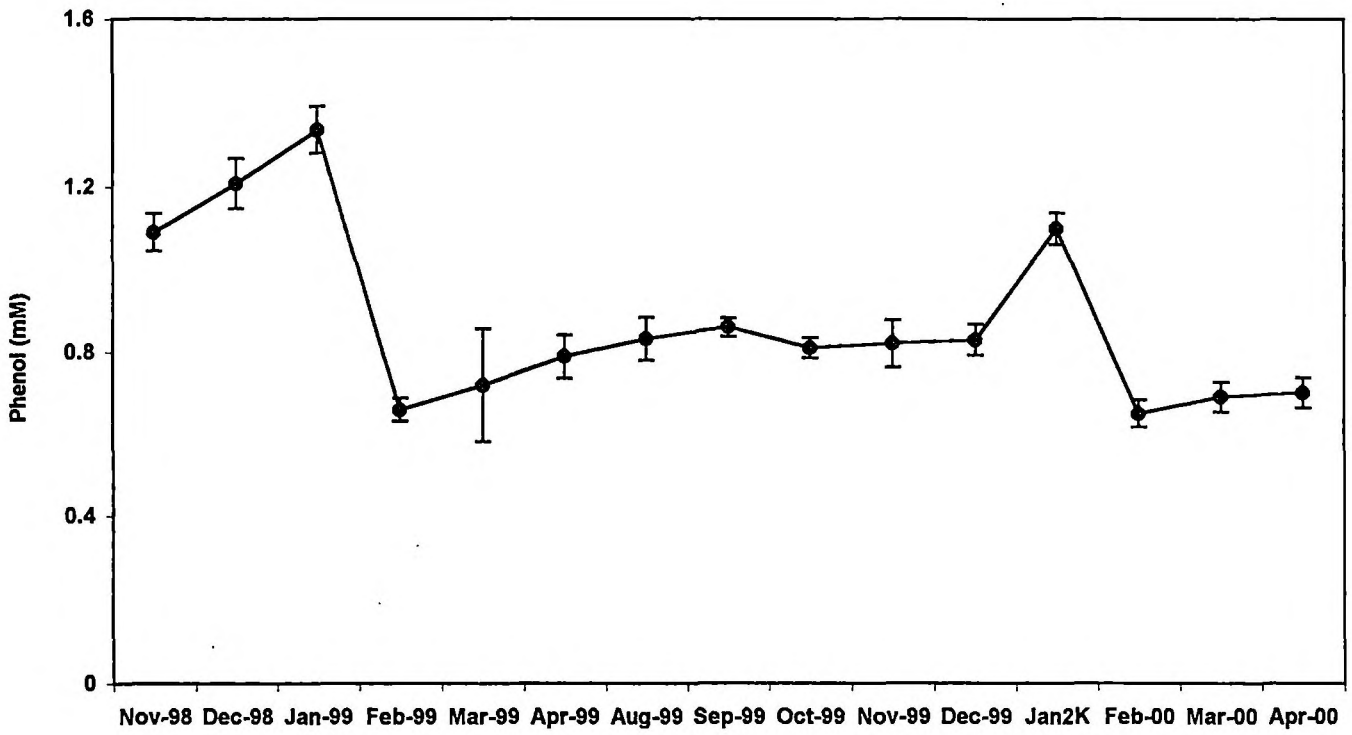


Figure- 5 Monthly variations in latex phenol in newly opened trees of clone RRII 105 under 1/2 S d/2 6 d/7 tapping system

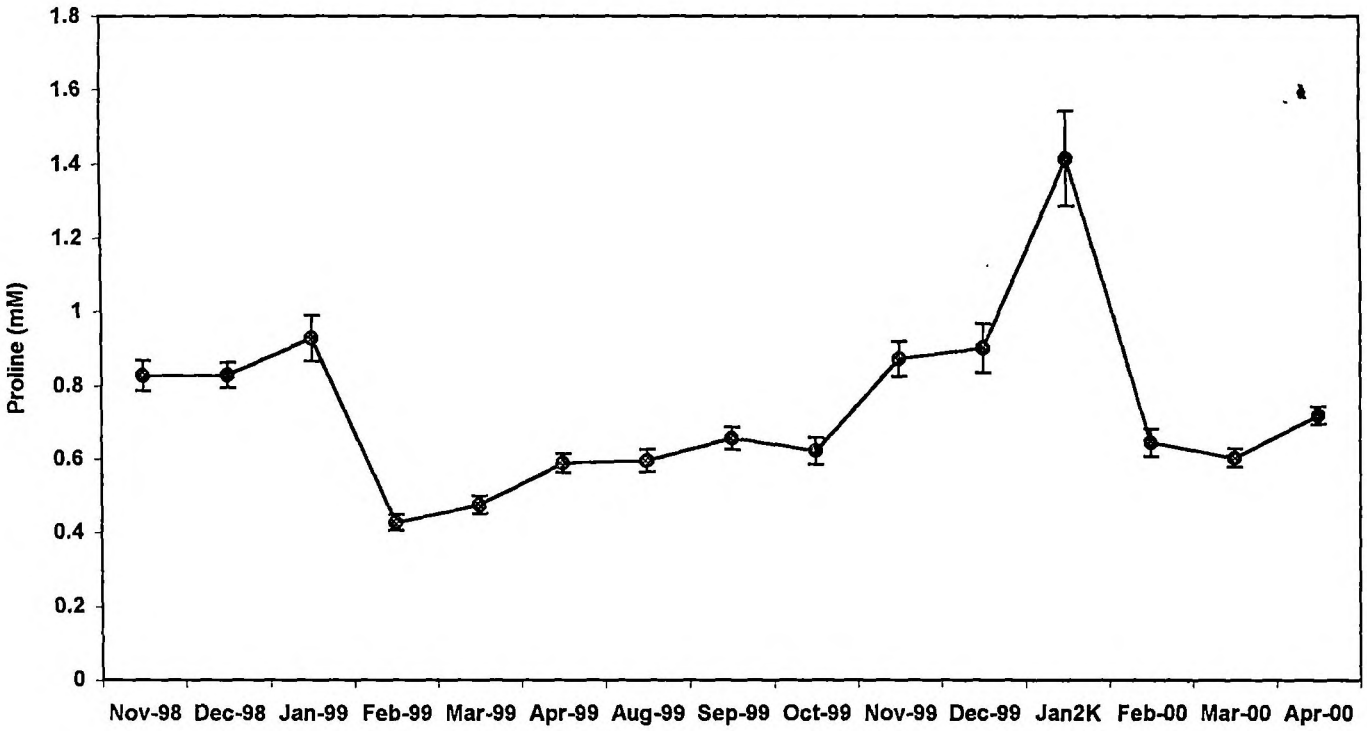


Figure-6. Monthly variations in latex proline in newly opened trees of clone RRII 105 under 1/2 S d/2 6d/7 tapping system

3.1.2 Seasonal variations in yield and physiological parameters

The results are presented in Table-1. Analysis of variance of the data showed significant differences in dry rubber yield and various physiological parameters. A significant reduction in dry rubber yield was observed during stress when compared to peak yielding period. TSC, DRC, Pi and phenol in latex were also reduced. Reduced thiols showed an increase during stress period. Proline content in latex did not show any significant difference between seasons.

Table-1 Seasonal variations in yield and latex physiological parameters in clone RRII 105 under 1/2S d/2 6d/7 system of tapping

Parameter	Peak season Sep-Oct-Nov (1999)	Stress season Feb-Mar-Apr (2000)	Significance
Yield ($g\ tree^{-1}\ tap^{-1}$)	93.27	45.36	12.11 **
Dry rubber content (%)	43.03	35.29	3.09 *
Total solid content (%)	46.12	39.66	2.66 *
Total thiols (mM)	0.13	0.25	0.03 **
Inorganic phosphorus (mM)	13.9	9.2	1.9 *
Phenol (mM)	0.82	0.70	0.07 **
Proline (mM)	0.63	0.65	0.06 ns

** CD (P=0.01) * CD (P=0.05) ns- not significant

3.1.3 Relationship between yield and physiological parameters

Results of the regression analysis of physiological parameters with dry rubber yield are presented in Table-2. Thiols, inorganic phosphorus and phenols in latex showed significant positive correlation with yield. Proline content in latex showed a negative correlation. TSC did not show any correlation with yield.

Table-2. Regression analysis of yield and latex physiological parameters in clone RRII 105 under ½S d/2 6d/7 system of tapping (pooled data of one year)

Variable	Coefficient	Standard error	T-Stat	
Constant (Yield)	10.942	82.071	0.1333	
Thiols	166.80	71.687	2.326	*
Inorganic phosphorus	3.48	1.049	3.318	**
Phenols	111.93	35.300	3.170	**
Proline	-63.558	22.893	-2.776	**
Total solids	0.137	1.640	0.083	ns

3.1.4 Seasonal variations in latex flow characteristics

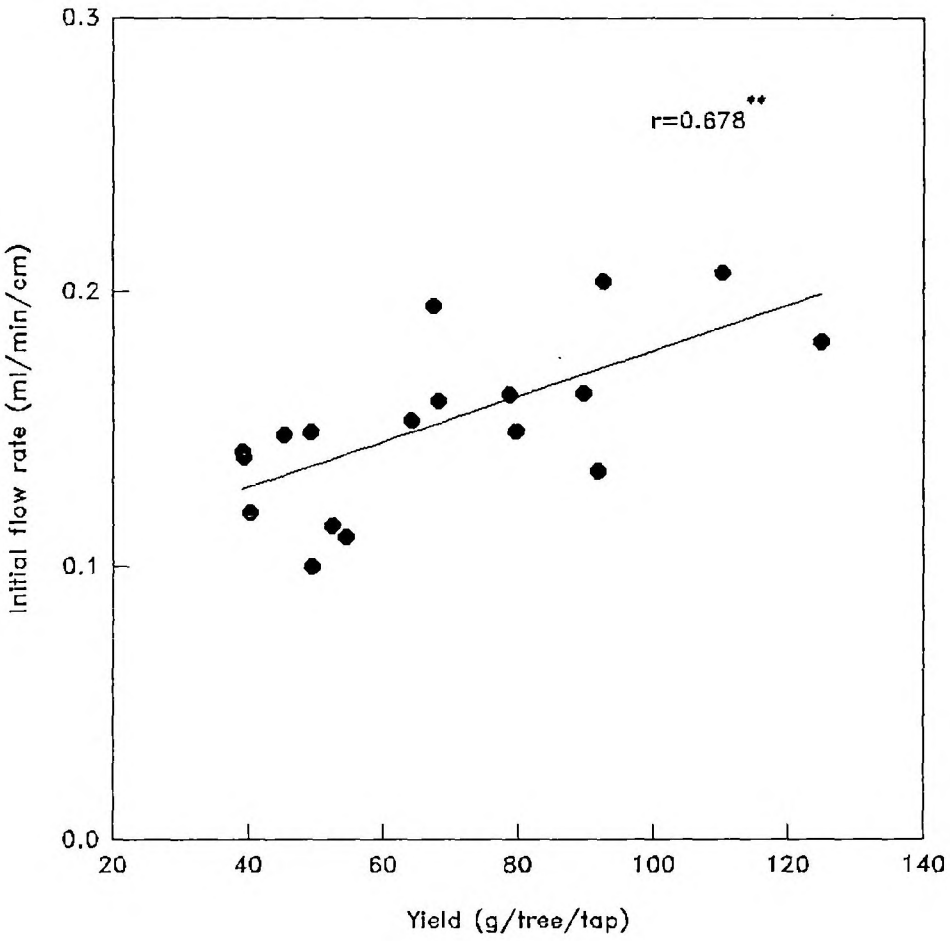
The results are presented in Table-3. A low initial flow rate of latex and significantly high plugging and bursting index was observed during stress season when compared to peak season. Pre tapping latex vessel turgor pressure did not show any significant variation between seasons.

Table-3. Seasonal variations in latex flow characteristics in clone RRII 105 under 1/2S d/2 6d/7 system of tapping

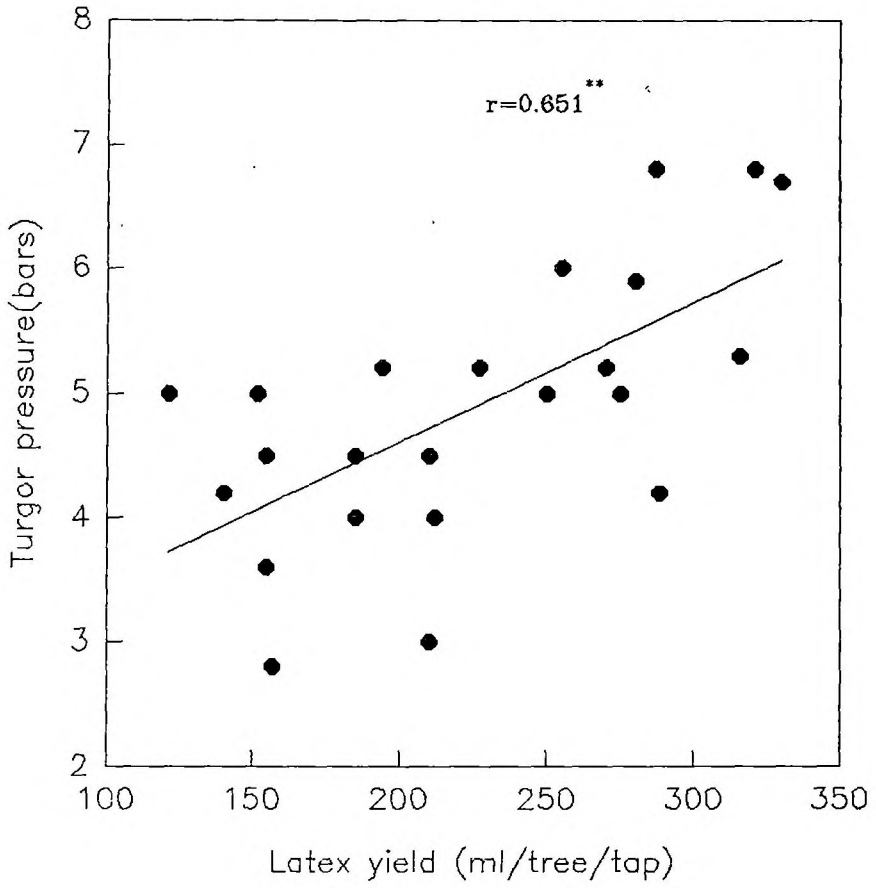
Parameter	Peak season (Sep-Oct-Nov 1999)	Stress season (Feb-Mar-Apr 2000)	Significance
Initial flow rate (ml min^{-1} cm^{-1})	0.096 ± 0.0049	0.064 ± 0.0068	*
Turgor pressure (bars)	4.5 ± 0.36	5.6 ± 0.26	ns
Plugging Index	3.27 ± 0.21	6.72 ± 0.42	*
Bursting Index	6.79 ± 0.38	12.93 ± 0.82	*

*significant at $p \leq 0.05$ ns-not significant

Relationships of these parameters with yield are presented in Figures 7-10. IFR and TP showed a positive correlation and PI and BI showed a negative correlation with yield.



Figure–7. Relationship between initial flow rate and yield in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak season)



Figure–8. Relationship between latex yield and turgor pressure in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak yielding season)

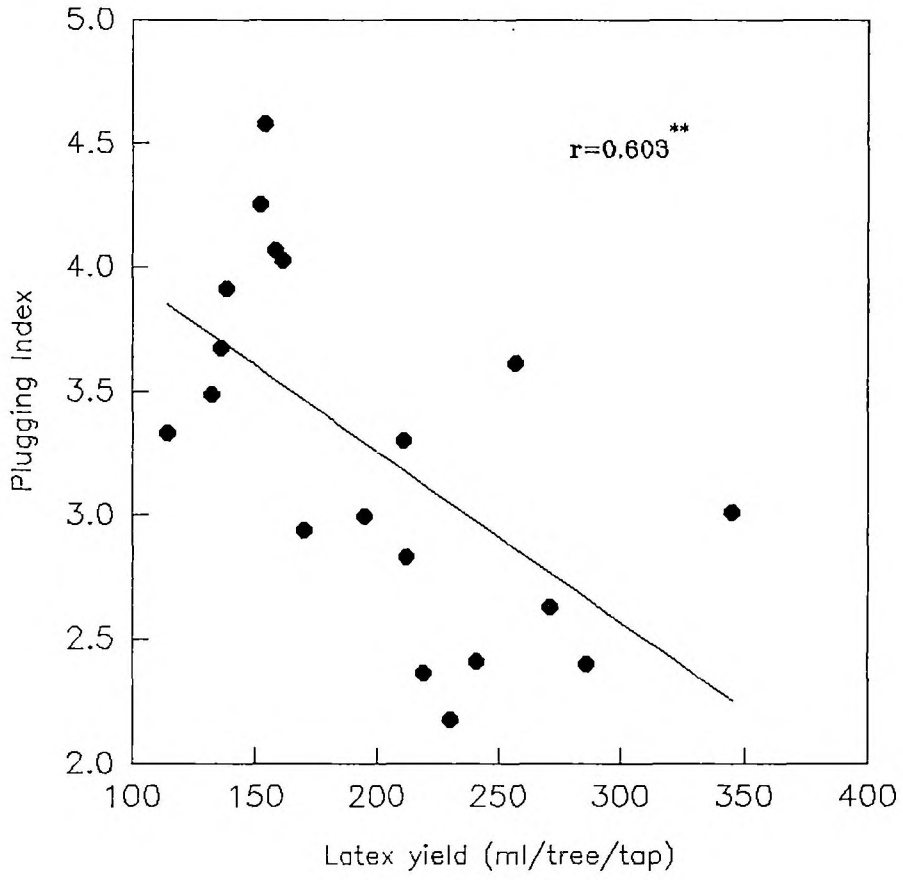


Figure-9 Relationship between latex yield and plugging index in clone RRII 105 under 1/2 S d/2 6 d/7 tapping system (peak yielding season)

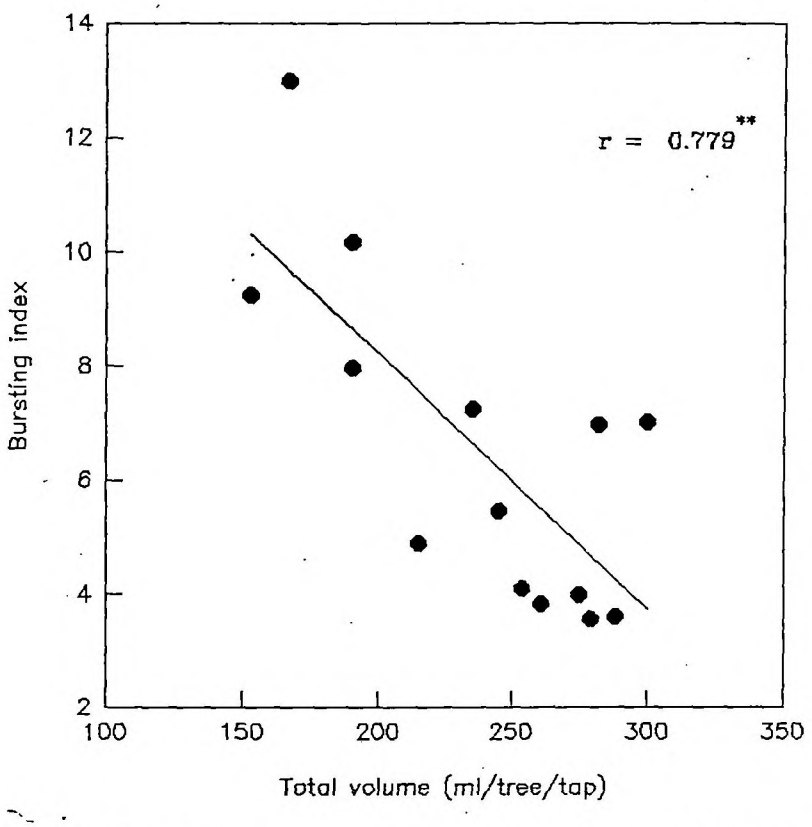


Figure-10. Relationship betwen total volume and bursting index in clone RRII 105 under 1/2 S d/2 6 d/7 tapping system (peak yielding season)

3.1.5 Seasonal variations in thiols

The results are shown in Table-4. A significant increase in reduced glutathione and glutathione reductase activity was observed in C- serum during stress period when compared to peak yielding season. Oxidised glutathione showed an increase during stress but not statistically significant. GSH/GSSG ratio was also high during stress period. A positive correlation between yield and thiols and glutathione reductase in C- serum was observed (Fig-11 &12).

Table-4. Seasonal variations in glutathione pool and glutathione reductase activity in C-serum of clone RRII 105 under 1/2S d/2 6d/7 system of tapping.

Parameter	Peak season Sep-Oct-Nov (1999)	Stress season Feb-Mar-Apr (2000)	Significance
Oxidised glutathione (GSSG) (mM) equivalent of GSH	0.045±0.003	0.06±0.008	ns
Reduced glutathione (GSH) (mM)	0.35±0.041	0.55±0.036	*
Glutathione reductase (units min^{-1} mg $protein^{-1}$)	0.81±0.07	1.12 ±0.04	*
GSH/GSSG	7.8 ±0.331	9.1 ± 0.495	*

*significant at $p \leq 0.05$ ns-not significant 1 mole of GSSG= 2 moles of GSH

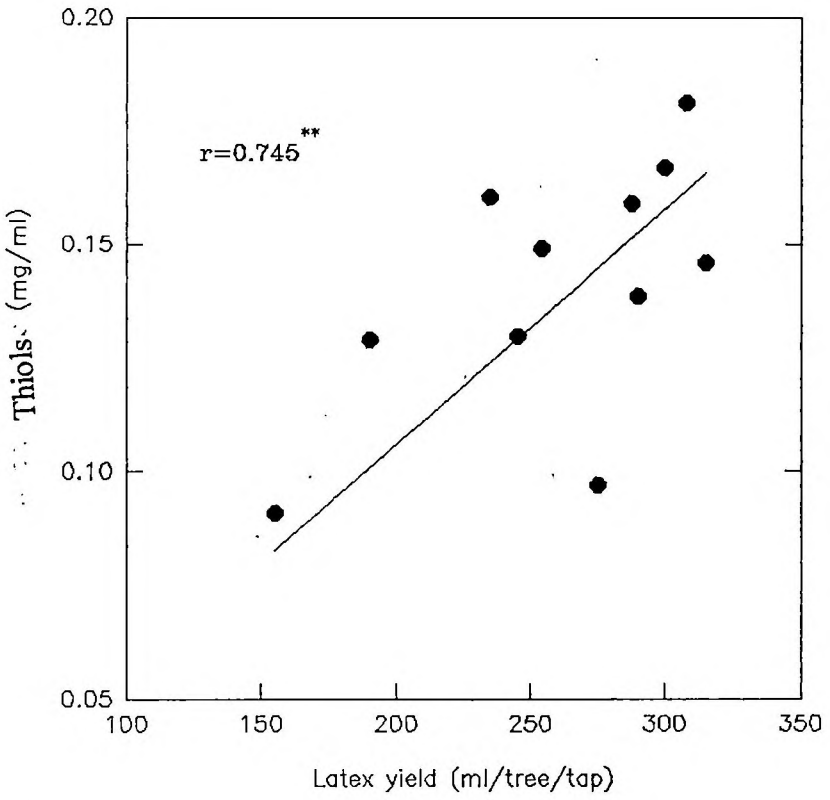


Figure-11. Relationship between latex yield and c-serum thiols in clone RRII 105 under 1/2S d/2 6 d/7 tapping system (peak season)

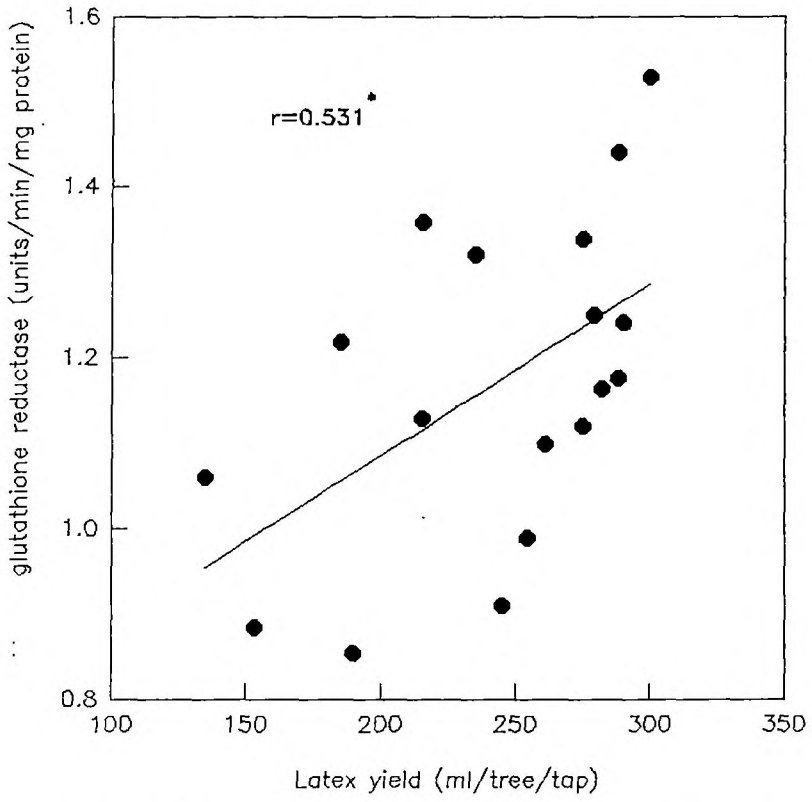


Figure-12.Relationship between latex yield and c-serum glutathione reductase activity in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak yielding season)

3.1.6 Seasonal variations in energy metabolism

The results are shown in Table-5. The ATP and ADP content of latex were significantly low during stress period when compared to peak season. But AMP content of latex increased during stress. There was no significant difference between ATP/ADP ratios. The adenylate energy charge of the system also showed a decrease during stress period..

Table-5. Seasonal variations in the adenylate pool in latex and adenylate energy charge in clone RRII 105 under 1/2S d/2 6d/7 system of tapping.

Parameter	Peak season Sep-Oct-Nov (1999)	Stress season Feb-Mar-Apr (2000)	Significance
ATP (μM gm fresh wt^{-1})	244.8±23.65	168.87±33.48	*
ADP (μM gm fresh wt^{-1})	234.4±27.62	189.4±34.4	*
AMP (μM gm fresh wt^{-1})	156.8±23.31	281.6±53.3	*
ATP/ADP	1.04±0.012	0.89±0.027	ns
AEC	0.58±0.03	0.41±0.06	*

*significant at $p \leq 0.05$ ns- not significant

3.1.7. Seasonal variations in antioxidant enzymes

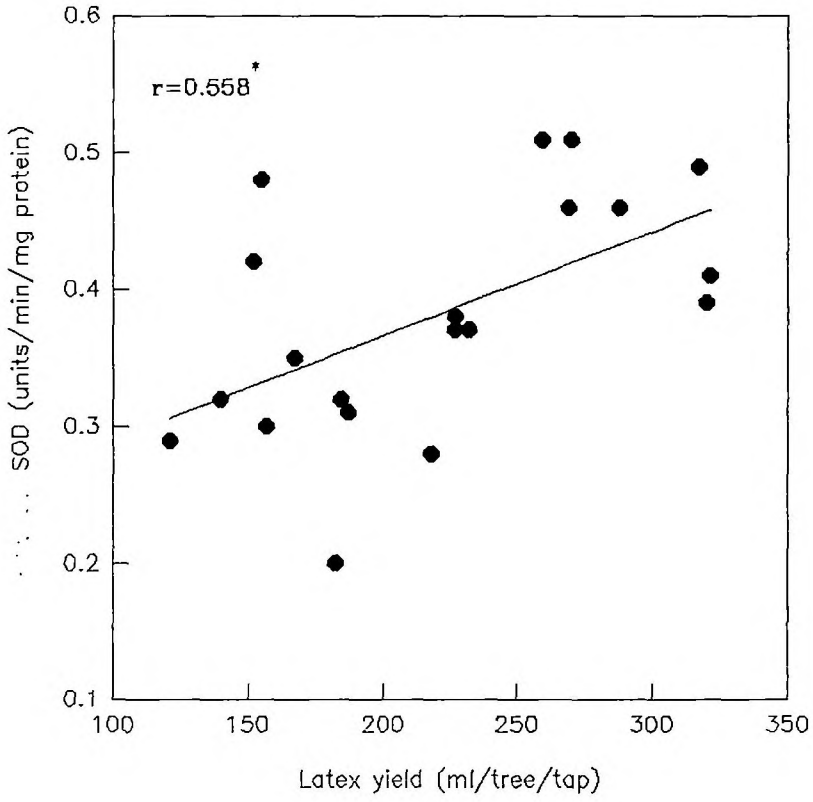
The variations in antioxidant enzymes are presented in Table-6. A significant increase in the activities of peroxidase in C- and B- serum was observed during stress period when compared to peak yielding season. SOD activity of C- and B- serum and catalase activity of C- serum decreased during stress period. Catalase activity in B- serum did not show any significant change between seasons.

Positive correlation between yield and SOD in C & B- serum was observed (Fig-13&14)

Table-6. Seasonal variations in antioxidant enzymes in clone RRII 105 under 1/2S d/2 6d/7 system of tapping.

Enzymes (units min^{-1} mg $protein^{-1}$)	Peak season Sep-Oct-Nov (1999)	Stress season Feb-Mar-Apr (2000)	Significance
Peroxidase (C-serum)	0.196±0.008	0.28±0.034	*
Peroxidase (B-serum)	0.07±0.004	0.12±0.008	*
Catalase (C-serum)	0.19±0.009	0.16±0.002	*
Catalase (B-serum)	0.22±0.028	0.23±0.014	ns
SOD (C-serum)	0.48±0.053	0.27±0.036	*
SOD (B-serum)	0.82±0.077	0.57±0.068	*

*significant at $p \leq 0.05$ ns- not significant



Figure–13 Relationship between latex yield and c–serum SOD in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak season)

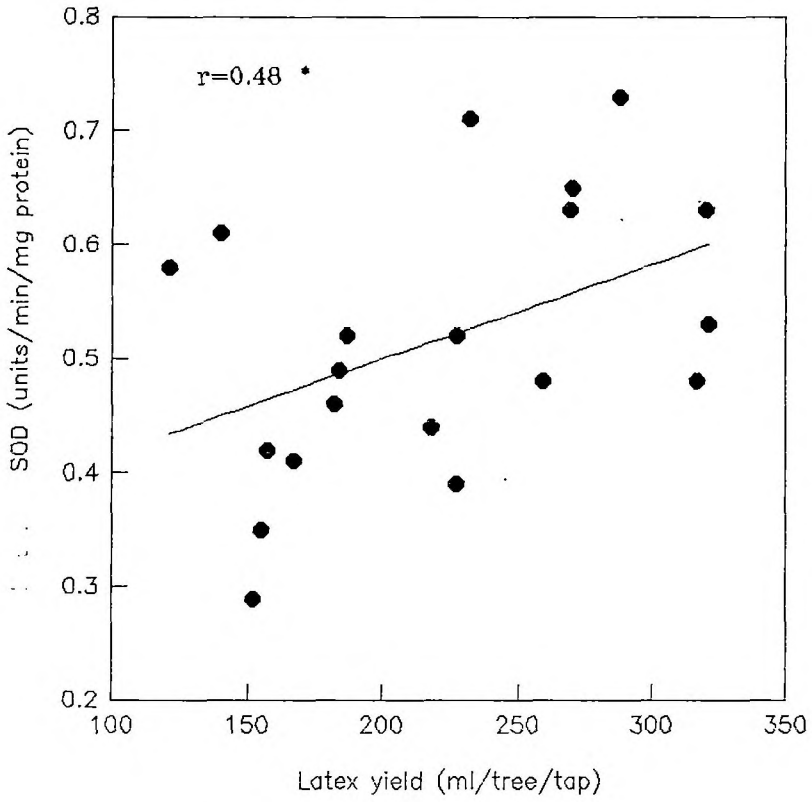


Figure-14 Relationship between latex yield and b-serum SOD in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak yielding season)

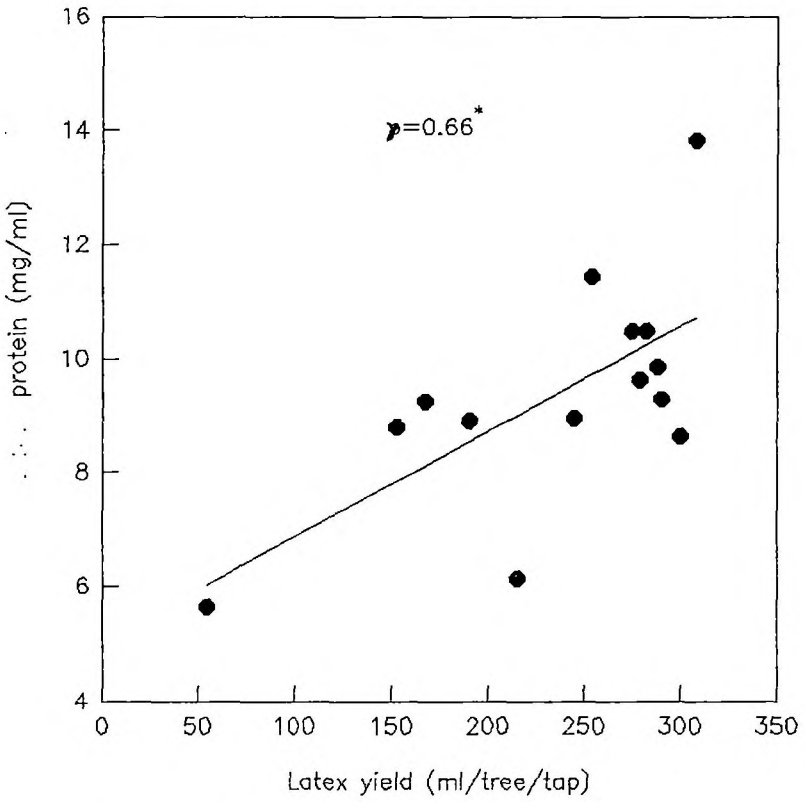
3.1.8 Seasonal variations in proteins and glutamine synthetase activity

The results are shown in Table-7. Protein content of C- and B- serum showed an increase and glutamine synthetase activity showed a decrease during stress period when compared to peak yielding period. Prorein content of C- serum and Glutamine synthetase activity showed a positive correlation with yield (Fig-15&16). No significant correlation was observed between B- serum protein and yield (Fig-17).

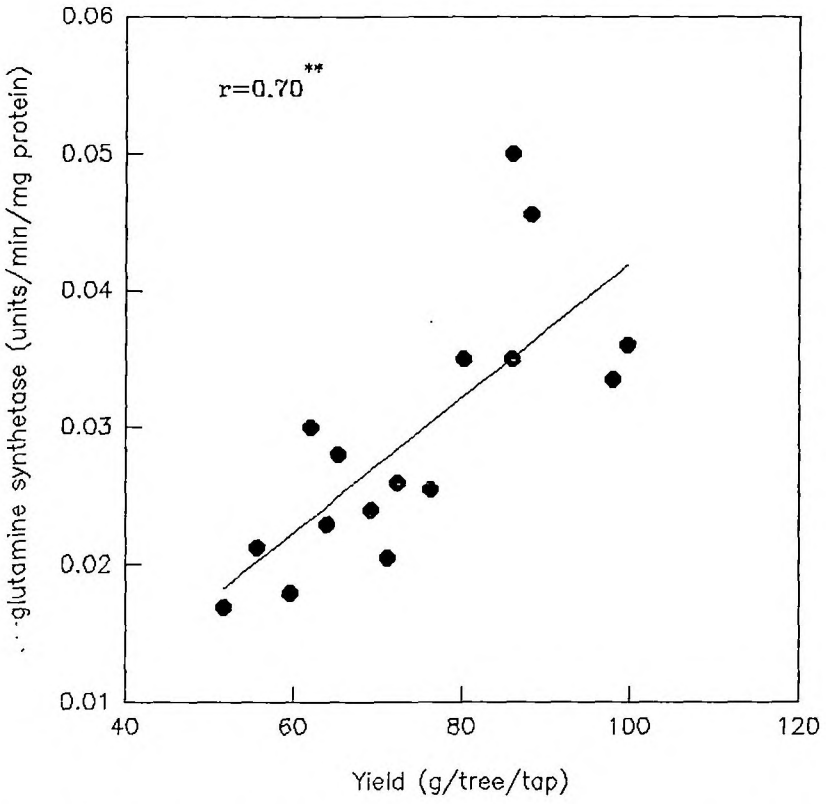
Table-7. Seasonal variations in protein and glutamine synthetase activity in clone RRII 105 under 1/2S d/2 6d/7 system of tapping.

Parameter	Peak season Sep-Oct-Nov (1999)	Stress season Feb-Mar-Apr (2000)	Significance
Protein (C-serum) (mg ml^{-1})	8.54±0.41	11.01±0.48	*
Protein (B-serum) (mg ml^{-1})	16.68±0.84	19.71±0.46	*
Glutamine synthetase activity (C-serum) (units min^{-1} mg $protein^{-1}$)	0.034±0.0013	0.011±0.0011	*

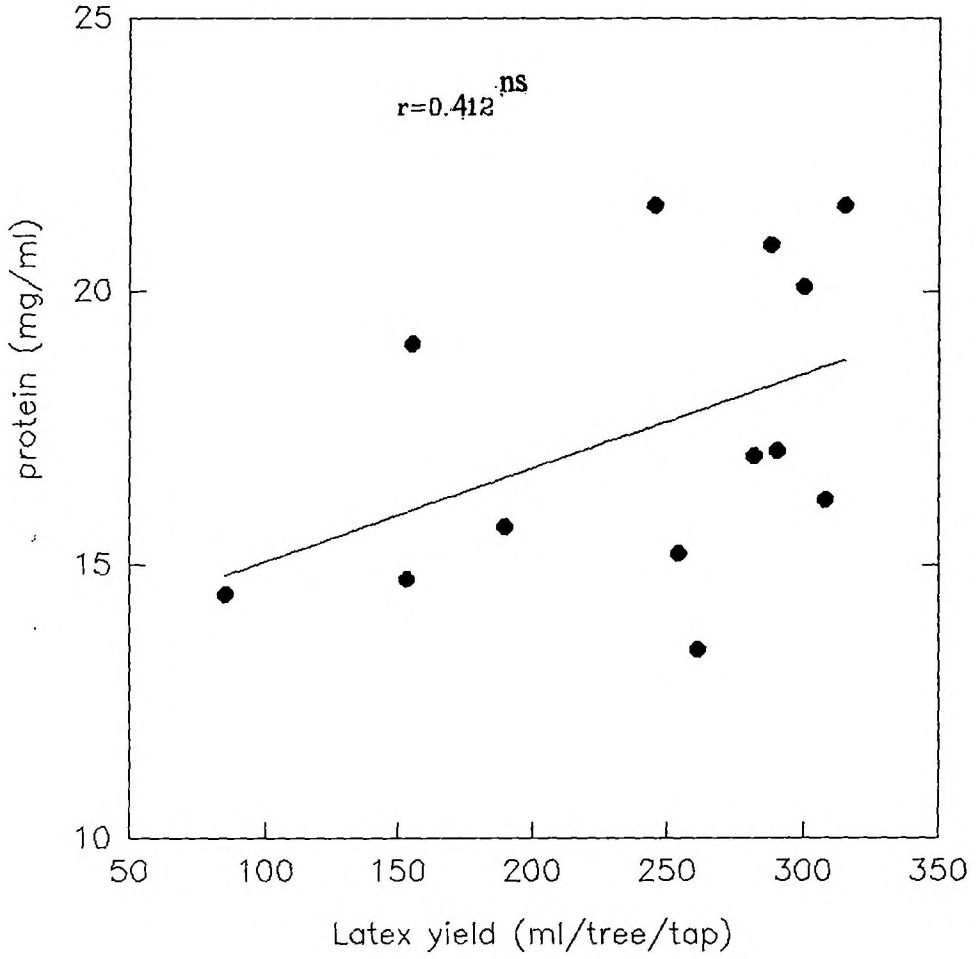
*significant at $p \leq 0.05$ ns- not significant



Figure–15 Relationship between latex yield and c– serum protein in clone RRII 105 under 1/2 S d/2 6 d/7 tapping system (peak yielding season)



Figure–16 Relationship between yield and c–serum glutamine synthetase in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak yielding season)



Figure–17 Relationship between latex yield and b–serum protein in clone RRII 105 under 1/2 S d/2 6d/7 tapping system (peak yielding season)

3.1.9 Seasonal variations in enzymes associated with cessation of latex flow

The results of variations in enzymes are presented in Table-8. A significantly high β- 1,3 glucanase and a low N- acetyl glucosaminidase activities were observed during stress period. Chitinase activity did not show any significant change between seasons.

Table-8. Seasonal variations in enzymes associated with cessation of latex flow in clone RRII 105 under 1/2S d/2 6d/7 system of tapping.

Parameter	Peak season Sep-Oct-Nov (1999)	Stress season Feb-Mar-Apr (2000)	Significance
β- 1,3 glucanase (B-serum) (mg glucose min^{-1} mg protein^{-1})	5.3±0.67	7.95±0.48	*
N-acetyl glucosaminidase (B-serum) (mg p-nitrophenol min^{-1} mg protein^{-1})	0.47±0.036	0.30±0.02	*
Chitinase (B-serum) (units min^{-1} ml B-serum	2.7±0.135	2.13±0.10	ns

*significant at $p \leq 0.05$ ns- not significant

3.1.10. Hevein content of B-serum

A higher hevein content was observed during stress when compared to peak period (Fig-18)

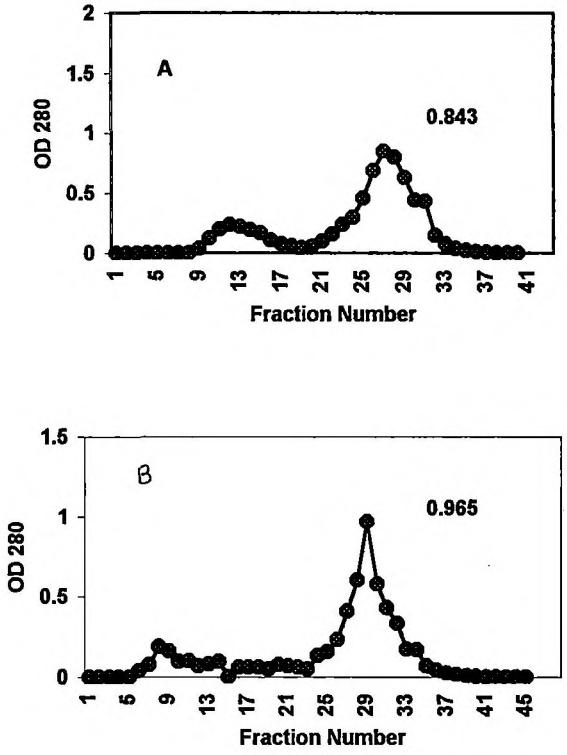


Fig- 18. Gel filtration of B-serum of clone RRII 105 (A) Peak season (October 1999) and (B) stress season (March 2000). Protein content of 5.00mg on a sephadex G-25 column (3x50 cm). Elution with 1.2 M acetic acid. Fractions of 3.0ml were collected.

3.2. Variations in yield, physiological and biochemical parameters associated with different tapping frequencies in clone RRII 105.

The results of the study conducted in clone RRII 105, tapped under high (1/2 S d/2 6d/7) and low (1/2S d/4 6d/7) frequency tapping systems are presented in this chapter. The data of peak yielding period was used to study the variations. Co-variance analysis was used to compare yield and physiological parameters. Independent t test was used for comparing data on latex flow characters and biochemical parameters.

3.2.1. Monthly variations in yield and physiological parameters

Monthly variations in yield and physiological parameters are presented in Fig-19-24.

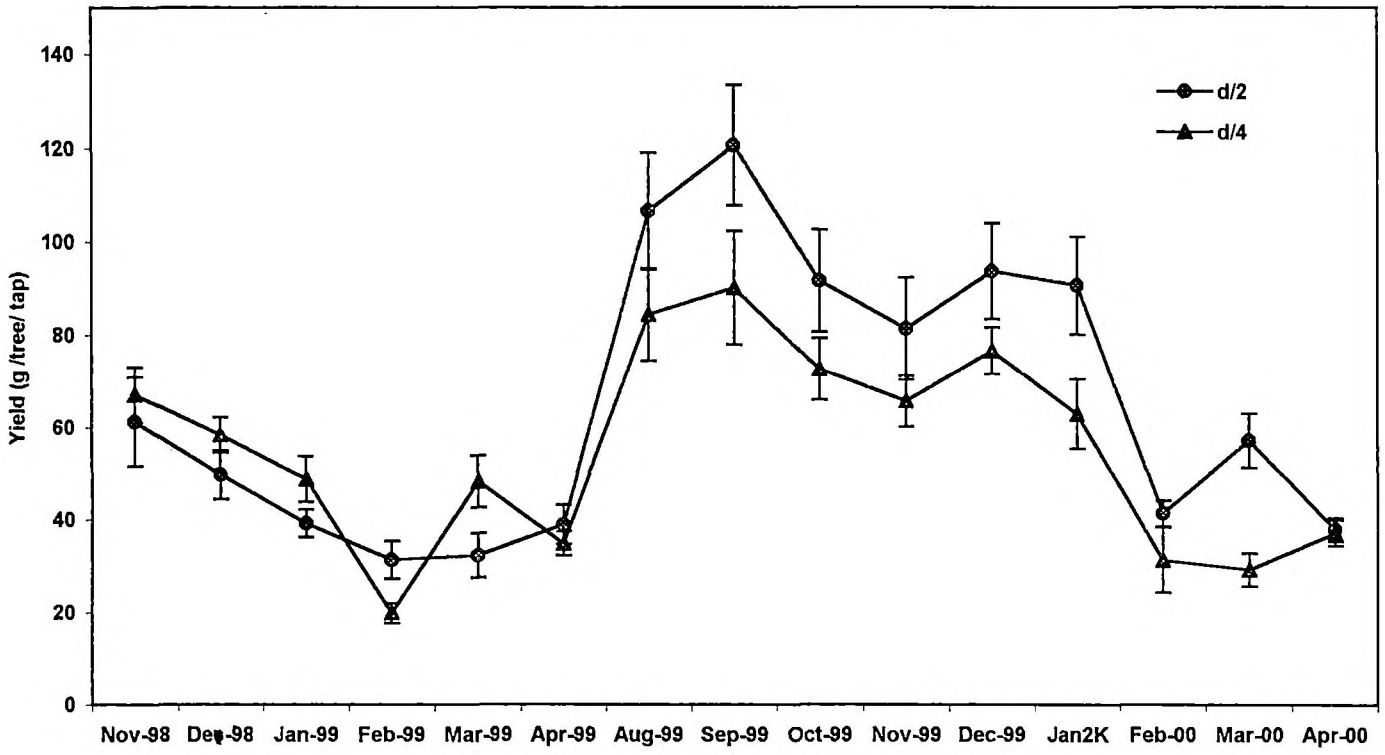


Figure-19 Monthly variations in dry rubber yield in newly opened trees of clone RRII 105 under 1/2 S d/2 6d/7 and 1/2 S d/4 6d/7 tapping systems

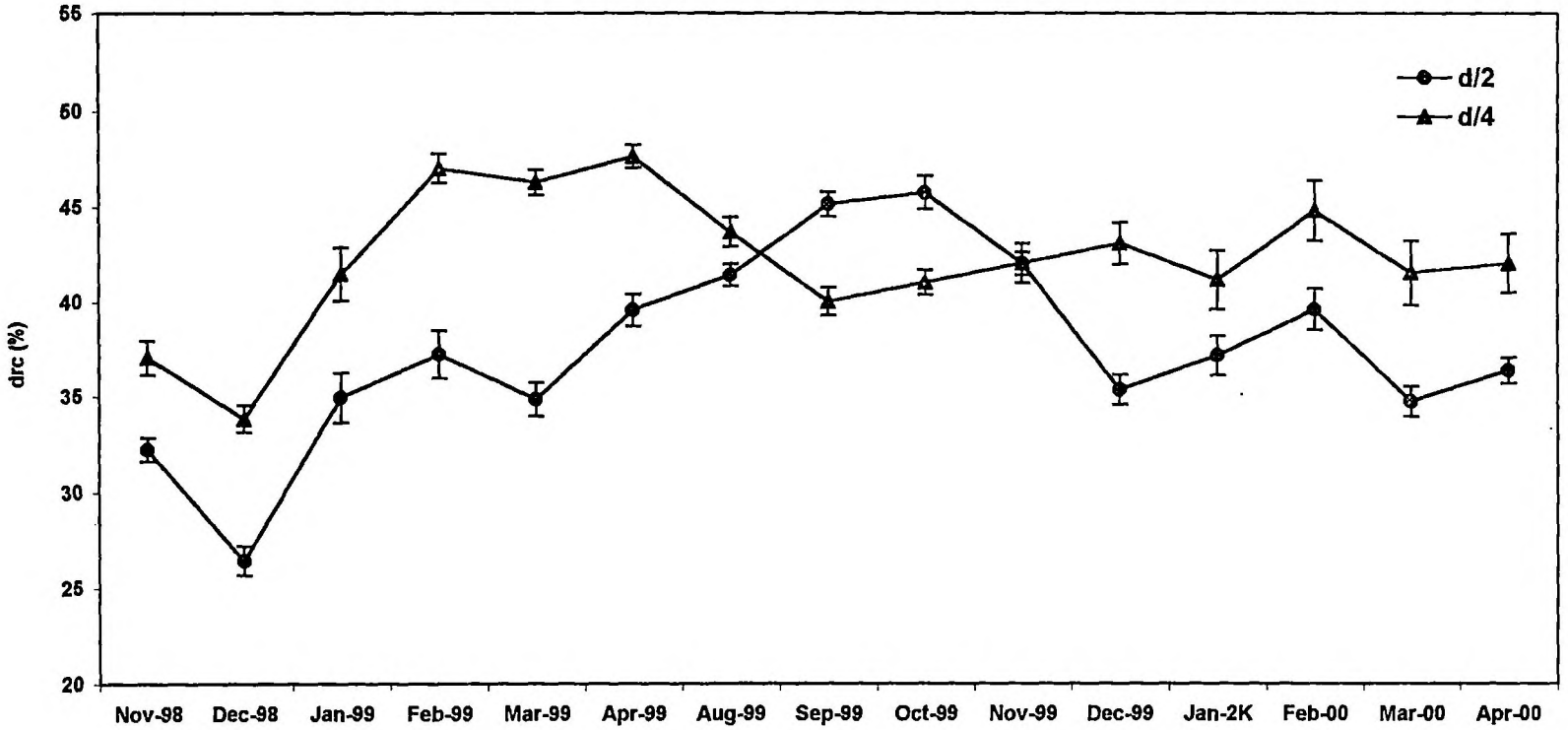


Figure-20 Monthly variations in dry rubber content in newly opened trees of clone RRII 105 under 1/2 S d/2 6 d/7 and 1/2 S d/4 6 d/7 tapping systems

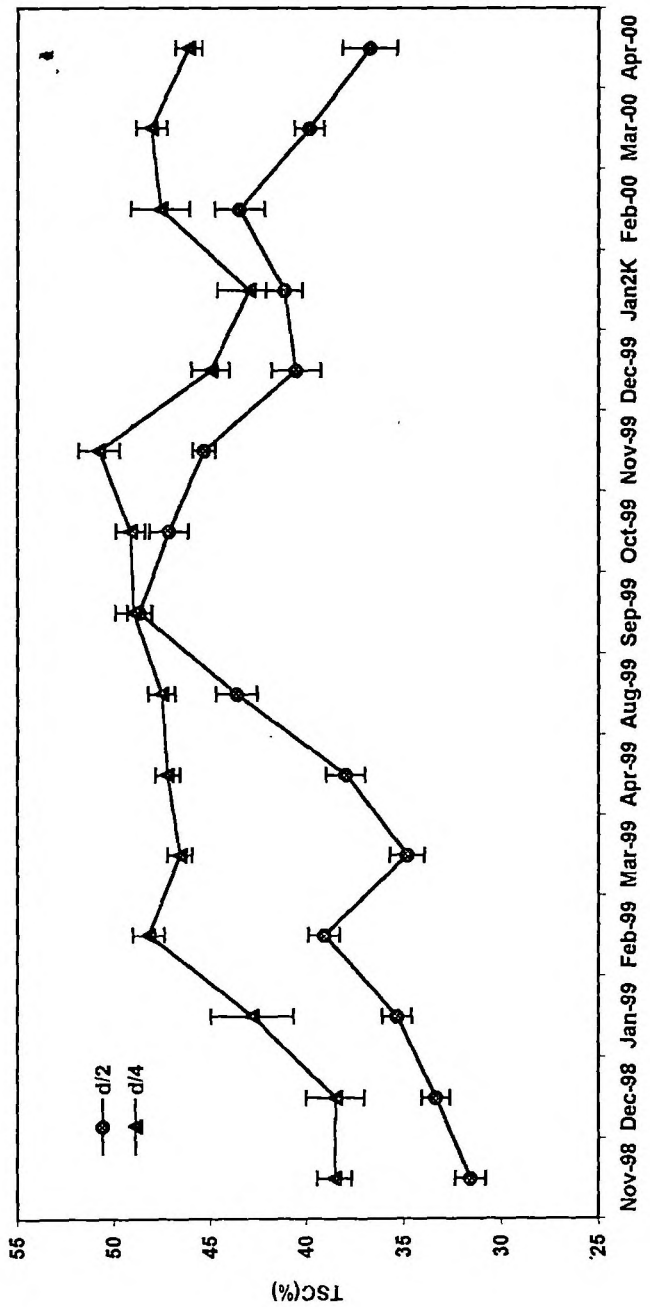


Figure 21. Monthly variations in total solid content in clone RRII 105 under 1/2 S d/2 6d/7 and 1/2 S d/4 6d/7 tapping systems

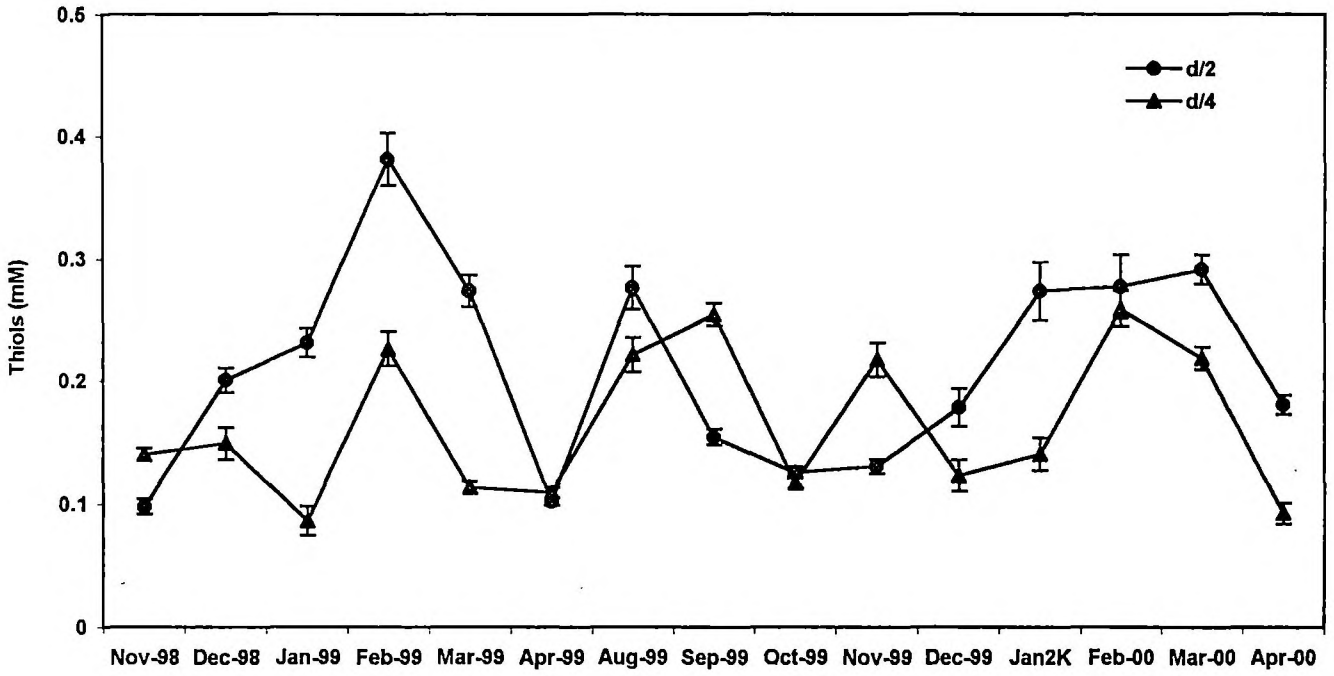


Figure-22Monthly variations in latex thiols in newly opened trees of clone RRII 105 under 1/2 S d/2 6 d/7 and 1/2 S d/4 6 d/7 tapping systems

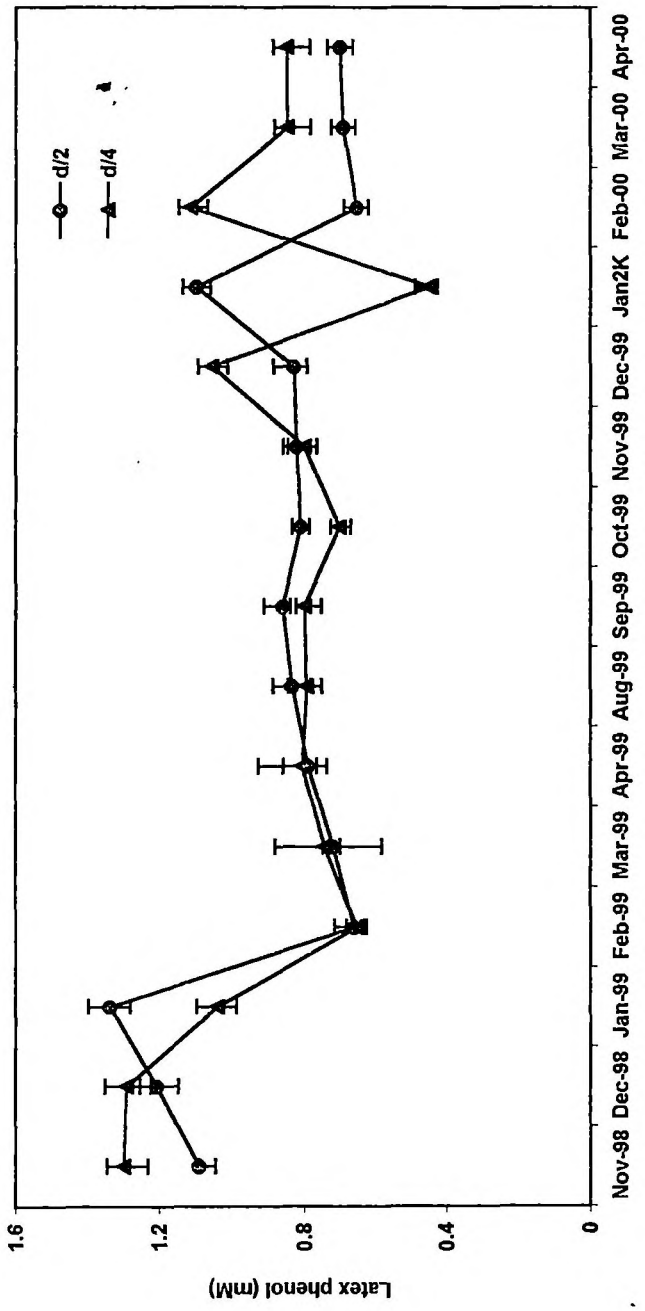


Figure:23 Monthly variations in latex phenol in newly opened trees of clone RRII 105 under 1/2 S d/2 6 d/7 and /2 S d/4 6 d/7 tapping systems

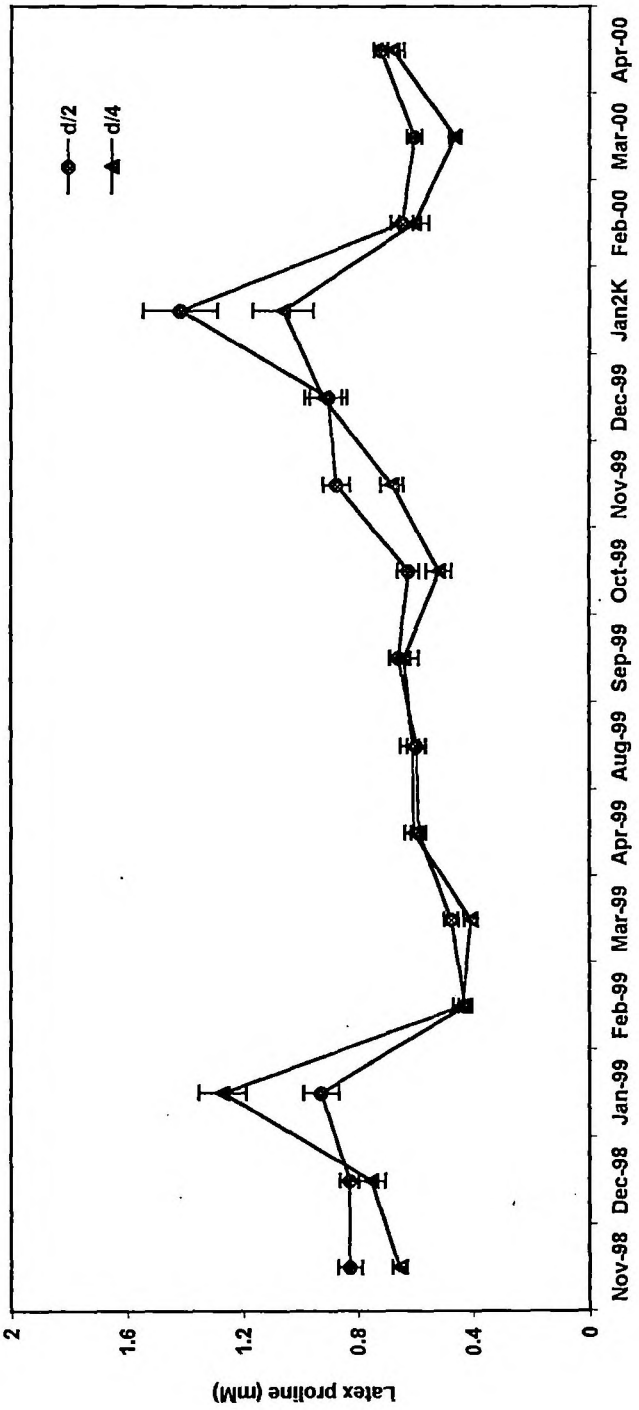


Figure- 24. Monthly variations in latex proline in newly opened trees of clone RRII 105 under 1/2 S d/2 6d/7 and 1/2 S d/4 6d/7 tapping systems

3.2.2 Effect of tapping frequency on yield and physiological parameters

The results are presented in Table-9. A significantly high dry rubber yield was observed in d/2-tapped trees when compared to d/4-tapped trees during the peak season of 1999. Reduced thiols were also high in d/2- tapped trees. Total solid content of latex was high in d/4- tapped trees. Phenol and proline content of latex did not show any variation between two treatments.

Table-9. Effect of tapping frequency on yield and latex physiological parameters in clone RRII 105 (peak yielding season- 1999).

Parameters	High frequency 1/2 S d/2 6d/7	Low frequency 1/2 S d/4 6d/7	Significance
Yield (g tree $^{-1}$tap^{-1})	93.27	67.21	10.36 **
Total solid content (%)	45.84	49.34	2.74 *
Thiols (mM)	0.2	0.12	0.062 *
Phenol (mM)	0.67	0.72	0.08 ns
Proline (mM)	0.76	0.71	0.067 ns

** CD (P=0.01) * CD (P=0.05) ns- not significant

3.2.3. Effect of tapping frequency on latex flow characteristics

Latex flow characteristics of d/2 and d/4- tapped trees are presented in Table-10. DRC showed a decrease in d/4-tapped trees compared to d/2. Pre tapping latex vessel turgor pressure was comparable in d/2 and d/4- tapped trees. A high initial flow rate, Plugging index, bursting index and latex ATP was observed in d/4-tapped trees.

Table-10. Effect of tapping frequency on latex flow characteristics in clone RRII 105 (peak yielding season-1999)

Parameters	High frequency (1/2 S d/2 6d/7)	Low frequency (1/2 S d/4 6d/7)	Significance
Dry rubber content (%)	43.3 ±0.41	41.5±0.67	*
Initial flow rate (ml min^{-1} cm^{-1})	0.10 ±0.0041	0.1447±0.0068	*
Turgor pressure (bars)	4 .3 ±0.368	4.2±0.26	ns
Plugging Index	3.28 ±0.21	4.8±0.17	*
Bursting index	6.89±0.83	13.03±0.58	*
ATP in latex	240.36±20.39	276.2±22.12	*

*significant at $p \leq 0.05$ ns- non significant

3.2.4. Effect of tapping frequency on thiols

The results are shown in Table-11. Significantly high reduced glutathione and glutathione reductase activity was observed in d/2- tapped trees when compared to d/4- tapped trees. Oxidised glutathione did not show any significant change between two treatments.

Table-11. Effect of tapping frequency on glutathione pool and glutathione reductase activity in C-serum of clone RRII 105 (peak season of 1999)

Parameters	High frequency (1/2 S d/2 6d/7)	Low frequency (1/2 S d/4 6d/7)	Significance
Oxidized glutathione (mM) Equivalents of GSH	0.049±0.006	0.035±0.0039	ns
Reduced glutathione (mM) (GSH)	0.38±0.041	0.195±0.023	*
Glutathione reductase (units min^{-1} mg protein $^{-1}$)	1.281±0.07	0.89±0.035	*

*significant at $p \leq 0.05$ ns- not significant 1 mol GSSG= 2 moles of GSH

3.2.5. Effect of tapping frequency on antioxidant enzymes

The results are presented in Table-12. Peroxidase activity of C- serum and catalase activities of C- and B- serum did not show any significant change between d/2- and d/4- tapped trees. A low peroxidase activity in B- serum was observed in d/4- tapped trees. SOD activities of C- and B- serum were significantly higher in d/2-tapped trees when compared to d/4.

Table-12. Effect of tapping frequency on antioxidant enzymes in clone RRII 105 (Peak yielding season-1999)

Enzymes (units min^{-1} mg $protein^{-1}$)	High frequency (1/2 S d/2 6d/7)	Low frequency (1/2 S d/4 6d/7)	Significance
Peroxidase (C-serum)	0.196±0.008	0.184±0.007	ns
Peroxidase (B-serum)	0.119±0.004	0.087±0.006	*
Catalase (C-serum)	0.163±. 021	0.17±0.009	ns
Catalase (B-serum)	0.14±0.03	0.16±0.011	ns
SOD (C-serum)	0.53±0.04	0.40±0.025	*
SOD (B-serum)	0.81±0.08	0.56±0.07	*

*significant at $p \leq 0.05$ ns- not significant

3.2.6. Effect of tapping frequency on proteins and glutamine synthetase activity

The results are presented in Table-13. A high protein content in C- and B-serum was observed in d/4- tapped trees when compared to d/2. Glutamine synthetase activity was also higher in d/4- tapped trees

Tabe-13. Effect of tapping frequency on proteins and glutamine synthetase activity in clone RRII 105 (peak yielding season-1999)

Parameters	High frequency (1/2 S d/2 6d/7)	Low frequency (1/2 S d/4 6d/7)	Significance
Protein (C-serum) (mg ml^{-1})	9.66±0.4	11.99±0.42	*
Protein (B-serum) (mg ml^{-1})	16.68±0.84	18.11±0.46	*
Glutamine synthetase (C-serum) (units min^{-1} mg $protein^{-1}$)	0.012±0.0018	0.036± 0.0026	*

*significant at $p \leq 0.05$ ns- not significant

3.2.7. SDS-PAGE profile of C- and B-serum proteins

SDS-PAGE profile of C- and B- serum proteins did not show any variation between high and low frequency tapped trees (Fig-25&26). Some bands appear to fluctuate quantitatively but no consistent correlation was observed between their accumulation and tapping frequency (Table-14&15).

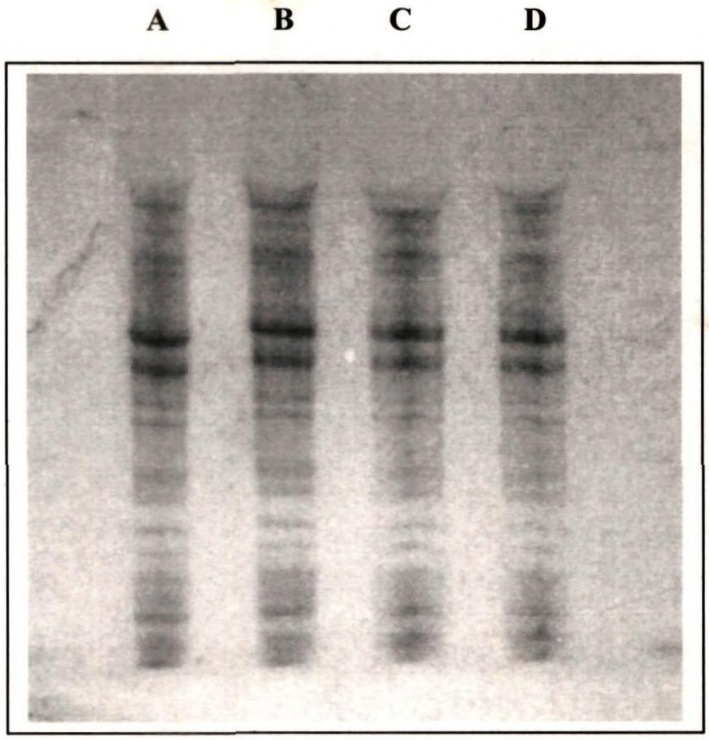


Fig-25 SDS-PAGE profile of C-serum protein in clone RRII 105 under ½ S d/2 6d/7 and ½ S d/4 d/7 tapping system (30µg protein per lane)

Lanes A&B - d/2 C&D - d/4

Table-14. Intensity of bands after SDS-PAGE of C- serum proteins of clone RRII 105 under ½ S d/2 6d/7 and ½ S d/4 6d/7-tapping systems

C-serum	d/2		d/4	
Lanes	A	B	C	D
Band No	OD	OD	OD	OD
1	0.094	0.0796	0.1325	0.1260
2	0.106	0.0798	0.1284	0.1307
3	0.086	0.0682	0.1520	0.1215
4	0.082	0.0573	0.1210	0.1123
5	0.1074	0.0591	0. 1245	0.1167
6	0.1145	0.0752	0. 1384	0.1351
7	0.0967	0.0855	0. 1500	0.1458
8	0.0876	0.0812	0.1375	0.1345
9	0.1044	0.0688	0.1318	0.1325
10	0.1114	0.0685	0.1360	0.1343
11	0.1615	0.0664	0.1512	0.1495
12	0.1729	0.0652	0.1959	0.2068
13	0.1622	0.0694	0.2174	0.2198
14	0.1388	0.0889	0.1910	0.2057
15	0.1287	0.1564	0.1637	0.1919
16	0.0910	0.1395	0.1414	0.1656
17	0.0830	0.1321	0.1297	0.1410
18	0.0789	0.0875	0.1230	0.1181
19	0.0518	0.0780	0.1155	0.1008
20	0.0544	0.0710	0.0865	0.0798
21	0.0567	0.0592	0.0842	0.0785
22	0.0650	0.0587	0.0777	0.0844
23	0.0656	0.0619	0.0923	0.1028
24	0.0583	0.0697	0.1043	0.1057
25	0.0439	0.0633	0.0989	0.0867
26	0.0475	0.0568	0.0854	0.0828
27	0.0462	0.0508	0.0833	0.0683
28	0.0594	0.0444	0.0691	0.0694
29	0.0771	0.0475	0.0680	0.0858
30	0.0828	0.0464	0.0954	0.1041
31	0.0959	0.0559	0.0986	0.1058
32	0.1160	0.0632	0.0987	0.1346

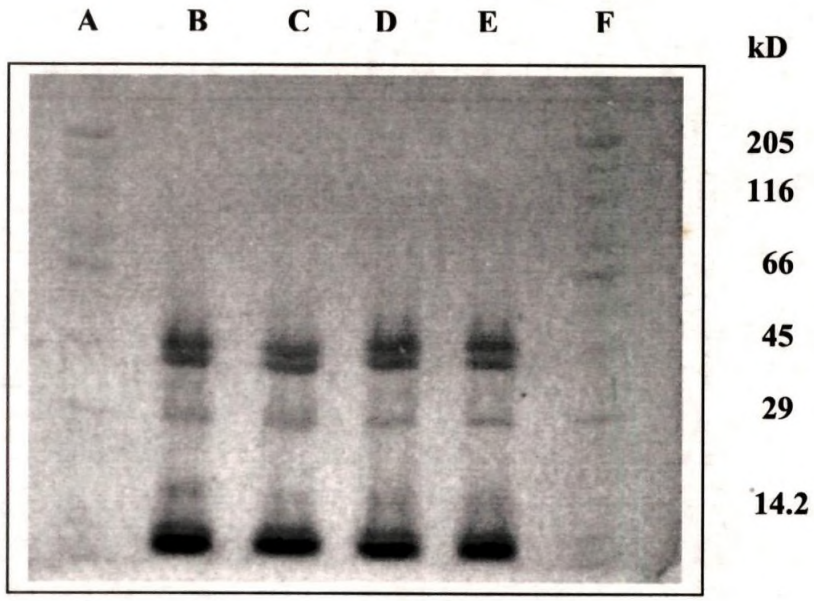


Fig- 26. SDS-PAGE profile of B-serum proteins of clone RRII 105 under 1/2S d/2 6d/7 and ½ S d/4 6d/7 tapping systems (15µg protein per lane)

Lanes	**A&F**	**- Mol.wt markers**
Lanes	**B&C**	**- d/2**
Lanes	**D&E**	**- d/4**

Table-15. Amount of protein in each band after SDS-PAGE of B- serum proteins of clone RRII 105 under ½ S d/2 6d/7 and ½ S d/4 6d/7 tapping system

Band No	Amount (µg) Lane B	Amount (µg) Lane C	Amount (µg) Lane D	Amount (µg) Lane E
1	0.75339	0.85502	0.08246	0.14568
2	0.30989	0.19139	0.04869	0.06285
3	1.1702	0.74384	0.10130	0.07916
4	1.3682	1.4399	1.7176	0.12670
5	0.69075	0.45268	1.1292	2.7285
6	0.56020	1.1251	0.98456	0.58844
7	0.56917	0.64992	0.57342	1.1703
8	0.87095	0.30442	0.38643	0.32891
9	0.31552	0.34311	0.66224	0.71577
10	0.90495	0.81615	0.45693	0.81790
11	0.33139	0.44450	0.49743	0.90620
12	0.46076	0.66334	1.584	1.5474
13	0.28505	1.1276	1.7573	1.6945
14	2.5825	1.8868	1.2490	1.3493
15	2.1869	2.8932	2.1413	2.3523
Sum	13.360	13.937	13.372	14.614

3.2.8. Effect of tapping frequency on enzymes associated with cessation of flow

The variations are shown in Table-14. A significantly high β-1,3 glucanase and a low N- acetyl glucosaminidase activities were observed in d/4-tapped trees compared to d/2. Chitinase activity did not show any significant difference between d/2 and d/4 tapped trees.

Table-16. Effect of tapping frequency on activities of enzymes associated with cessation of latex flow in clone RRII 105 (peak yielding season-1999)

Parameters	High frequency 1/2 S d/2 6d/7	Low frequency 1/2 S d/4 6d/7	Significance
β 1,3 glucanase (B-serum) (mg glucose liberated min^{-1} mg $protein^{-1}$)	7.95±0.66	11.26±0.82	*
N-Acetyl glucosaminidase (B-serum) (mg p-nitrophenol liberated min^{-1} mg $protein^{-1}$)	0.48±0.036	0.276±0.016	*
Chitinase (B-serum) (unis min^{-1} ml B-serum	1.8 ± 0.22	1.1± 0.15	ns

*significant at $p \leq 0.05$ ns- not significant

3.2.9. Hevein content of B- serum of high and low frequency tapped trees.

Hevein content in the B-serum of high (d/2) and low (d/4) frequency tapped trees are shown in Fig-27. A high hevein content was observed in d/4 tapped trees.

3.2.9. Hevein content of B-serum

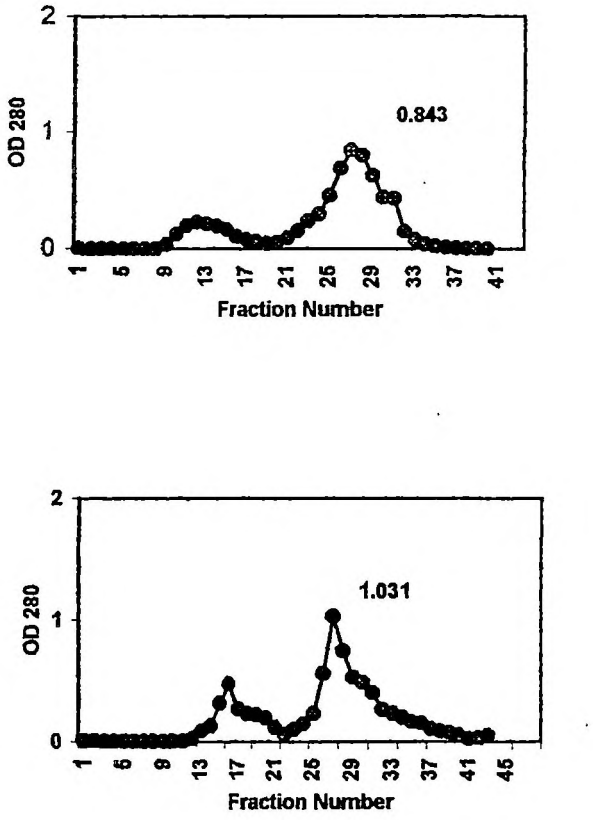


Fig- 27. Gel filtration of B-serum of clone RRII 105 under d/2 (A) and d/4 (B) system of tapping Peak season (October 1999). Protein content of 5.00mg on a sephadex G-25 column (3x50 cm). Elution with 1.2 M acetic acid. Fractions of 3.0ml were collected.

3.2.1.1. Biochemical changes associated with Tapping Panel Dryness

During the course of the experiment on high and low frequency-tapping system, observations were also made on the occurrence of the natural development of TPD. Two trees tapped under high frequency (d/2) showed symptoms of partial dryness after two years. The dry portion was marked and the severity of TPD was expressed as % (The length of dry portion to the total length of tapping cut).

Changes in the physiological and biochemical parameters of these trees (35%TPD) are presented in Fig-28-34. The mean values of ten healthy trees were used as control for individual partial dry trees. After one year these trees were completely dry with no latex flow. Bark samples were then collected for analysis and the data on changes in phenol, protein and thiols are presented in fig-35-37.

Latex yield prior to the appearance of partial dryness showed no change but after that the volume decreases in both the trees when compared to healthy trees (Fig-28).

Partial dry trees did not show any diffefrence in DRC prior to or aftere the incidence as against the average values of normal trees (Fig-29).

TSC increases after partial dryness in both the trees when compared to normal trees. Prior to the onset of dryness no change was observed (Fig-30).

No difference was observed in latex thiols before the incidence of partial dryness. After partial dryness, the thiol content of both the trees decreased (Fig-31).

Phenol content of latex did not show any change before in both the trees but after partial dryness, phenol content was high (Fig-32).

Protein content of C- serum showed an increase after the incidence in both the trees but tree No.2 showed a higher value (Fig-33).

Proline content in latex increases after partial dryness in both the trees (Fig-34). Protein and thiol content in the bark decreased after partial dryness (Fig-35&37). No consistant pattern was observed in the case of phenols in bark (Fig-36).

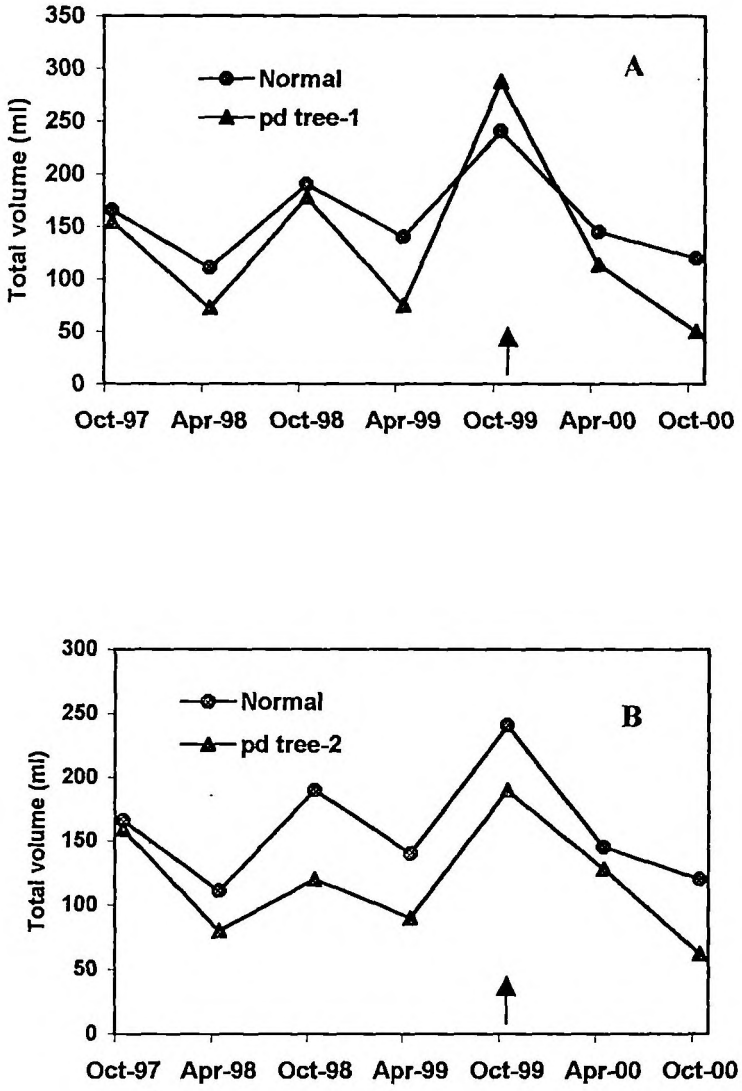


Fig- 28. Latex yield prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees. Arrow shows the appearance of partial dryness. A- partial dry (pd) tree-1 and B-pd tree-2

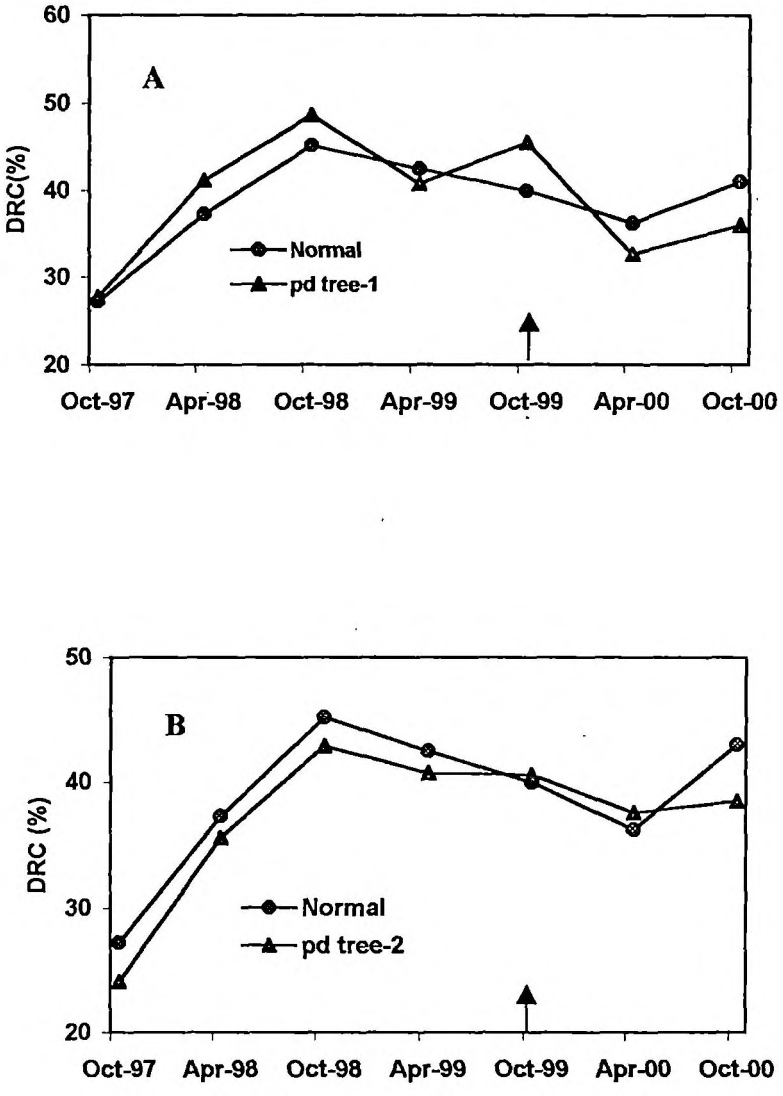


Fig.29 Dry rubber content in latex prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees. Arrow shows the appearance of partial dryness. A- partial dry (pd) tree-1 and B-pd tree-2

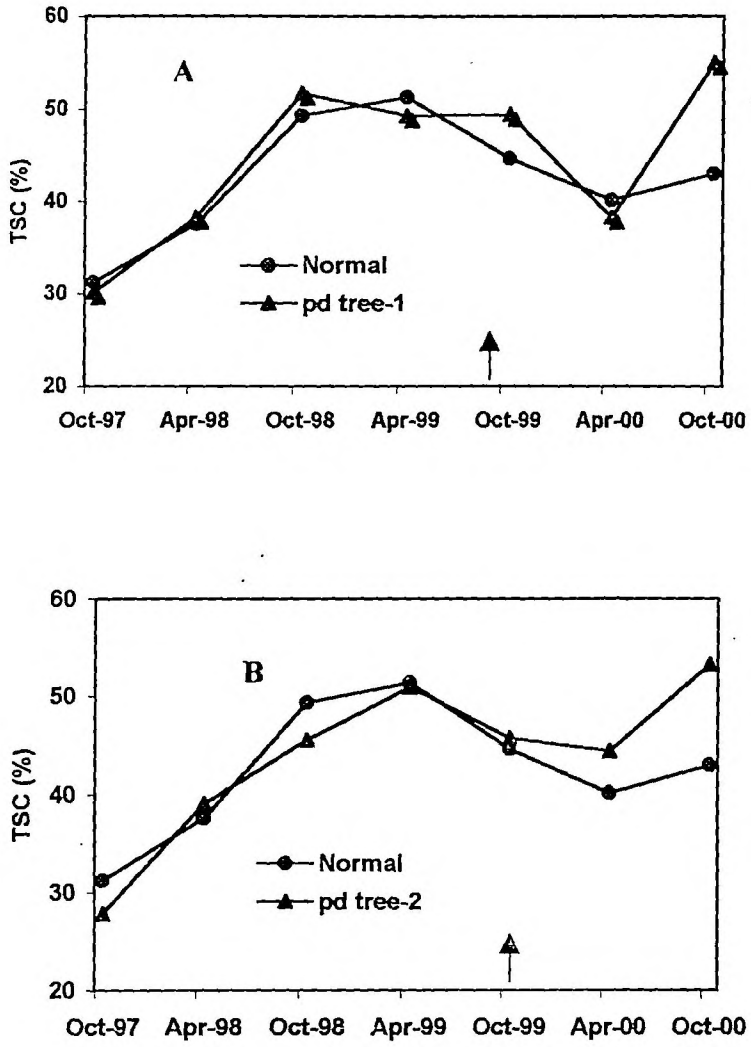


Fig- 30. Total solid content in latex prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees Arrow shows the appearance of partial dryness. A- partial dry (pd) tree-1 and B-pd tree-2

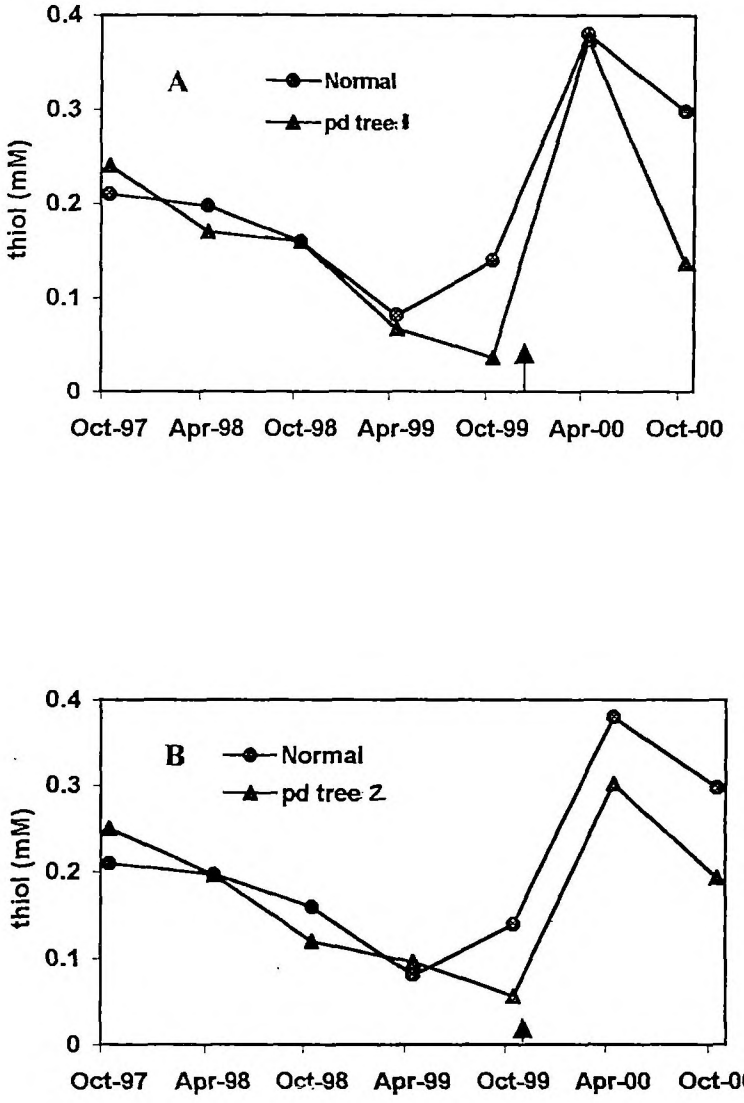
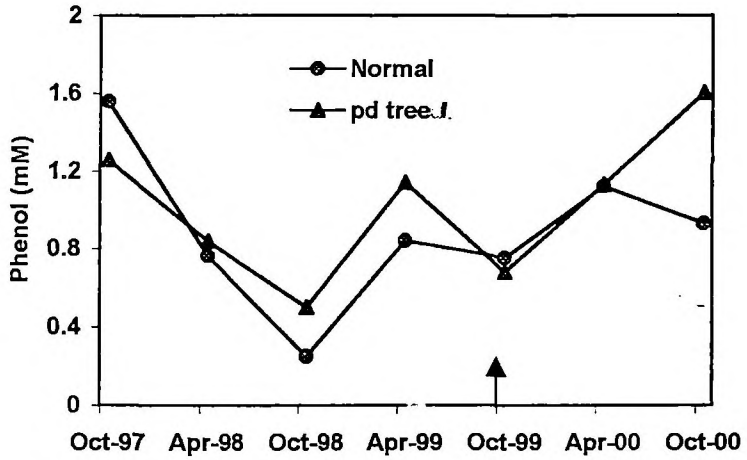


Fig-31.Thiol content in latex prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees. Arrow shows the appearance of partial dryness. A-partial dry (pd) tree-1 and B-pd tree-2



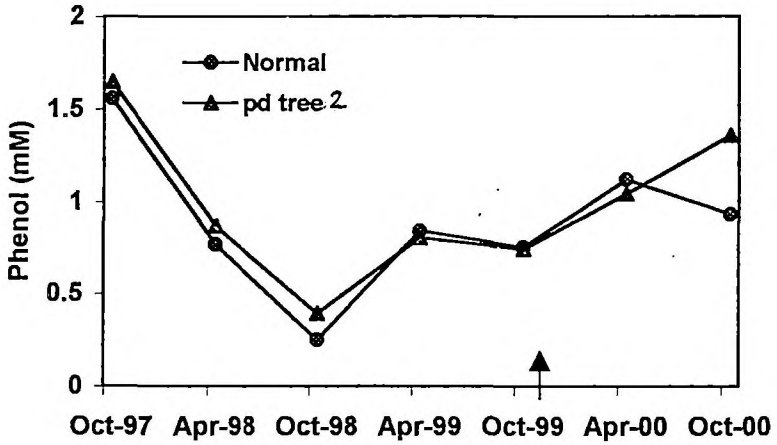
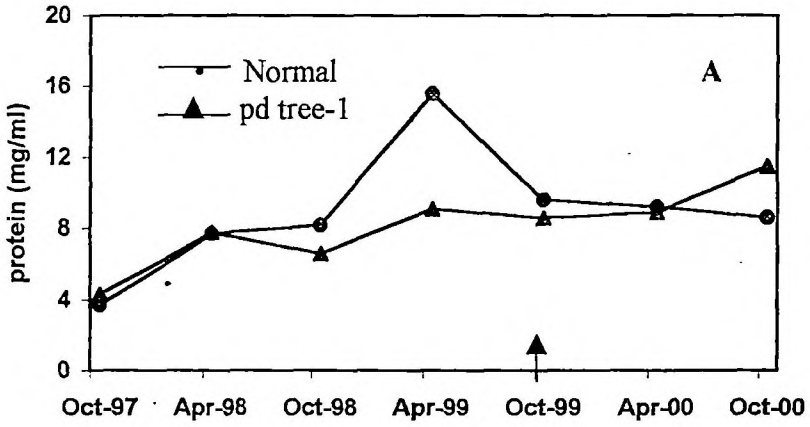


Fig- 32. Phenol content in latex prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees. Arrow shows the appearance of partial dryness. A- partial dry (pd) tree-1 and B-pd tree-2



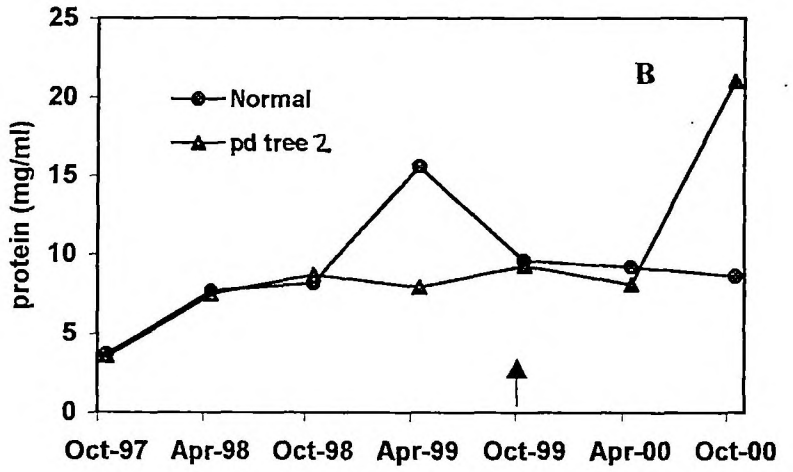


Fig- 33. Protein content of C-serum prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees. Arrow shows the appearance of partial dryness. A- partial dry (pd) tree-1 and B-pd tree-2

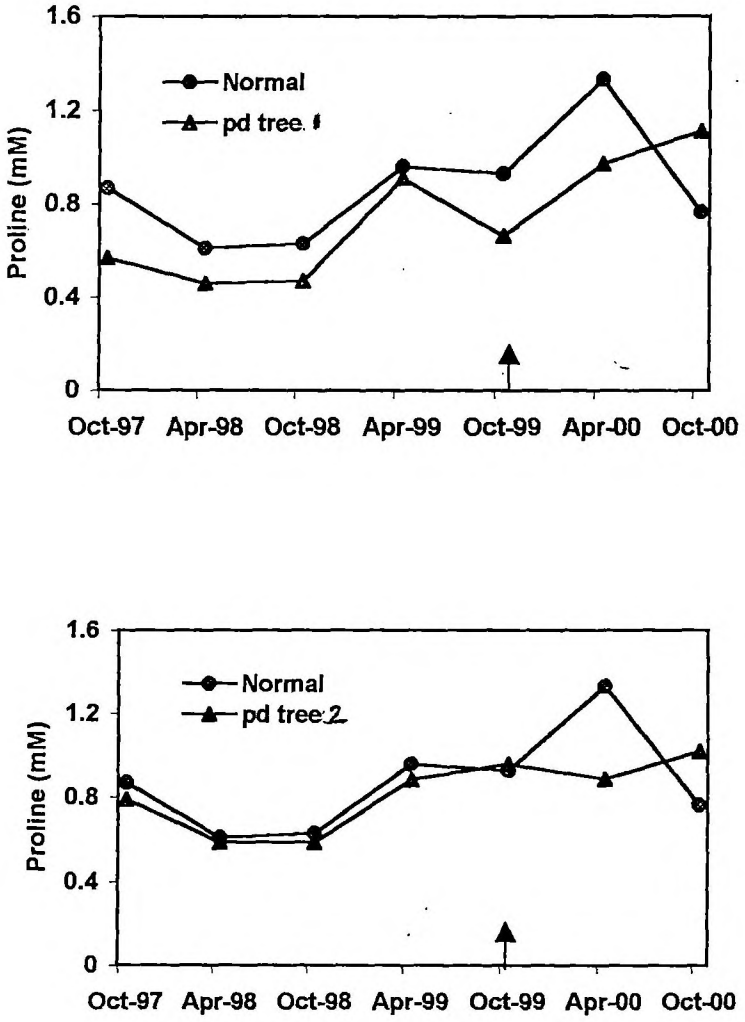


Fig-34. Proline content in latex prior to the onset of partial dryness and after the incidence. Normal- mean values of ten healthy trees. Arrow shows the appearance of partial dryness. A-partial dry (pd) tree-1 and B-pd tree-2

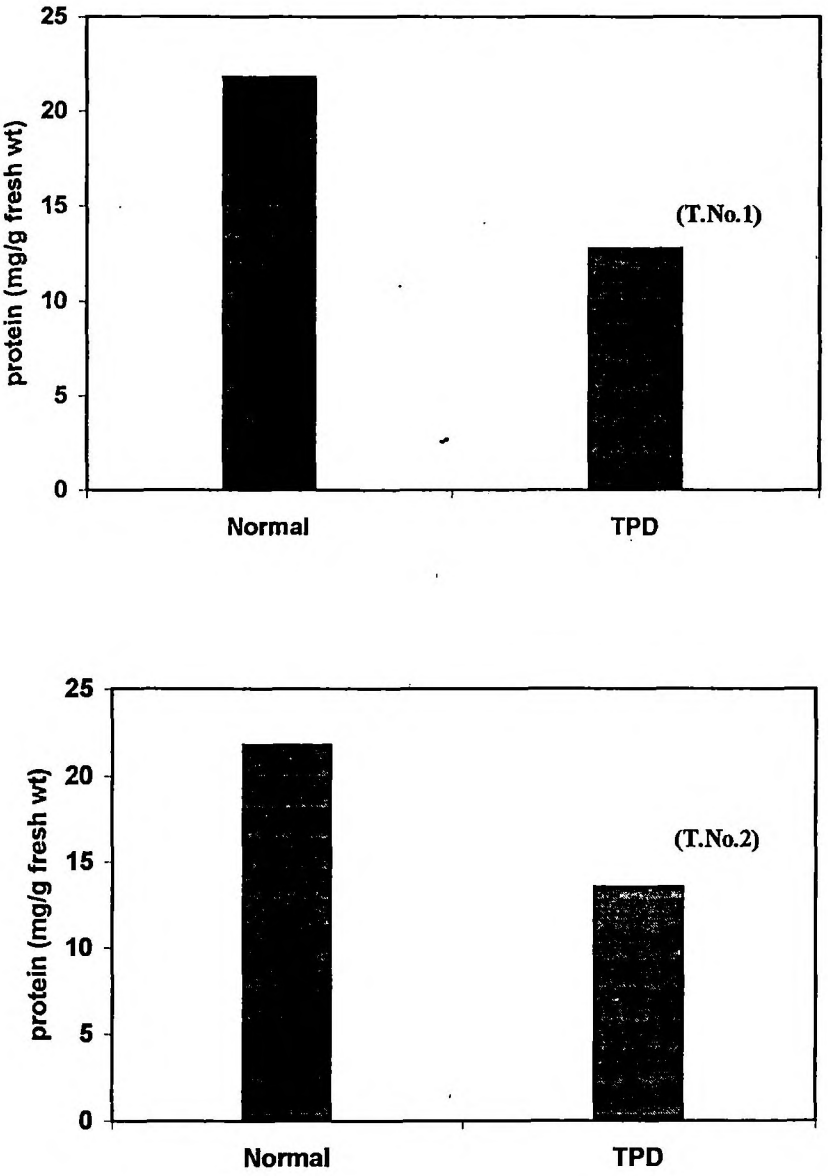
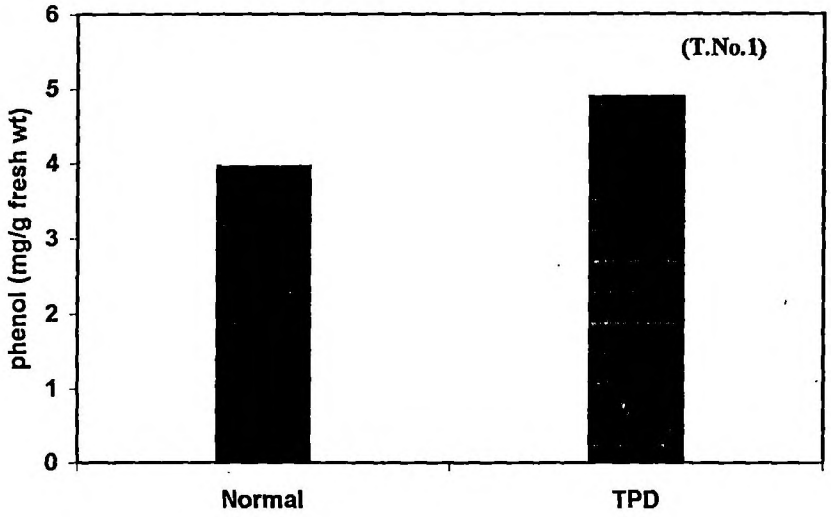


Fig-35. Protein content in the bark of normal and TPD affected trees (tree No. 1&2)



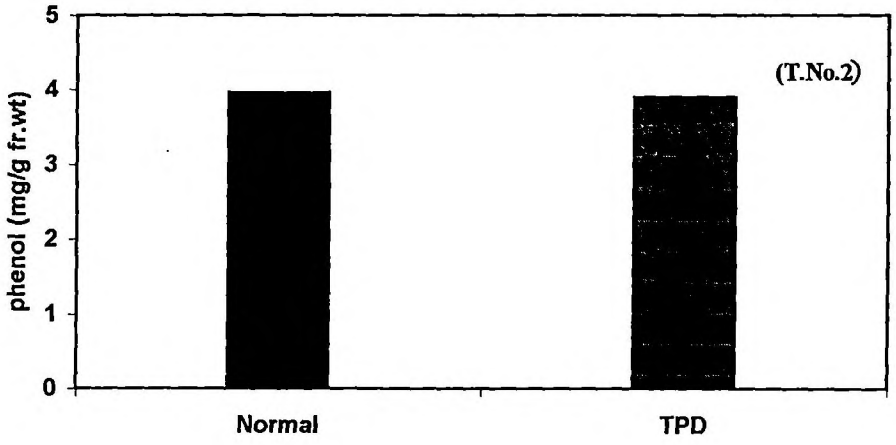


Fig- 36. Phenol content in the bark of normal and TPD affected Trees (Tree No.1&2)

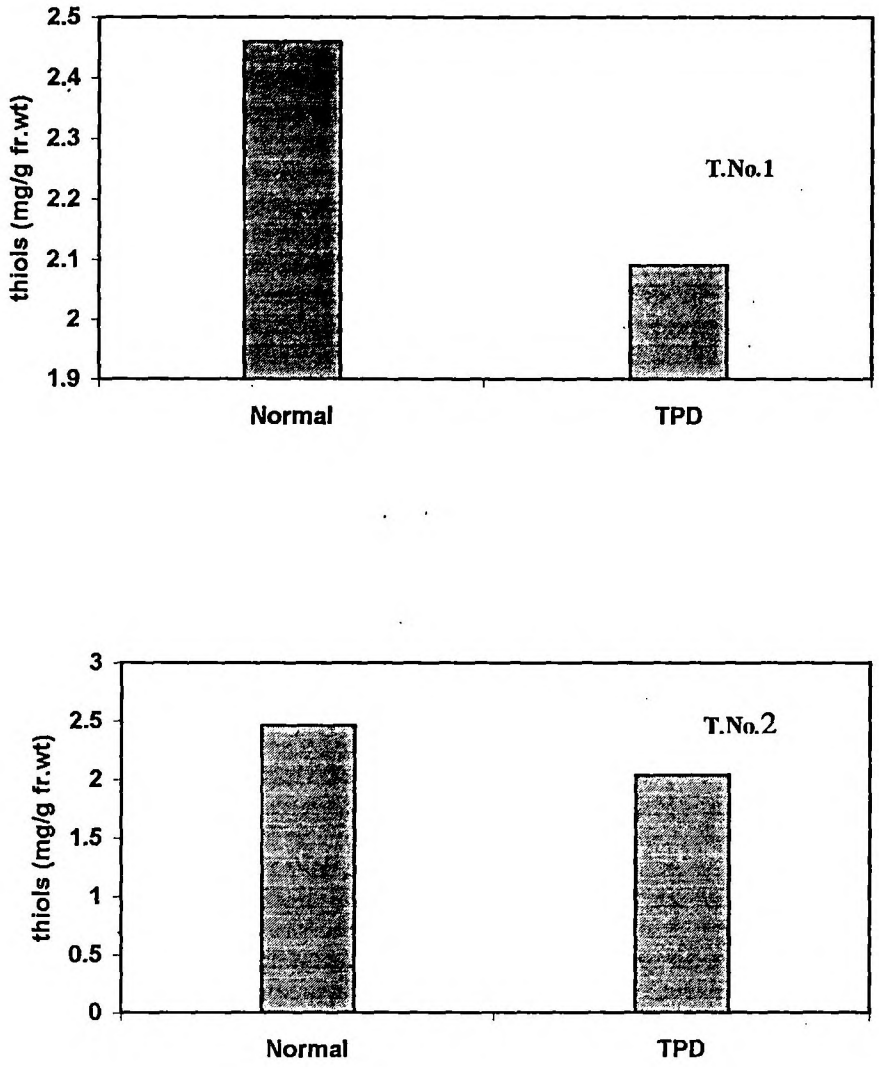


Fig-37. Thiol content in the bark of normal and TPD trees (T.No.1&2)

3.3. STIMULATION EXPERIMENTS

Results of the three experiments are given in this chapter.

3.3.1. Latex yield

Control and stimulated

Latex yield of stimulated trees were higher when compared to control trees on all tapping days. The maximum increase was on second and third tapping after stimulation and a slight reduction during fourth tapping but still higher than that of control (Fig- 38).

Stimulation and intensive tapping

Latex yield increased on all tapping days after stimulation and intensive tapping. Maximum yield was obtained on second and third intensive tapping. Then the latex yield decreases but higher than that before stimulation (Fig-39)

Stimulation and tapping rest

Maximum increase in latex yield was observed in trees with six-day rest after stimulation and then decreases. After fourteen-day rest the latex yield was same as that of unstimulated trees (Fig-40).

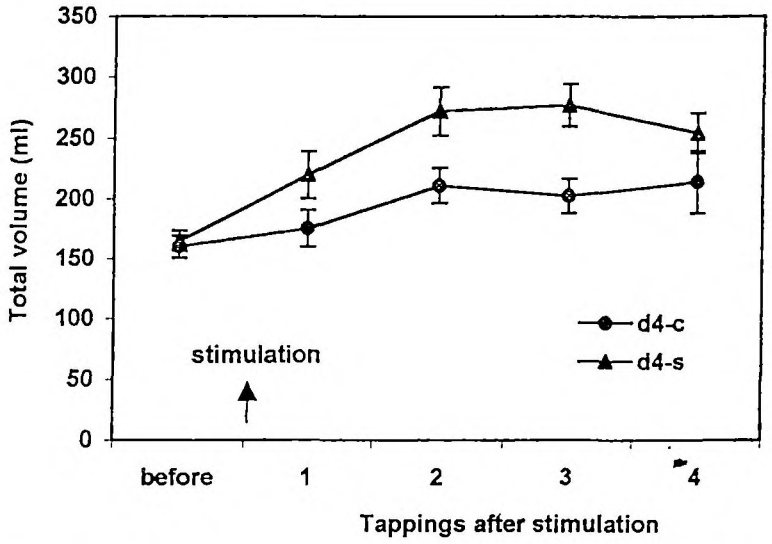


Fig-38. Effect of stimulation on latex yield in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d4-c : control d4-s : stimulated

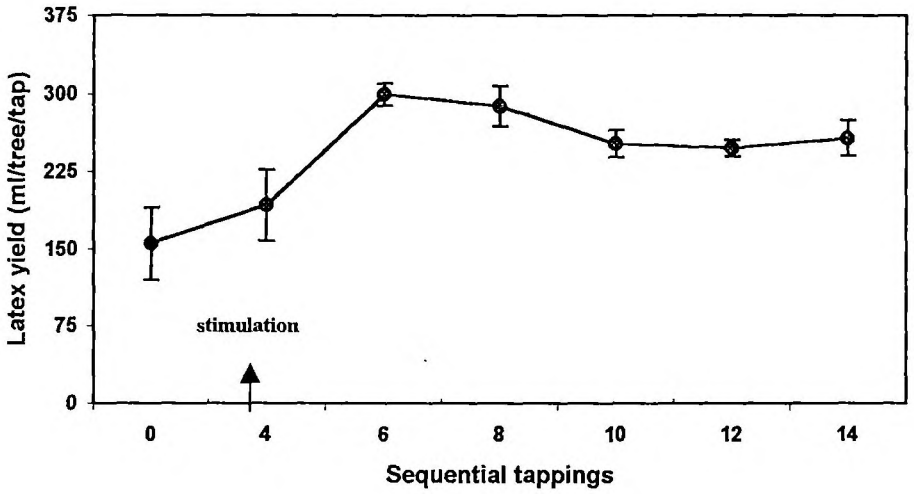


Fig-39 Effect of intensive tapping after stimulation on latex yield in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

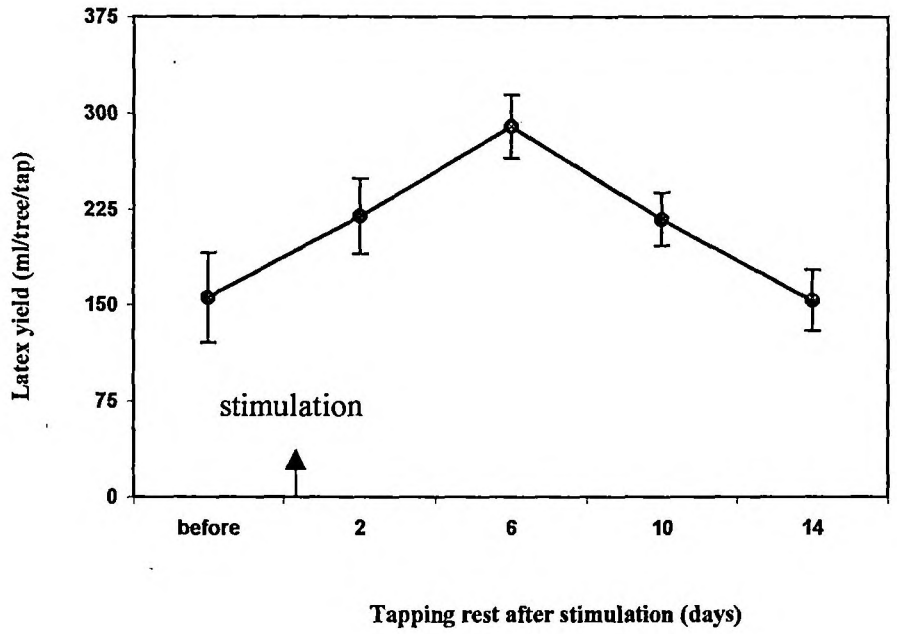


Fig-40 Effect of tapping rest after stimulation on latex yield in clone RRII 105 under ½ S d/4 6d/7 tapping system

3.3.2 Turgor pressure

Control and stimulated

No change in pre tapping latex vessel turgor was observed in stimulated trees when compared to control trees (Fig-41).

Stimulation and intensive tapping

No variation in latex vessel turgor was observed after stimulation and intensive tapping (Fig-42).

Stimulation and tapping rest

No change in pre tapping latex vessel turgor was observed in trees with different periods of tapping rest after stimulation (Fig-43).

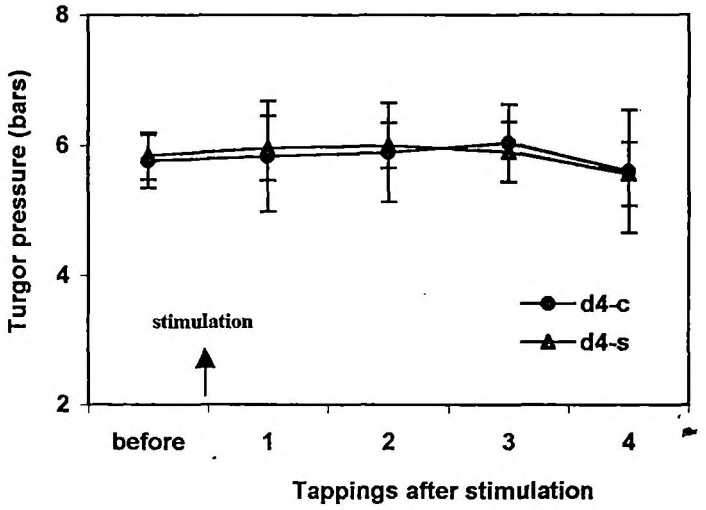


Fig- 41. Effect of stimulation on turgor pressure in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d4-c: unstimulated control d4-s: stimulated

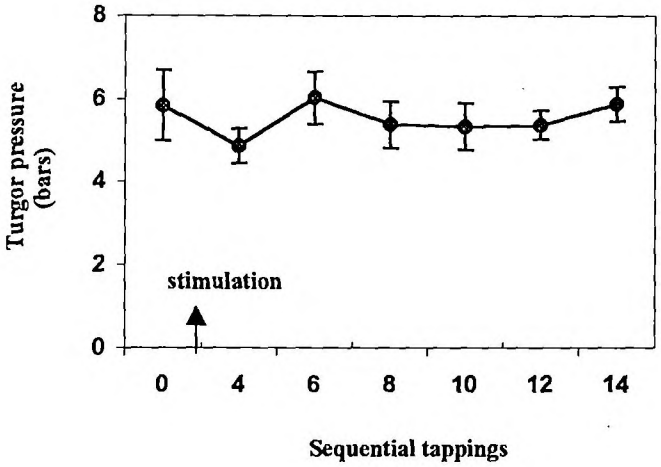


Fig- **42** Effect of intensive tapping after stimulation on turgor pressure in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

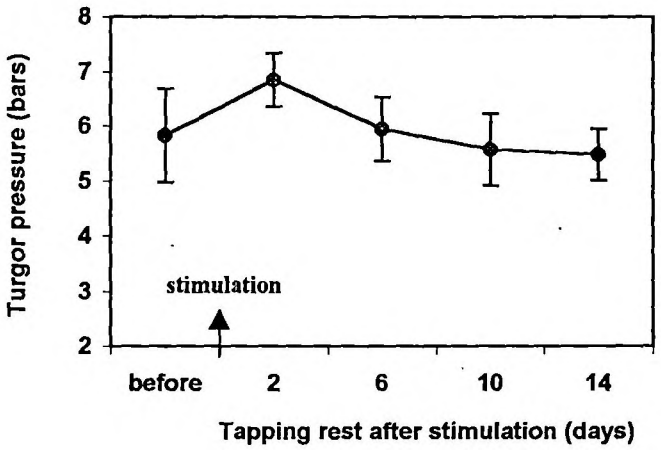


Fig- **43** Effect of tapping rest after stimulation on turgor pressure in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.3.Initial flow rate

Control and stimulated

Initial flow rate of stimulated trees were higher than that of control trees on all tapping days (Fig-44).

Stimulation and intensive tapping

Initial flow rate did not show any variation after stimulation and intensive tapping (Fig-45).

Stimulation and tapping rest

Maximum initial flow rate was observed in trees with six-day rest after stimulation and then decreases in trees with 10 and 14 day rested trees (Fig-46).

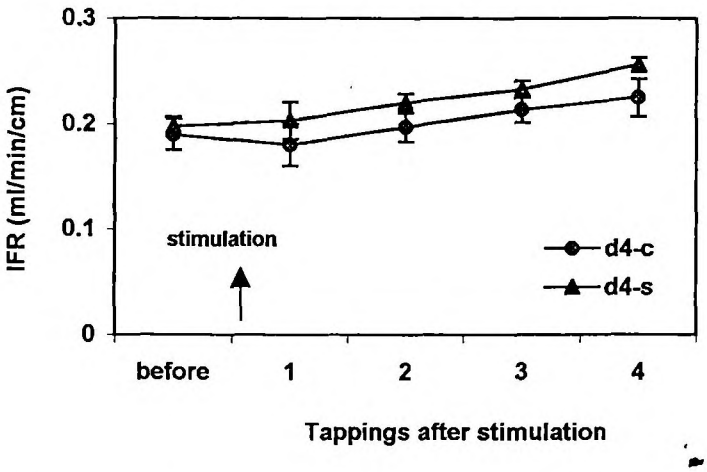


Fig-44. Effect of stimulation on IFR in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

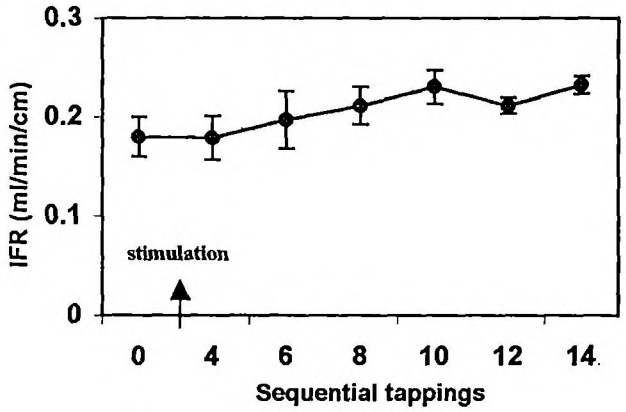


Fig-45 Effect of intensive tapping after stimulation on initial flow rate in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

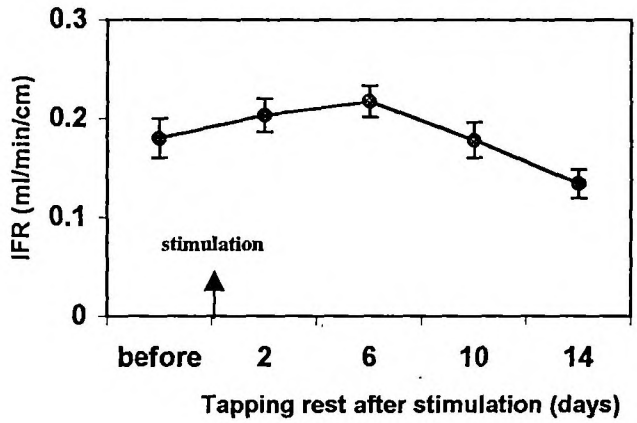


Fig-46 Effect of tapping rest after stimulation on initial flow rate in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.4. Plugging Index

Control and stimulated

Plugging index of stimulated trees was lower than that of control trees. It was minimum on second and third tapping after stimulation. On fourth tapping plugging index of stimulated trees were same as that of control trees (Fig-47).

Stimulation and intensive tapping

A general decrease in plugging index was observed on all intensive tapping days after stimulation. Maximum decrease was on second intensive tapping (Fig-48).

Stimulation and tapping rest

Plugging index was minimum in trees with six-day rest after stimulation and then increases. On fourteen-day rest it reached same as that of unstimulated trees (Fig-49).

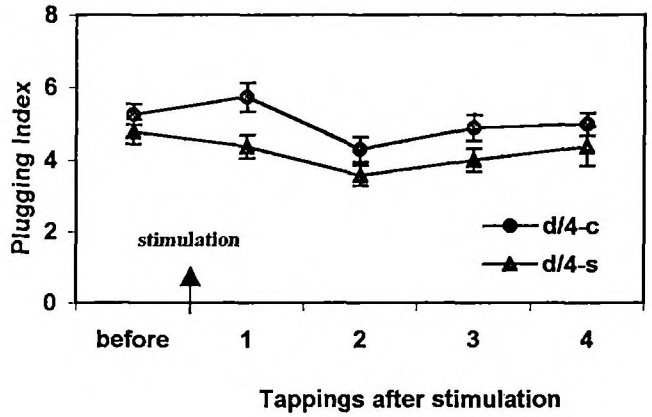


Fig-47. Effect of stimulation on plugging index in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

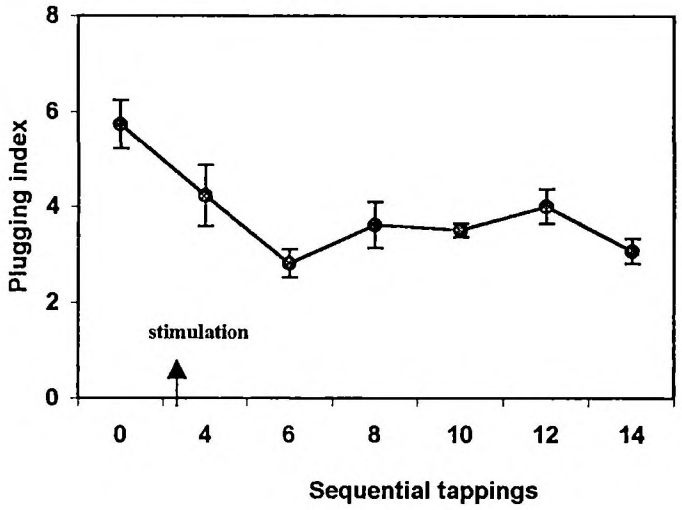


Fig- 48 Effect of intensive tapping after stimulation on plugging index in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

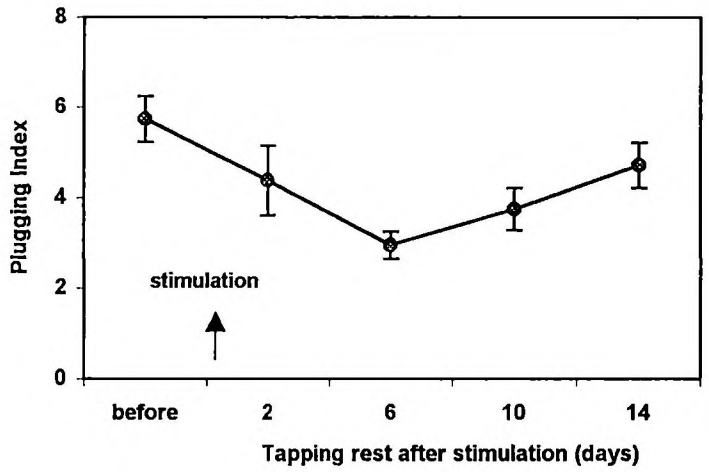


Fig- 49 Effect of tapping rest after stimulation on plugging index in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.5 Bursting Index

Control and stimulated

A decreased BI was observed in stimulated trees after the first tapping when compared to control trees (Fig-50).

Stimulation and intensive tapping

The bursting index showed an increase up to third intensive tapping and then decreases (Fig-51).

Stimulation and tapping rest

The bursting was minimum on six day rest after stimulation and then increases (Fig-52)

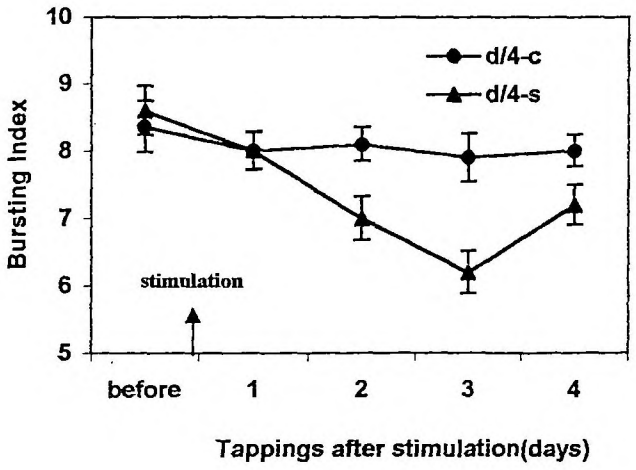


Fig- 50. Effect of stimulation on bursting index in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

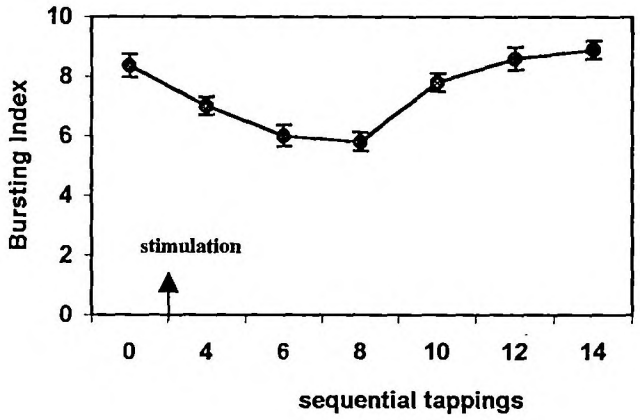


Fig- 51 Effect of intensive tapping after stimulation on bursting index in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

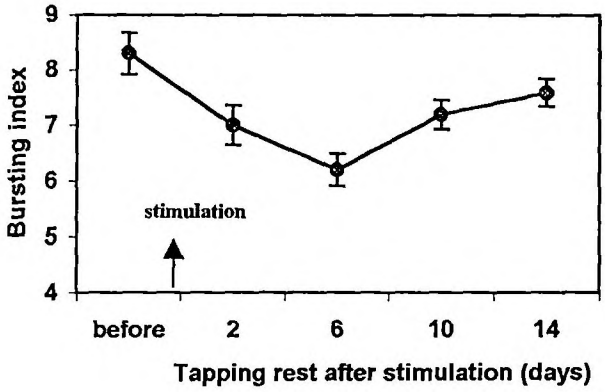


Fig-52 Effect of tapping rest after stimulation on bursting index in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.6 Dry rubber content

Control and stimulated

DRC of stimulated trees were always lower than that of control trees. The reduction was observed in the first tapping onwards after stimulation (Fig-53).

Stimulation and intensive tapping

A decrease in DRC was observed after second intensive tapping onwards (Fig-54).

Stimulation and tapping rest

DRC was minimum in trees with six and ten day tapping rest after stimulation and then increased in trees with 10 day rest and on fourteen-day rest it was higher than that of unstimulated trees (Fig-55).

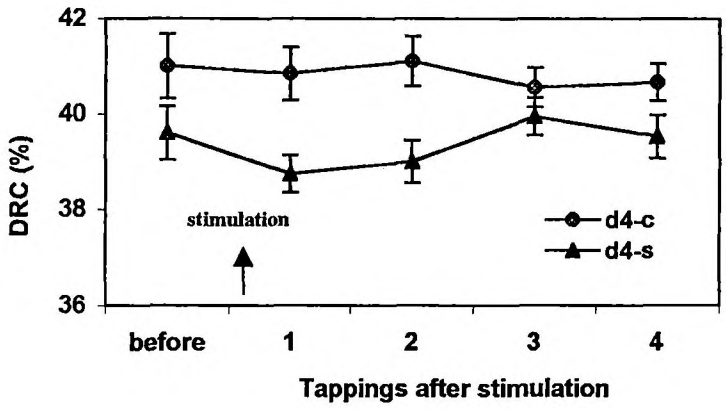


Fig- 53. Effect of stimulation on DRC in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

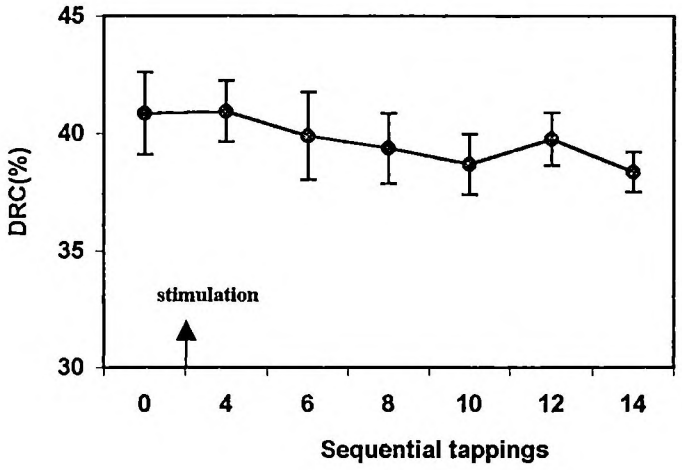


Fig-54 Effect of intensive tapping after stimulation on DRC in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE).

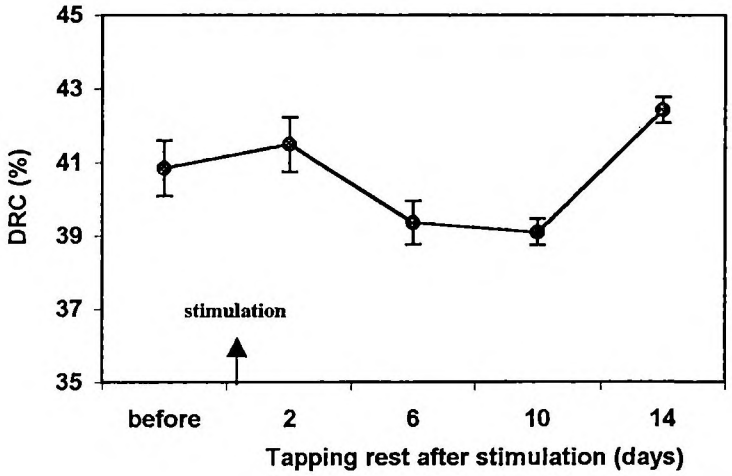


Fig- 55 Effect of tapping rest after stimulation on DRC in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.7. Total solid content

Control and stimulated

A general decrease was observed in the total solid content of stimulated trees when compared to control trees on all tapping days (Fig-56).

Stimulation and intensive tapping

A general decrease in TSC was observed on all intensive tapping days after stimulation (Fig-57).

Stimulation and tapping rest

Total solid content decreases in trees with different periods of tapping rest after stimulation. Minimum TSC was observed in trees with ten and 14-day tapping rest (Fig-58).

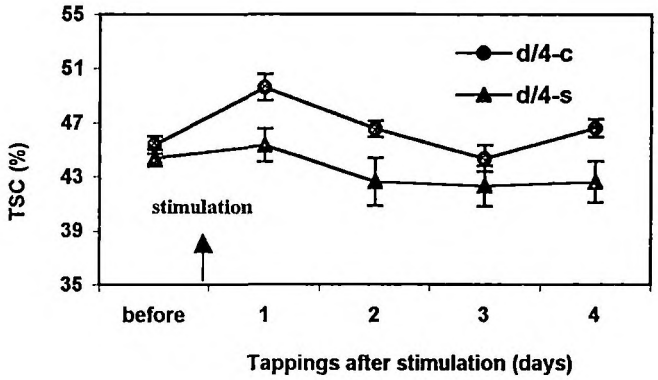


Fig- 56. Effect of stimulation on TSC in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

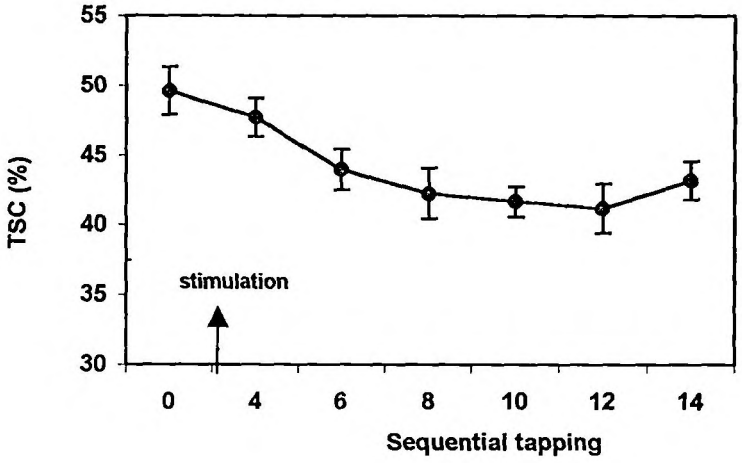


Fig- 57 Effect of intensive tapping after stimulation on TSC in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE).

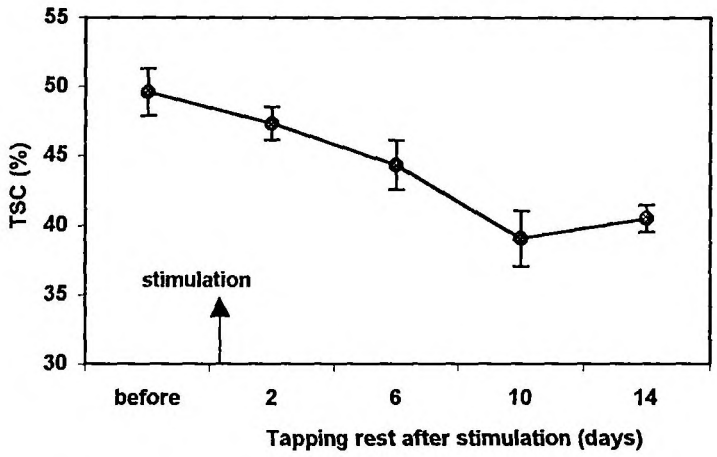


Fig-58 Effect of tapping rest after stimulation on TSC in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.8. Phenols

Control and stimulated

From second tapping onwards, latex phenol content in the lattices of stimulated trees showed an increase when compared to control trees (Fig-59).

Stimulation and intensive tapping

A higher level of latex phenol was observed after second intensive tapping onwards. The maximum increase was on third intensive tapping after stimulation (Fig-60).

Stimulation and tapping rest

Maximum latex phenol was observed in the latex of trees with six and ten day rest after stimulation. On fourteen-day rest it was same as that of un stimulated trees. Trees with 2-day rest it was same as before stimulation (Fig-61).

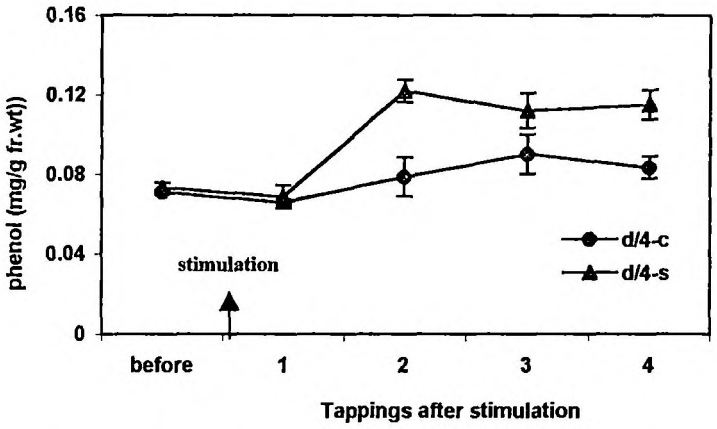


Fig-59. Effect of stimulation on phenol (latex) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

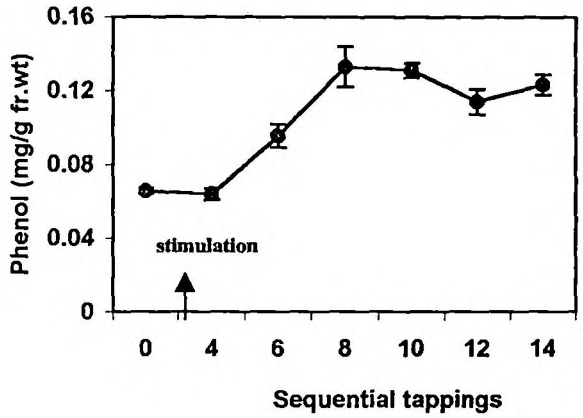


Fig-60 Effect of intensive tapping after stimulation on latex phenol in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

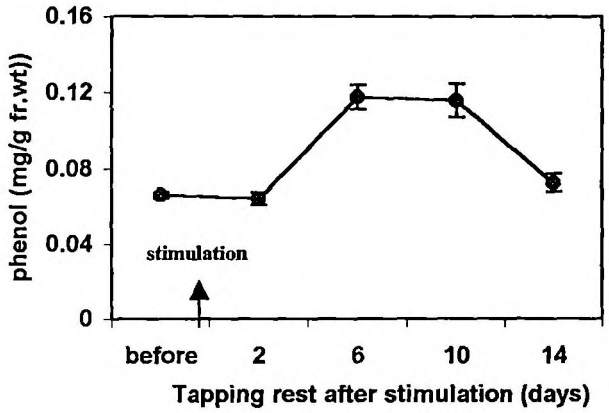


Fig-61 Effect of tapping rest after stimulation on latex phenol in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.9. Proline

Control and stimulated

A general decrease in proline content of latex was observed in stimulated trees when compared to control trees on all tapping days (Fig-62).

Stimulation and intensive tapping

Latex proline content increases after stimulation and intensive tapping up to fourth intensive tapping and showed a decrease on subsequent tappings (Fig-63).

Stimulation and tapping rest

Maximum proline content of latex was observed in trees with two-day rest after stimulation. Then it decreases in trees with six and ten day rest after stimulation. On fourteen-day rest it was same as that of unstimulated control trees (Fig-64).

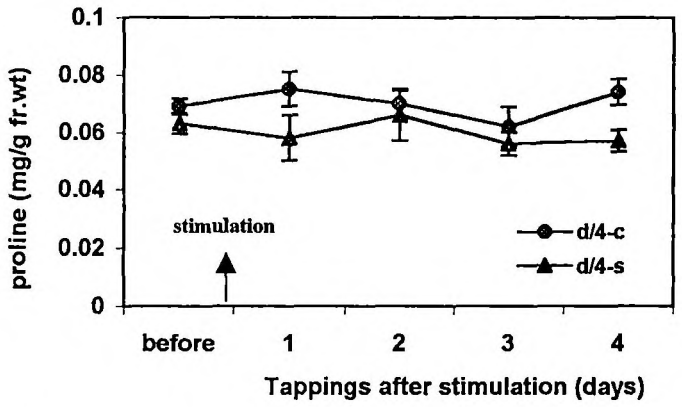


Fig-62. Effect of stimulation on latex proline in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

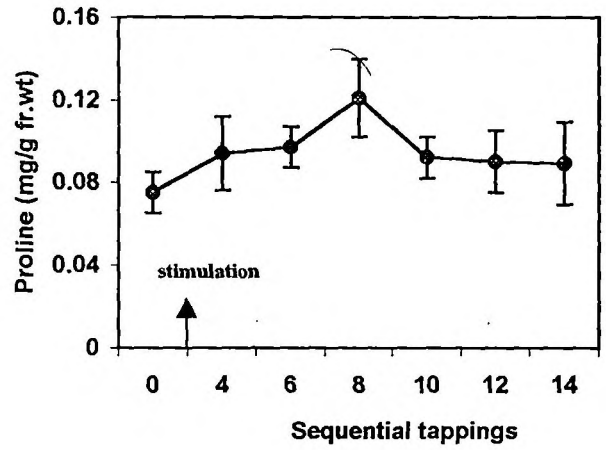


Fig 63 Effect of intensive tapping after stimulation on latex proline in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

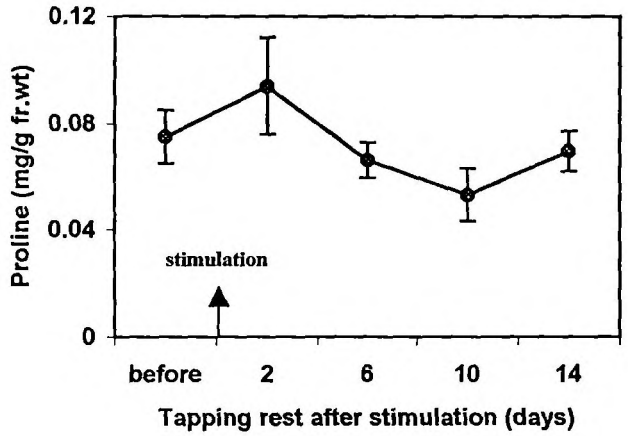


Fig64 Effect of tapping rest after stimulation on latex proline in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.10. Protein (C-serum)

Control and stimulated

The first and second tapping after stimulation was characterized by an abrupt rise in the content of C-serum proteins in stimulated trees compared to control trees. Then a sudden decrease was observed during third tapping and increases but it was almost same as that of control trees on fourth tapping (Fig-65).

Stimulation and intensive tapping

A general increase in the content of C- serum protein was observed on all intensive tapping days. The maximum protein content was on fourth intensive tapping (Fig-66).

Stimulation and tapping rest

The protein content of C- serum protein was maximum in trees with six day rest after stimulation and then decreased to the same level of proteins in un stimulated trees (Fig-67).

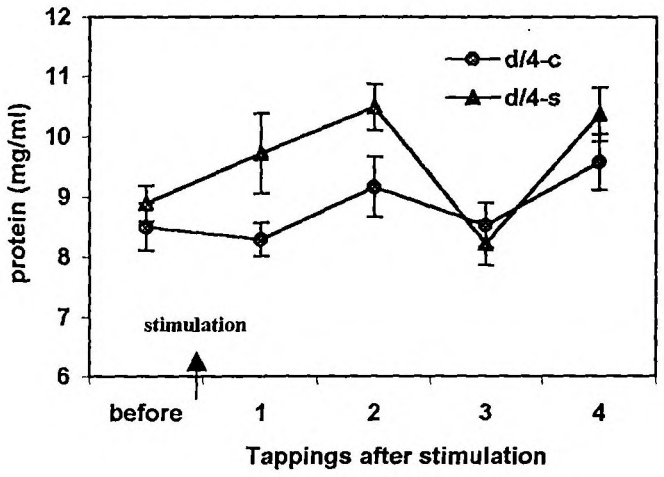


Fig-65. Effect of stimulation on protein (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

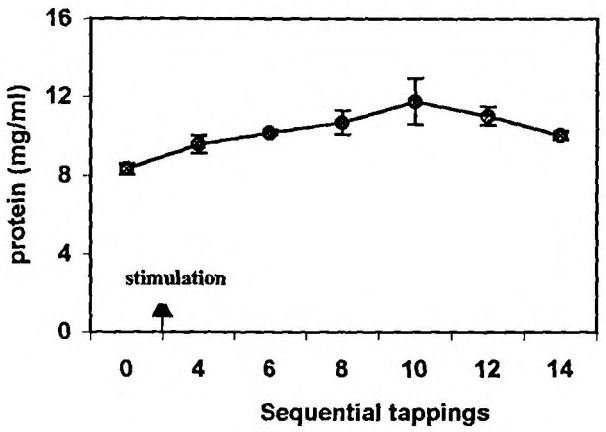


Fig-66 Effect of intensive tapping after stimulation on c-serum protein in clone RRII 105 under 1/2S d/4 6d/7 tapping system (mean of six trees ±SE)

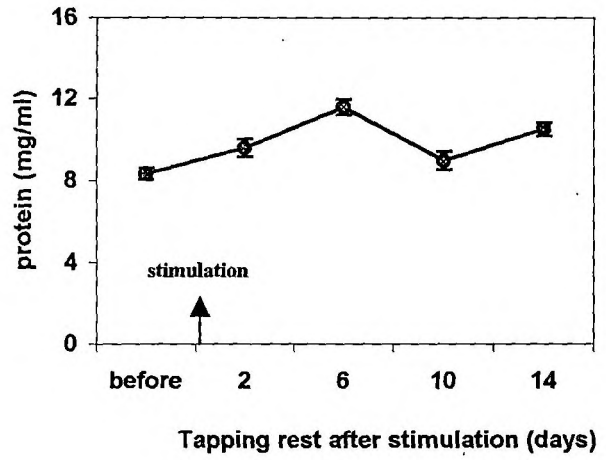


Fig-67 Effect of tapping rest after stimulation on c-serum protein in clone RRII 105 under 1/2S d/4 6d/7 tapping system (mean of six trees ±SE)

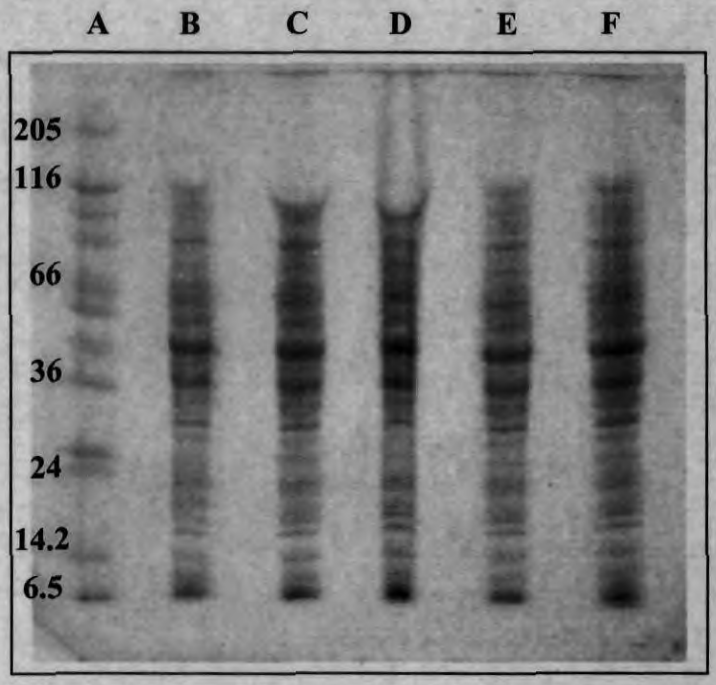


Fig-68. SDS-PAGE profiles of C-serum proteins in clone RRII 105 under ½ S d/4 6d/7 tapping system (30 µg protein per lane)

Lanes
A- Mol.wt markers
B- before stimulation
C- 2-day rest after stimulation
D- 6-day rest after stimulation
E- 10-day rest after stimulation
F- 14-day rest after stimulation

3.3.11. B-serum proteins

Control and stimulated

An overall increase in the content of B-serum protein was observed in stimulated trees for the first three tapping and then decreased to the same level as control trees on fourth tapping (Fig-69).

Stimulation and intensive tapping

A general increase in the protein content of B-serum was observed on all sequential tapping days after stimulation (Fig-70).

Stimulation and tapping rest

A general increase in the content of B-serum protein was observed in trees with different periods of tapping rest when compared to unstimulated control trees (Fig-71).

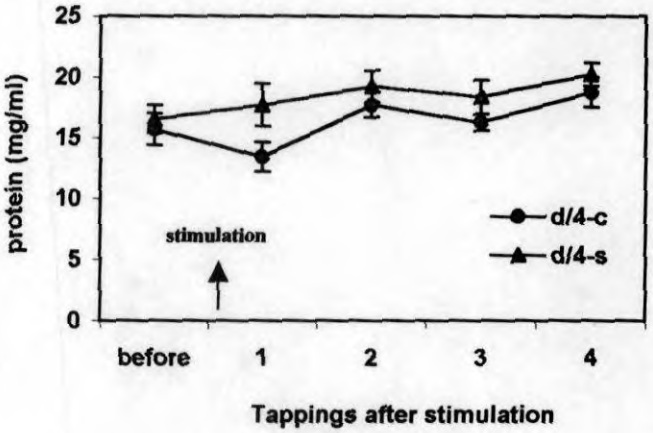


Fig-69. Effect of stimulation on protein (B-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

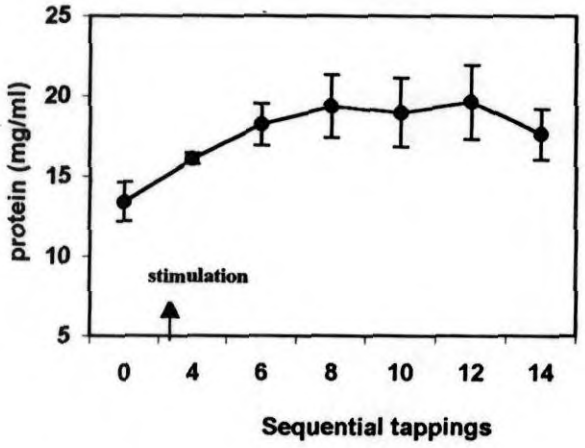


Fig-70 Effect of intensive tapping after stimulation on b-serum protein in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

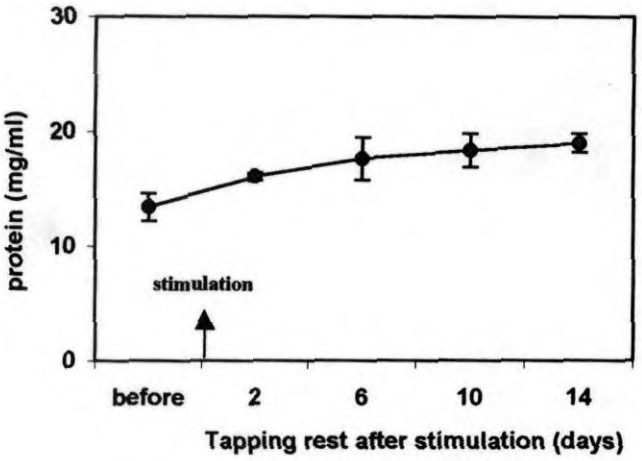


Fig-71 Effect of tapping rest after stimulation on b-serum protein in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

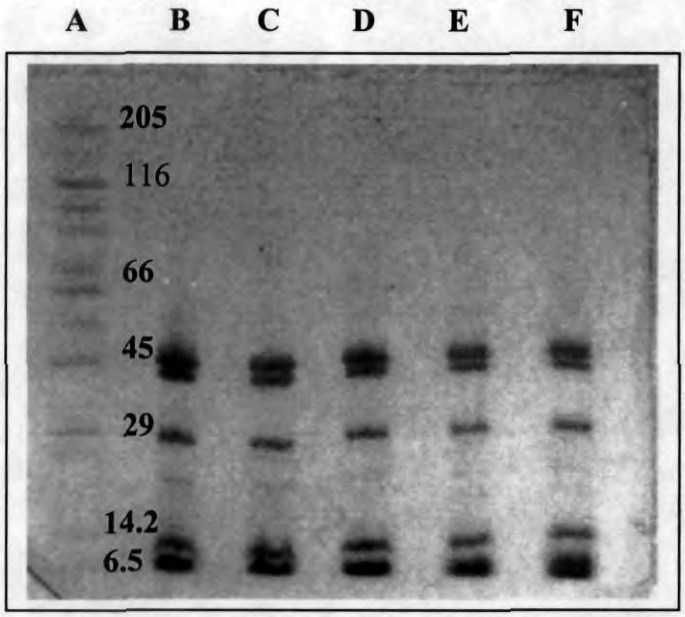


Fig- 72 SDS-PAGE profiles of B-serum proteins in clone RRII 105 under ½ S d/4 6d/7 tapping system (15 µg protein per lane)

Lanes: A- Mol.wt markers
B- before stimulation
C- 2-day rest after stimulation
D- 6-day rest after stimulation
E- 10-day rest after stimulation
F- 14-day rest after stimulation

3.3.12 Glutamine synthetase (C-serum)

Control and stimulated

Glutamine synthetase activity of C-serum was always higher in stimulated trees when compared to control trees on all tapping days (Fig-73).

Stimulation and intensive tapping

Glutamine synthetase activity in C- serum increased after stimulation and intensive tapping. The maximum activity was on second and third intensive tapping and then decreases but higher than that before stimulation (Fig-74).

Stimulation and tapping rest

Maximum glutamine synthetase activity was observed in the C-serum of trees with two and six-day rest after stimulation and then decreases and reached the same as that of unstimulated control trees after 10 and 14 day rest (Fig-75).

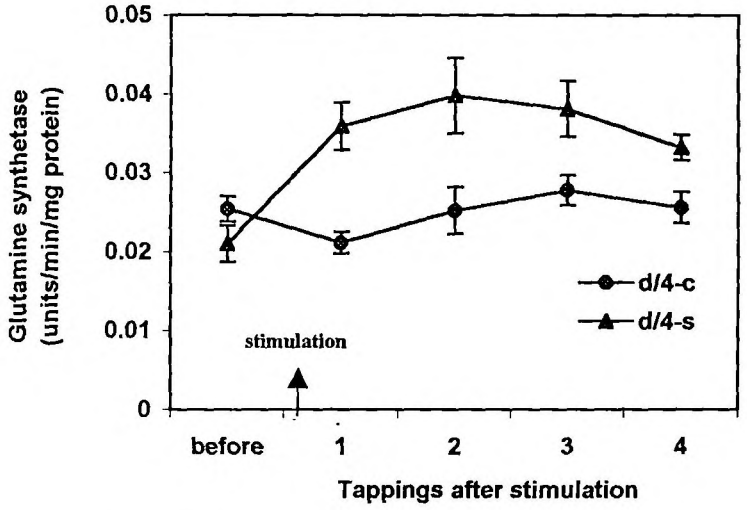


Fig- 73. Effect of stimulation on glutamine synthetase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

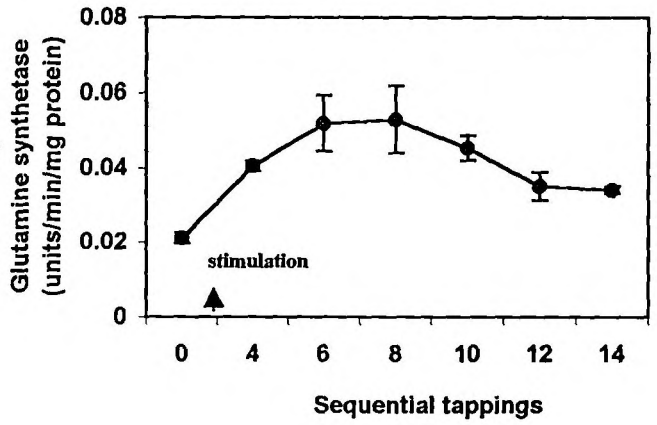


Fig-74 Effect of intensive tapping after stimulation on glutamine synthetase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

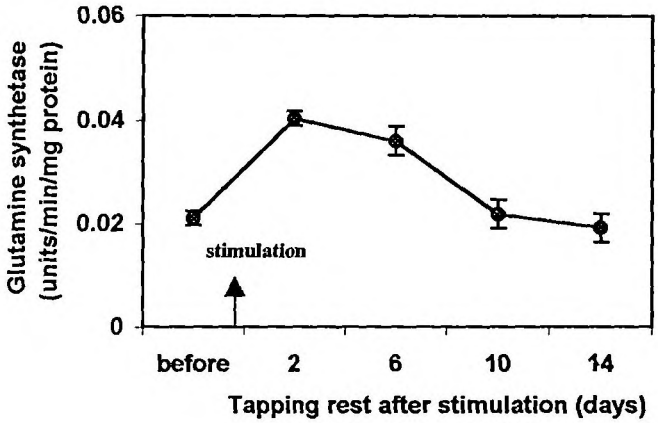


Fig-75 Effect of tapping rest after stimulation on glutamine synthetase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.13. ATP in latex

Control and stimulated

ATP content in latex increases on second tapping onwards after stimulation compared to control trees (Fig-76).

Stimulation and intensive tapping

Maximum ATP content was observed on second and third intensive tapping after stimulation and then decreases (Fig-77).

Stimulation and tapping rest

No variation in ATP content was observed after stimulation and tapping rest (Fig-78)

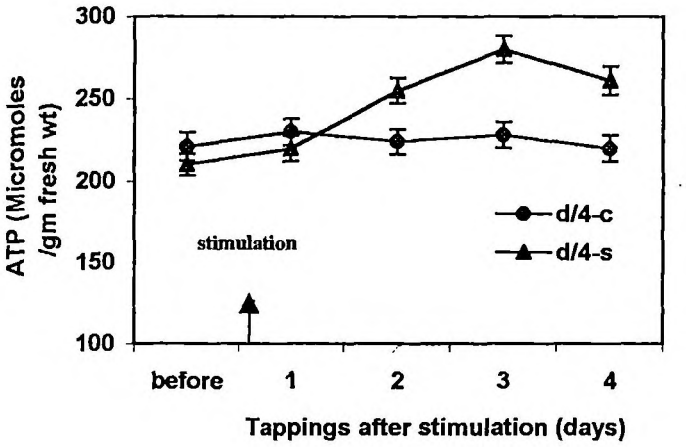


Fig- 76. Effect of stimulation on latex ATP in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

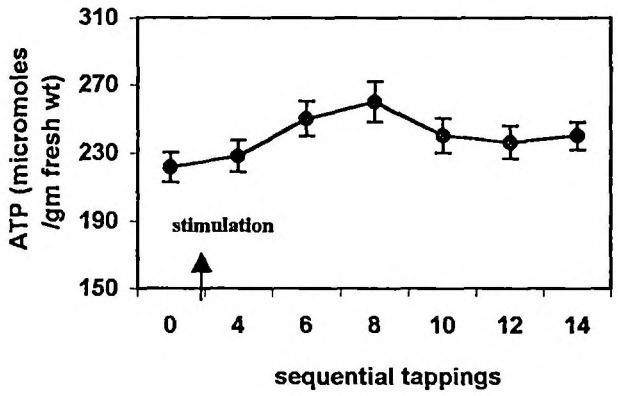


Fig-77Effect of intensive tapping after stimulation on latex ATP in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

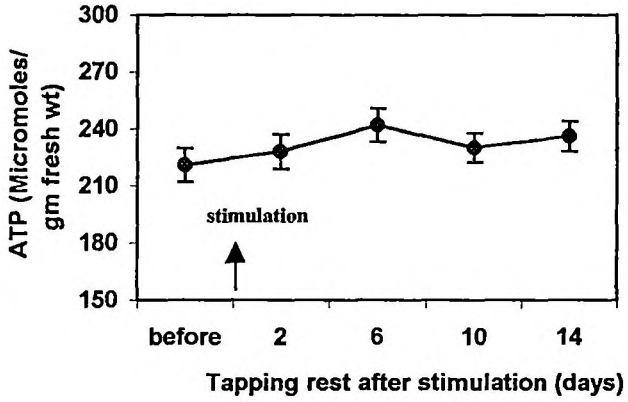


Fig-78Effect of tapping rest after stimulation on latex ATP in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.14 Latex thiols

Control and stimulated

Thiols in latex showed an increase in stimulated trees than control trees on all tapping days. Maximum increase was on second tapping and then slightly decreases but higher than that of control trees (Fig-79)

Stimulation and intensive tapping

Up to second intensive tapping there was no change in thiols in the latex and then a significant increase was observed on the next two tappings and then decreases but higher than that before stimulation (Fig-80)

Stimulation and tapping rest

Maximum thiols in latex was observed in trees with six and ten day rest after stimulation and then decreases in trees with 14 day rest but higher than that of un stimulated trees (Fig-81)

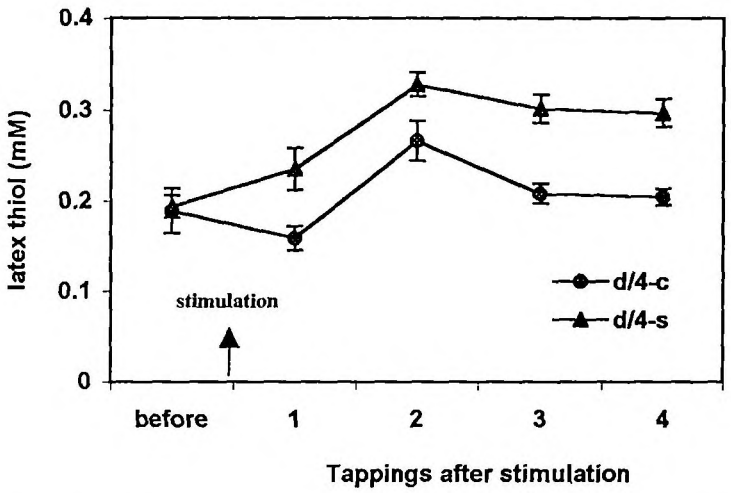


Fig- 79. Effect of stimulation on thiol (latex) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

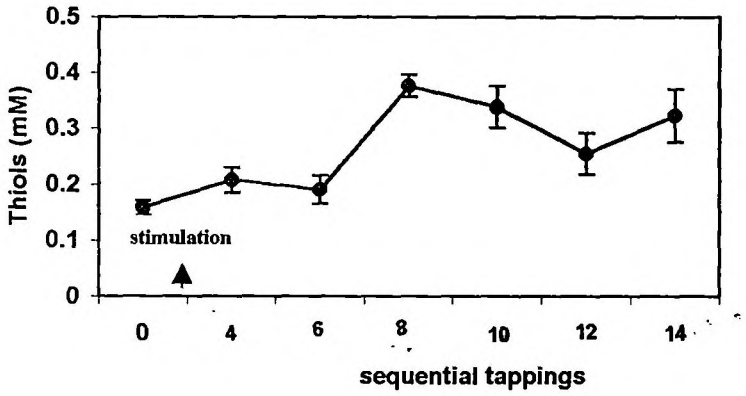


Fig-80 Effect of intensive tapping after stimulation on latex thiols in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

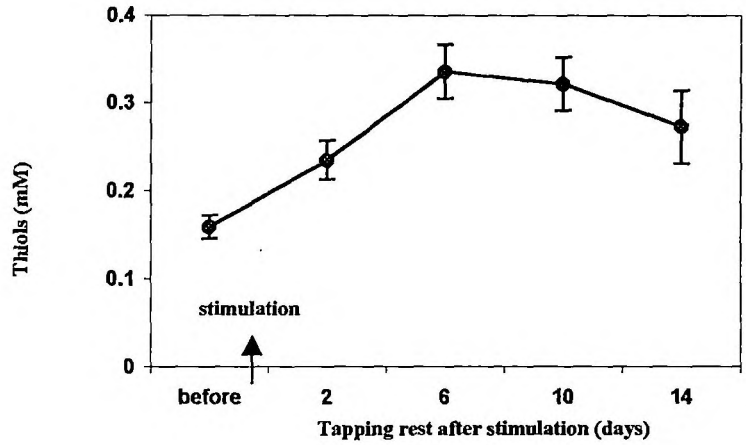


Fig-81 Effect of tapping rest after stimulation on latex thiols in clone RRII under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.15 Thiols (C-serum)

Control and stimulated

Thiols of C- serum in stimulated trees increased on second tapping after after stimulation. Up to second tapping thiol levels were same as that of control trees (Fig-82)

Stimulation and intensive tapping

A general increase in thiol content of C-serum was observed after stimulation and intensive tapping. Maximum thiol was on third intensive tapping (Fig-83)

Stimulation and tapping rest

Thiols in C- serum was maximum in trees with six-day rest after stimulation and then decreased in trees with ten and fourteen- day rest and reached the same level as that of unstimulated control trees (Fig-84)

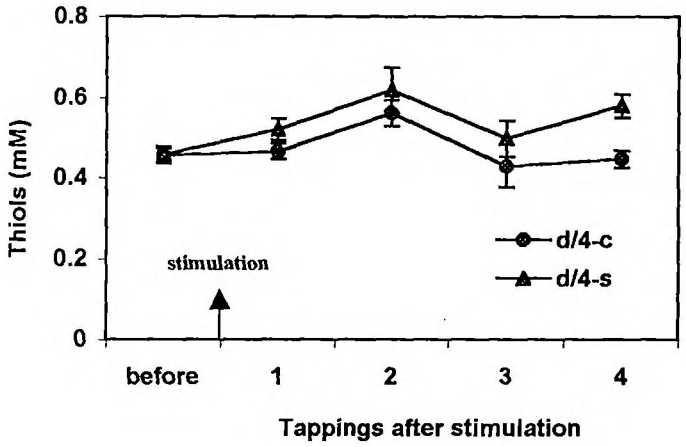


Fig- 82 Effect of stimulation on thiols (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

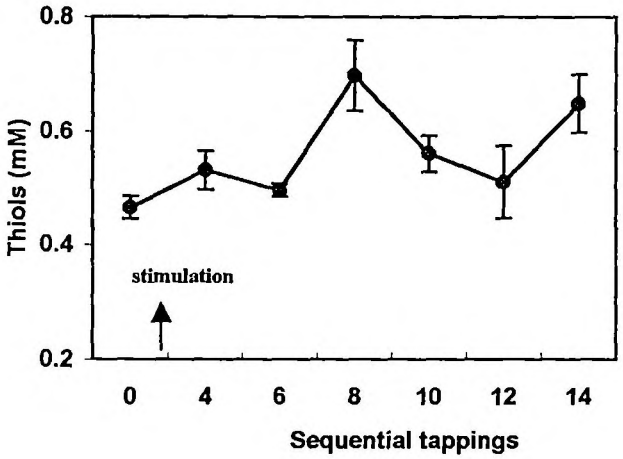


Fig-83 Effect of intensive tapping after stimulation on thiols (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

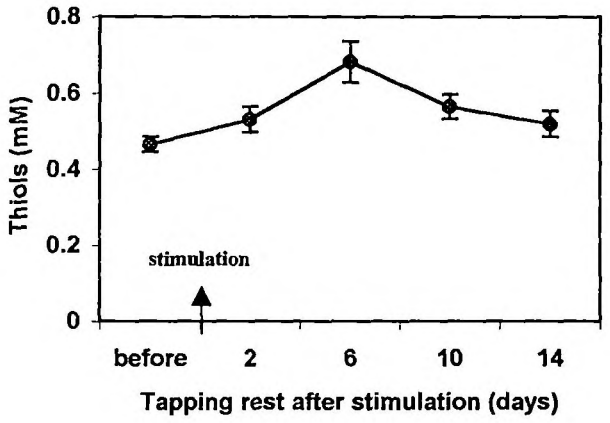


Fig-84 Effect of tapping rest after stimulation on thiols (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.16. Glutathione reductase

Control and stimulated

An increase in glutathione reductase activity in C-serum was observed in stimulated trees after second tapping onwards (Fig-85).

Stimulation and intensive tapping

Maximum glutathione reductase activity in C-serum was observed in second intensive tapping and a drastic reduction was observed after subsequent tappings (Fig-86).

Stimulation and tapping rest

Glutathione reductase activity decreases in the C- serum of trees with 6,10 and 14 day tapping rest. In trees with 2 day rest it was almost same as unstimulated trees (Fig-87).

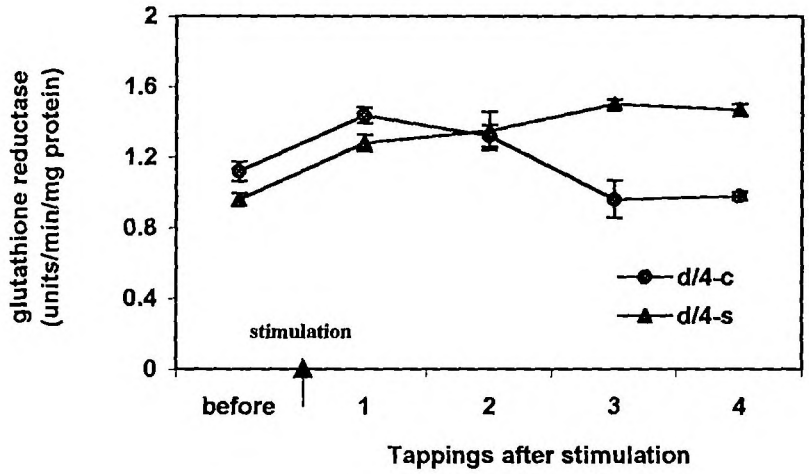


Fig- 85. Effect of stimulation on glutathione reductase (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

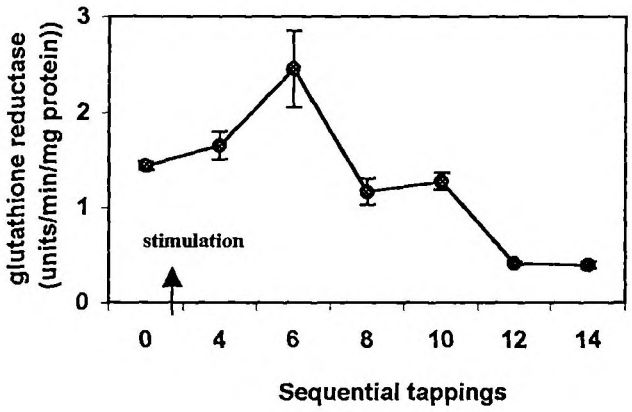


Fig-86 Effect of intensive tapping after stimulation on glutathione reductase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

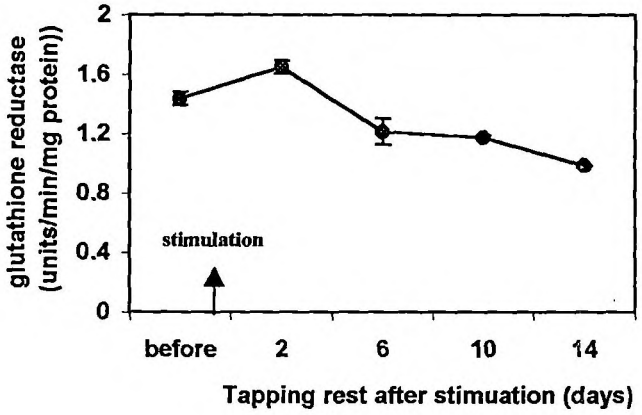


Fig-87 Effect of tapping rest after stimulation on glutathione reductase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.17. Peroxidase

Control and stimulated

A sudden decrease in the peroxidase activity of C- serum was observed in stimulated trees during the first tapping and then increases in the second and third tapping and on fourth tapping it remains the same as control trees (Fig-88).

Stimulation and intensive tapping

Peroxidase activity in C- serum decreases up to fourth intensive tapping and then increases (Fig-89).

Stimulation and tapping rest

A general decrease in the peroxidase activity of C- serum was observed in trees with different periods of rest after stimulation (Fig-90).

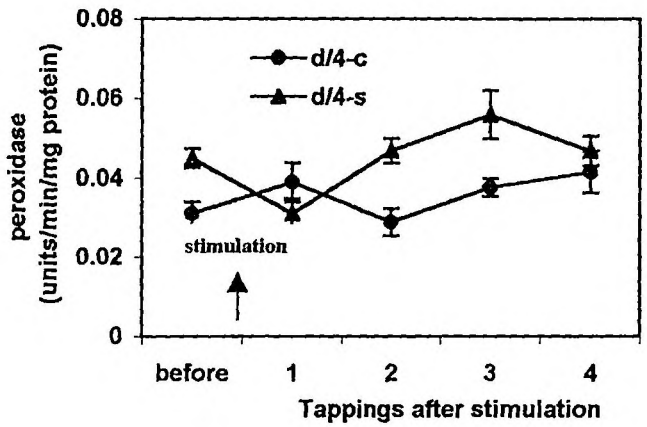


Fig- 88. Effect of stimulation on peroxidase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)
d/4-c: control d/4-s: stimulated

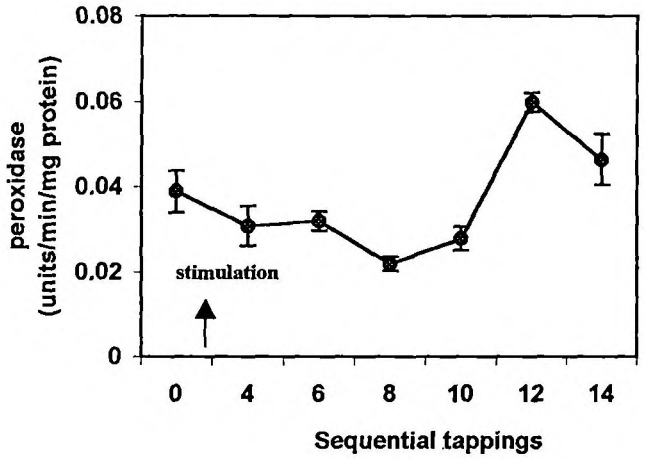


Fig-89 Effect of intensive tapping after stimulation on peroxidase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

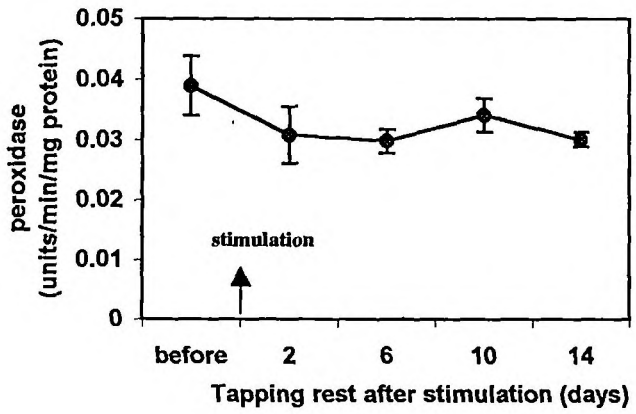


Fig-90 Effect of tapping rest after stimulation on peroxidase activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ± SE)

3.3.18 Super oxide dismutase

Control and stimulated

SOD activity of C-serum of stimulated trees was higher than that of control trees up to third tapping. Then the activity decreases and it was almost same as that of control trees. Maximum activity was observed on second tapping after stimulation (Fig-91).

Stimulation and intensive tapping

Maximum activity of SOD in C-serum was observed on third intensive tapping and then decreases (Fig-92).

Stimulation and tapping rest

Up to six day tapping rest after stimulation SOD activity of C- serum was higher. Trees with ten and fourteen-day rest, the activity was same as that of un stimulated trees (Fig-93).

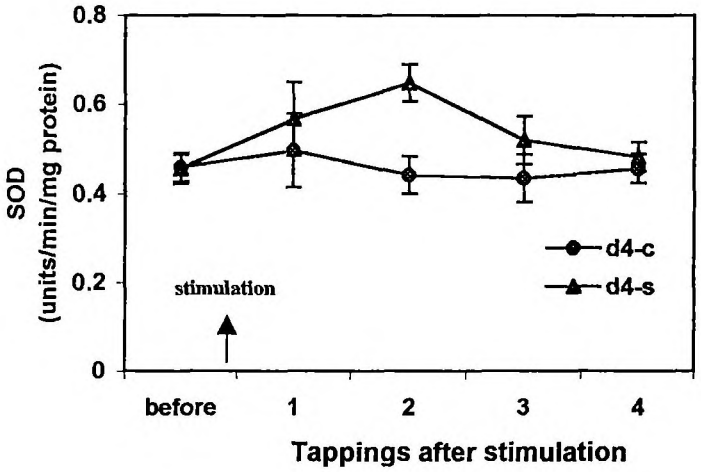


Fig- 91. Effect of stimulation on SOD (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

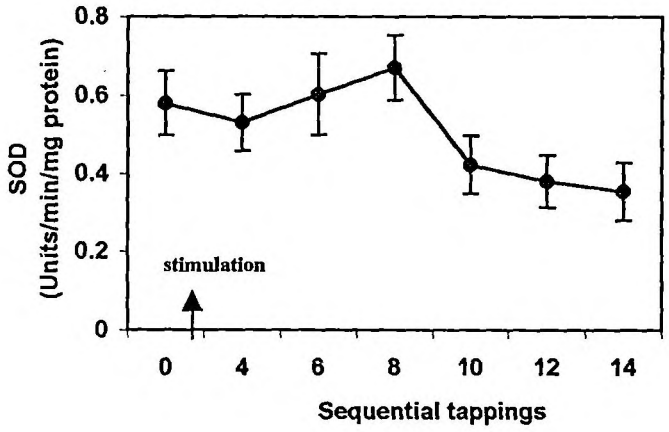


Fig.92 Effect of intensive tapping after stimulation on SOD activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

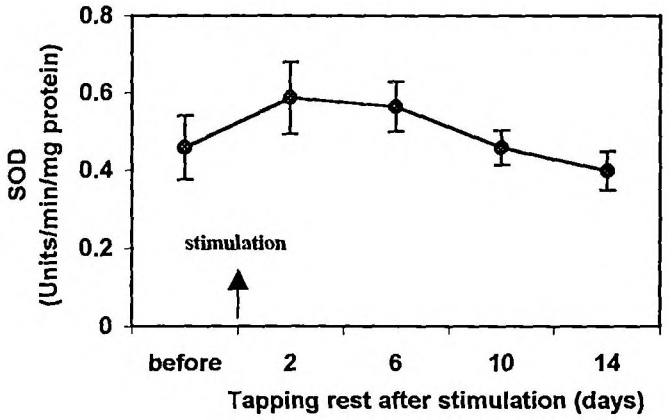


Fig93 Effect of tapping rest after stimulation on SOD activity (C-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.3.19.β-1,3 glucanase

Control and stimulated

A higher β-1,3 glucanase activity in the B-serum was observed in stimulated trees on the first tapping and a decrease was observed on the next tapping and then remained same as that of control trees (Fig-94)

Stimulation and intensive tapping

β-1,3 glucanase activity decreases after stimulation and intensive tapping. Maximum decrease was on fifth intensive tapping (Fig-95)

Stimulation and tapping rest

A general decrease in β-1, 3 glucanase activity was observed when the trees were subjected to different periods of tapping rest after stimulation. In the lattices of trees with 10 and 14-day rest, the activity was less (Fig-96)

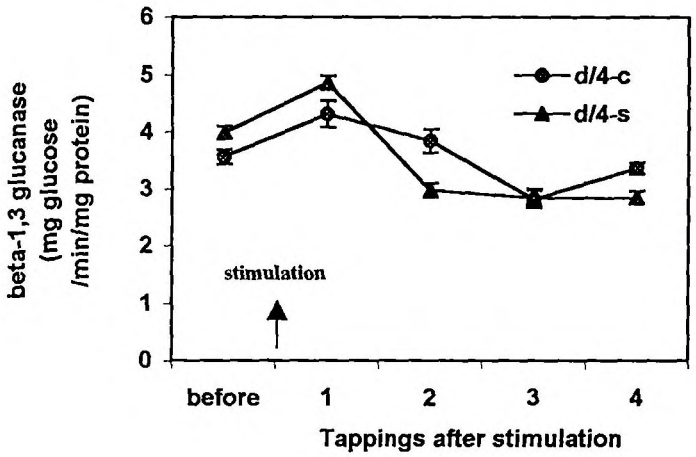


Fig- 94. Effect of stimulation on beta 1,3 glucanase activity (B-serum) in clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)
d/4-c: control d/4-s: stimulated

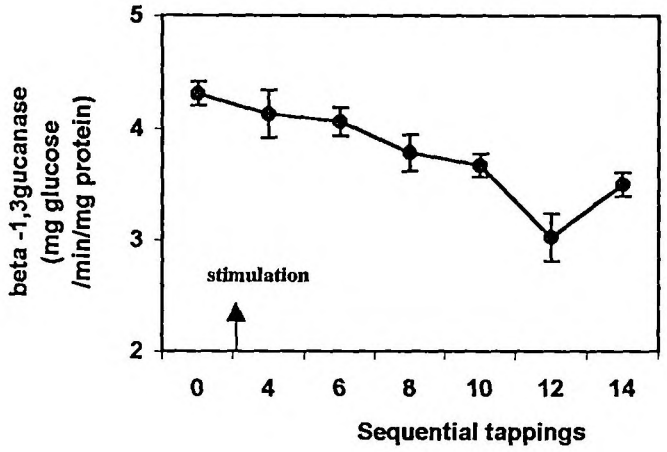


Fig-95 Effect of intensive tapping after stimulation on beta 1,3 glucanase activity (B-serum) of clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

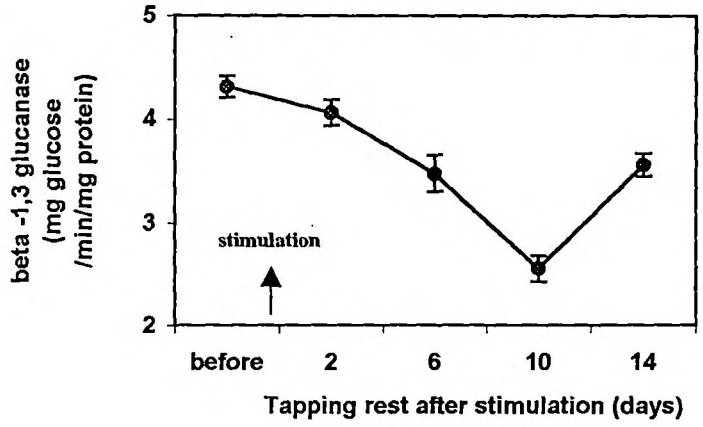


Fig-96 Effect of tapping rest after stimulation on beta 1,3 glucanase activity (B-serum) of clone RRII 105 under ½ S d/4 6d/7 tapping system (mean of six trees ±SE)

3.4. Clonal variations in biochemical parameters associated with latex flow

The results of the study carried out in high and low yielding clones are presented in this chapter. Co variance analysis was used to compare the clonal variations

3.4.1. Clonal variations in yield, DRC and plugging index

The variations in these parameters are presented in Table-17. A significantly high latex yield was observed in high yielding clones when compared to low yielders. A high plugging index was observed in low yielders. DRC did not show any significant change between high and low yielding clones.

Table-17. Total volume, DRC and plugging index of high and low yielding clones of *Hevea* under ½ Sd/2 6d/7 system of tapping.

Category	Clones	Total volume (ml/tree/tap)	DRC (%)	Plugging Index
High yielding	RRII 105	163.66	33.02	3.26
	RRIM 600	152.5	33.08	3.78
Low yielding	HP 20	66.66	38.03	7.52
	RRII 38	75.36	36.06	6.02
CD (0.05)		33.13	4.633	1.25

3.4.2. Clonal variations in thiols, inorganic phosphorus and glutathione reductase activity of C-serum.

The results are presented in Table-18. A high thiol and inorganic phosphorus content in latex was observed in high yielding clones when compared to low yielders. Glutathione reductase activity was higher in clone RRII 105 when compared to low yielders. But in clone RRIM 600 which is also a high yielder, the activity was comparable with the low yielding clones.

Table-18. Variations in thiols and Pi in latex and glutathione reductase activity in C-serum of high and low yielding clones of *Hevea* under ½ Sd/2 6d/7 system of tapping..

Category	Clones	Latex thiol (mM)	Latex Pi (mM)	Glutathione reductase (units min^{-1} mg $protein^{-1}$)
High yielding	RRII 105	0.202	7.9	0.270
	RRIM 600	0.196	5.6	0.211
Low yielding	HP 20	0.132	2.6	0.150
	RRII 38	0.151	3.1	0.142
CD (0.05)		0.041	3.2	0.076

3.4.3. Clonal variations in proteins, glutamine synthetase and β- 1,3 glucanase activities

The results are presented in Table-19. The protein content of C- serum did not show any clonal variation. But a higher protein content of B- serum was observed in low yielding clones when compared to high yielders. β- 1,3 glucanase activities were also higher in these clones. Glutamine synthetase activity was higher in clone RRII 105 but in the other high yielding clone RRIM 600, the activity was comparable with the low yielders.

Table-19. Variations in proteins, glutamine synthetase and β- 1,3 glucanase activity in high and low yielding clones of *Hevea* under ½ Sd/2 6d/7 system of tapping.

Category	Clones	Protein C-serum (mg ml^{-1})	Protein B-serum (mg ml^{-1})	Glutamine synthetase activity in C-serum (units min^{-1} mg $protein^{-1}$)	β- 1,3 glucanase in B-serum (mg glucose min^{-1} mg $protein^{-1}$)
High yielding	RRII 105	13.21	17.48	0.042	2.426
	RRIM 600	13.11	15.97	0.0252	2.243
Low yielding	HP 20	12.23	22.41	0.0254	3.604
	RRII 38	13.25	20.49	0.0138	2 .854
CD (0.05)		1.93	2.52	0.0028	0.250

3.4.4. Clonal variations in antioxidant enzymes

The results are presented in Table-20. Peroxidase activities of C- and B-serum did not show any significant variation between high and low yielding clones. SOD activities of C- and B- serum were higher in high yielding clones when compared to low yielders.

Table-20. Variations in activities of antioxidant enzymes in high and low yielding clones of *Hevea* under ½ Sd/2 6d/7 system of tapping.

Category	Clones	Peroxidase (C-serum) (units /min/ mg protein)	Peroxidase (B-serum) (units /min / mg protein)	SOD (C-serum) (units /min/ mg protein)	SOD (B-serum) (units/min/ mg rotein)
High yielding	RRII 105	0.1485	0.041	0.56	0.85
	RRIM 600	0.1594	0.036	0.65	0.63
Low yielding	HP 20	0.1576	0.023	0.36	0.48
	RRII 38	0.1441	0.013	0.41	0.50
CD (0.05)		0.064	0.0083	0.11	0.09

3.4.5. Clonal variations in adenine nucleotides

The variations are presented in Table-21. High yielding clones showed a high ATP content in latex. No significant difference was noticed in ADP and AMP content of latex between high and low yielding clones. But a higher value of AMP was observed in low yielding clones.

Table-21. Variations in adenine nucleotides in latex of high and low yielding Clones of *Hevea.* Under ½ Sd/2 6d/7 system of tapping

Category	Clones	ATP (µ moles g fr.wt^{-1})	ADP (µ moles g fr.wt^{-1})	AMP (µ moles g fr.wt^{-1})
High yielding	RRII 105	224.53	330.98	126.87
	RRIM 600	244.86	279.84	102.86
Low yielding	HP 20	174.79	150.01	286.72
	RRII 38	129.82	198.51	135.52
CD (0.05)		37.7	79.17	63.26

3.4.6. Variations in ATP/ADP, AEC and ATP ase activities

The results are presented in Table-22. No consistent variations in ATP/ADP or AEC were observed between high and low yielding clones. Significantly high ATPase activities were observed in high yielding clones.

Table-22. Variations in ATP/ADP, AEC and ATPase activities of high and low yielding clones of *Hevea* under ½ S d/2 6d/7 tapping system.

	Clones	ATP/ADP	AEC	ATPase (μ mole Pi/min/mg protein
High yielding	RRII 105	0.677	0.575	5.06
	RRIM 600	0.875	0.615	4.08
Low yielding	HP 20	1.166	0.408	2.31
	RRII 38	0.654	0.494	2.65
CD (0.05)		0.26	0.071	0.92

CHAPTER 4

DISCUSSION

4.1. Seasonal differences in physiological and biochemical parameters associated with latex flow, cessation of flow and production

Yield output of rubber tree varies over years and the yield depressions during stress periods are of particular significance to rubber planters. In India, the period of peak yield is from September to January and low yield is from February to April. In the present study, a significant reduction in dry rubber yield was observed during stress period (Table-1; Figure-1). Several researchers established that at low soil moisture levels, the rate and duration of flow as well as yield were reduced (Buttery and Boatman, 1976; Sethuraj *et al.*, 1984; Devakumar *et al.*, 1988; Vijayakumar *et al.*, 1988 and Rao *et al.*, 1990). Rao and Vijayakumar, (1992) reported that the lower yield in March- April is due to the combined effect of high temperature and soil moisture stress, in addition to the effects of defoliation and refoliation.

Latex flow characteristics such as initial flow rate, plugging index and turgor pressure and bursting index were also altered during stress (Table-3). A low initial flow rate and a high plugging index observed during stress period were

in confirmation with the results of Milford *et al.*, 1969; Paardekooper and Somosorn, 1969; Yeang and Paranjothy, 1982).

Premakumari *et al.*, (1982) reported that seasonal variation in PI could be related to changes in the phospholipid content of lutoid membrane. PI is easily influenced by environmental factors especially soil moisture content (Saraswathyamma and Sethuraj, 1975; Sethuraj and George, 1976).

The high bursting index during stress period indicate that lutoids become less stable and easy flow of latex was restricted due to the bursting of lutoids and plugging of the laticiferous vessels. This result is in confirmation with the earlier reports (Jacob *et al.*, 1985; Dey *et al.*, 1995).

No change in latex vessel turgor was observed during stress period. Buttery and Boatman (1965); Gomez, (1983), Pakianathan *et al.*, (1988) reported that turgor pressure is a dynamic factor easily influenced by the components of the water relations of the plant. Devakumar *et al.*, (1988) reported that summer yield drop was found to be low in clone RRII 105 and it showed a higher latex vessel turgor and low solute potential during stress period when compared to other clones.

The significant reduction in dry rubber content and total solid content observed during stress period (Table-1; Figure 2&3) in clone RRII 105 is in conformation with the result of Dey *et al.*, (1995).

Decreased latex phenol during stress period (Table1, Figure 5) indicates that the phenols may be oxidized to quinones under the catalysis of peroxidase in

latex. Maximum decrease was observed in February. No significant change in latex proline was observed between peak and stress season (Table-1; Figure-6). Many studies have proved that water stress caused proline accumulation within plants and help to maintain the normal function of plant cell through its osmotic adjustment (Tang Zhang Cheng, 1984). Maximum proline content was observed in January.

Increased latex thiol content (Table-1; Figure-9) during stress indicating a certain amount of protection of laticiferous system from peroxidative degradation. Thiols trap the toxic oxygen species produced during stress and prevent the damage. A general activation of glutathione pool in C-serum was also observed during stress (Table-4). Higher activities of glutathione reductase indicate that the oxidized glutathione formed during stress are converted to reduced glutathione and maintain the GSH/GSSG ratio. GSH regeneration requires energy (ATP) and partly depends on in situ sucrose availability. Sucrose rich laticifers regenerate RSH more easily. There is competition between isoprene synthesis and GSH regeneration.

A decrease in latex ATP and ADP during stress (Table-6) revealed a lower activation of metabolism. AMP accumulates during stress and is not completely converted to ATP. Adenylate Energy Charge (AEC) of the system, which reflects the status of the cellular metabolic activity, is also decreased during stress.

In situ latex renewal especially isoprene synthesis requires a great deal of energy. The biological mechanism linked to flow during tapping also requires

biochemical energy (Jacob *et al.*, 1997). ATP is also the specific sustrate for the proton pumping ATPase located on the lutoid membrane, which is involved in the regulation of cytosolic pHand energises the transport and accumulation of various solutes inside the lutoids.

When ATP level is low, the ATPase proton pump functions slowly and the cytosol pH decreases and a reduction in anabolic processes i.e rubber synthesis slows down.

High activities of C and B- serum peroxidase and a low catalase in C-serum were observed during stress period. B-serum catalase did not show any variation between seasons. Both C- and B- serum SOD was lower during stress period (Table-6). Coupe *et al.*, (1972) reported that 60-80 % of the peroxidase activity was located in lutoids. The increased peroxidase activity in C- serum during stress indicates that the enzyme was released from lutoids and entered into the cytosolic serum. C- serum peroxidase produce quinones, which inactivate certain enzymes, clustering of proteins and it is one of the factors responsible for the peroxidative damage of lutoid membranes as evidenced by Chrestin *et al.*, (1984) and Chrestin and Bengratz, (1984). In the present study lutoid damage was observed during stress as indicated by the increase in bursting index of lutoids. A low SOD activity in C- and B- serum and a low catalase activity in C-serum leads to the accumulation of superoxide radicals and the formed hydrogen peroxide is removed partly by B-serum peroxidase. They consume hydrogen peroxide and prevent the production of more toxic $OH^{\cdot}$. And thus provide a certain

amount of protection. There was no significant difference between B-serum catalase between two seasons.

Rubber production is closely related to the tolerance of laticiferous system to stress. Latex exploitation by successive tapping induces wounding stress in cells and metabolic stress related to latex regeneration between two tappings and other abiotic stresses. During these situations, toxic oxygen species are generated and involved in the proteolytic degradation and damage of lutoid membrane and release of destabilizing factors from lutoids in to the cytosol and cause early cessation of flow. Also the presence of an NADH quinone reductase in the lutoid membrane, which produces super oxide radical, was reported by d' Auzac *et al.*, (1986). When the generation active oxygen species exceeds that the rate of their removal, peroxidative damage of lutoid membrane occurs that destabilizes latex. Even though thiol metabolism is activated during stress (Table-7) it is not enough to prevent the damage since antioxidant enzymes are less.

Higher protein content in C- and B- serum indicates loss of protein during stress (Table-7) and results in a greater metabolic stress in the panel. The acidic protein hevein is the major protein of B-serum of latex (Archer, 1969). Stabilizing and destabilizing effect of hevein in latex coagulation was reported by Gidrol *et al.*, (1994); Ukun *et al.*, (1999). Jacob *et al.*, (1997) reported that latex coagulation is under the control of hevein and β-1, 3 glucanase (which induce coagulation) and N-acetyl glucosaminidase and chitinase (which slows down coagulation) of latex. In this study, a higher hevein, β -1,3 glucanase and a low N-acetyl

glucosaminidase activity in lutoids were observed during stress (Table-8, Fig-18.). Chitinase did not show any significant variation between seasons (Table-8). This implies that under normal conditions hevein does not interact with rubber particles immediately but during stress due to high lutoid instability, the contents of lutoids were released earlier into the cytosol and destabilize latex.

4.2. Variations in yield, physiological and biochemical parameters associated with different tapping frequency in clone RRII 105.

A significant increase in dry rubber yield was observed in trees under high frequency tapping system when compared to low frequency tapped trees (Table-9; Figure-19). This reduction may be due to the fact that the trees were newly opened and un stimulated. High per tap yield in unstimulated d/4 tapped trees of clone RRIM 600 were reported by Do KimThan *et al.*, (1996). The physiological and biochemical factors related to latex flow and its cessation were also affected by tapping frequency. In this study yield, physiological and biochemical parameters are based on the mean of peak yielding period of 1999 (Trees were at the second year of tapping).

Trees tapped on d/4 frequency in contrast to d/2 recorded consistently higher readings for the various physiological parameters associated with latex flow namely PI, IFR, TSC and BI (Table7&10). This implies that trees tapped on d/4 frequency have flow limiting factors such as high PI and BI. These results are in confirmation with the earlier reports (Do Kim Than *et al.*, 1996). Higher values of TSC in d/4 trees (Table-9, figure-21) showed that these trees have enough time

to regenerate the cell contents. Also a high TSC have an effect on latex viscosity and limit latex flow (d' Auzac and Jacob, 1984; Vangils, 1951). DRC showed a decrease in d/4-tapped trees compared to d/2 (Table-9; figure-22). Variation in latex yield due to tapping frequency could not be explained by variations in latex vessel turgor (Table- 10)

A high-reduced thiols in C-serum in d/2 tapped trees (Table-11). Reduced thiols can play an important role in the mechanism governing lutoid stability and hence latex flow. A high thiol content activates enzymes involved in carbohydrate metabolism and detoxification of active oxygen species and protect the lutoid membrane from degradation. A significantly high glutathione reductase indicates active conversion of oxidized glutathione to its reduced form.

In the present experiment, there were no significant differences in C-serum peroxidase and catalase activities between the two treatments (Table-12). B- serum peroxidase was higher in d/2-tapped trees this may be due to induction of the enzyme due to high frequency tapping and higher drain of latex. A high C- and B- serum SOD in d/2 tapped trees (Table-12) indicates active removal of superoxide radicals to hydrogen peroxide and it is removed using B- peroxidase and thus protects the membranes from degradation.

Higher protein content in d/4-tapped trees was observed when compared to d/2-tapped trees. Glutamine synthetase activity in C- serum was also higher (Table-13). So the removed protein during tapping was efficiently regenerated before the next tapping in d/4 tapped trees. The latex regeneration metabolism

involves not only rubber biosynthesis but also the reconstitution of the lost sub cellular components during tapping. The main enzyme necessary for this regeneration is glutamine synthetase which provides glutamine, which is the major nitrogeneous intermediate involved in the formation of aminoacids and nucleotides necessary for the synthesis of proteins and nucleic acids. Latex ATP content, which is the physiological activator of glutamine synthetase, is significantly high in d/4-tapped trees when compared to d/2-tapped trees (Table-10).

SDS-PAGE profile of C and B- serum proteins did not show any variation between high and low frequency tapped trees (Fig-25&26). Some bands appear to fluctuate quantitatively but no consistent correlation can be drawn between their accumulation and tapping frequency (Table14&15).

The activity of destabilizing enzyme β- 1,3 glucanase was high and N-acetyl glucosaminidase activity was low in d/4 tapped trees compared to d/2 tapped trees. Hevein content is also high in d/4-tapped trees (Figure-27). No significant difference was observed in chitinase activity between treatments (Table-16). This is related to the higher plugging index observed in the d/4 trees. Subronto *et al.*, (1996) presented evidence that purified preparations of hevamine and glucanase destabilize rubber particles with a greater destabilizing capacity of glucanase. It is apparent from this study that different factors were limiting latex flow in d/2 and d/4 tapped trees. Under d/2 the limitation is the short interval between tapping and in d/4 the physiological and biochemical factors that cause high PI and BI.

Stimulation is necessary to remove the flow limiting factors to get comparable yield from low frequency tapped trees.

4.3.Biochemical changes associated with Tapping Panel Dryness

TPD has long been recognized as a serious problem in rubber plantations, which have serious economic implications. TPD incidence was higher in high yielding clones. Paranjothy *et al.*, (1975) and Chrestin, *et al.*, (1985) reported that TPD development could be linked to changes occurring in certain physiological and biochemical parameters in latex. Most of the earlier investigations on TPD were on trees, which were induced to become dry with either intensive tapping or by over stimulation. The study reported here was on newly opened trees, which developed dryness naturally when subjected to a normal tapping system without stimulation. Differences in proline , proteins of C-serum and thiols were observed in both the affected trees after the incidence of dryness. Increased level of proline, phenol and reduction in thiols are in agreement with the earlier reports (Faridah Yusof *et al.*, 1985: Yang Shaoquing *et al.*, 1997). Increase in protein content of C-serum was not reported earlier. There were no abnormal changes in these parameters before the onset of partial dryness compared to normal trees. Reduction in thiols leads to damage of lutoid membrane by peroxidative degradation. Increased protein content in C- serum indicates more loss of protein. In the bark protein content is low after the incidence.

4.4. Immediate effects of stimulation on yield , physiological and biochemical parameters in clone RRII 105 under 1/2S d/4 6d/7 tapping system.

It is known that when high frequency tapping is combined with continuous stimulation, there is progressive decline in response to ethephon stimulation (Sivakumaran *et al.*, 1981). So low frequency-tapping system with ethephon stimulation have gained importance to get more yield and to reduce the cost of production.

The reduced number of tappings obtained with low frequency systems would results in lower yield per hectare per year. Thus it is necessary to couple adequate stimulation to enhance yield per tapping and yield per hectare by enhancing the latex flow and overcome flow-limiting factors (Gohet, 1991). Stimulation played a key role in achieving the desired productivity from low frequency tapping system (Sivakumaran and Ismail Hashim, 1986; Sivakumaran, 1994; Vijayakumar *et al.*, 2000; Karunaichamy *et al.*, 2001). But immediate changes in biochemical factors influencing latex flow and its cessation under low frequency tapping system with stimulation has not been attempted.

An increased latex yield was observed after stimulation on all tapping days (figure-38). In the present study turgor pressure was not affected by stimulation (Fig-41). Pakianathan *et al.*, (1982) showed that TP of latex producing tissue diminishes in stimulated trees. Low, (1978) reported that fall in turgor pressure is correlated with a fall in total cyclitols.

A decreased bursting index (Fig-50) observed after stimulation indicates better lutoid stability. Ribaillier, (1972) reported that lutoids from stimulated trees gained new properties and destabilizing activity of B- serum was considerably reduced by stimulation.

Decrease in TSC (figure-56) is caused by the dilution of latex. This result is in confirmation with the results of Prevot *et al.*, (1986); De jonge, (1955); Primot *et al.*, (1979). TSC of stimulated trees were always lower than un stimulated trees up to third tapping. After that it increases but still lower than control. The lowering of TSC was due to the increased influx of water into the laticifers. The recovery of TSC after third tapping could be due to the decline in effectiveness of the stimulant.

In this study the thiol content of latex and C-serum increases after ethephon treatment (figures 79&82). This result is in conformation with the result of Koshy, (1997). Prevot *et al.*, (1986) and Lacrotte *et al.*, (1988) reported a decrease in thiol content 24 hours after treatment and it tends to return to normal and then exceeds the initial level.

A suitably high thiol content is necessary to protect the intactness of lutoid membrane from the peroxidative damage caused by the production of toxic oxygen species by the activation of NADH quinone reductase in the tonoplast when ethephon was applied.

Glutathione reductase activity of C-serum increased after stimulation (figure-85). The capacity of the cellular antioxidant system is determined by the pool sizes of GSH and GSSG and by the ability of the metabolism to keep them in an active reduced form. Glutathione reductase, which converts, oxidized glutathione (GSSG) into its reduced form (GSH). This enzyme has a great affinity for its cofactor NADPH. The NADPH availability in the cytosol is probably a major regulatory factor. Certain quinoid compounds generated during peroxidation are strongly inhibits the enzyme (Prevot *et al*, 1986).

The rise in ATP level after ethephon treatment (figure-76) indicates general metabolic activation. ATP is the physiological regulator in the whole metabolic pathway leading to rubber biosynthesis (Jacob *et al.*, 1997, Kekwick, 1989). ATP is the specific substrate for H+ pumping ATP ase implicated in the regulation of pH of the cytosol. High ATP content in stimulated trees leads to more activation of ATP ase and maintain the pH and increases the yield. Early disturbance of ATP might result from enhancement of the utilization of this in the synthesis of nucleic acids and proteins. The increase in ATP of latex was observed from second tapping onwards. ATP content of latex remained fairly constant through out the experiment in un stimulated control trees.

The increase in phenol content after stimulation (Fig-59) is a common effect of wounding by tapping and stimulation. Phenols are accumulated in tissues at the time when the plants are in a state of stress. Xu wenxian *et al.*, (1986)

reported a positive correlation between phenol and production and an increase in phenol content due to stimulation in clone PR 107.

An overall increase in C- and B- serum protein and C-serum GS (Fig-65,69&73) indicates that ethylene triggers a general activation of protein synthesis in latex. Protein content of latex is efficiently controlled with full regeneration of the lost protein under conditions of ethylene induced latex removal. This result is in confirmation with the result of Valerie Pujade *et al.*, (1994). The increase in GS activity may not be the direct action of ethylene but through secondary messages. ATP, which is the physiological activator of GS, is also enhanced after stimulation (Fig-76).

Chrestin *et al.*, (1984); Chrestin and Bengratz, (1983) reported that stimulation causes an activation of NADH- quinone reductase present in the lutoid tonoplast. This enzyme produces toxic superoxide radicals, which may destroy the lutoid membranes by lipid peroxidation unless this toxic species are removed by protective enzymes. Molly Thomas *et al.*, (1999) reported an increased peroxidative damage and accumulation of malondialdehyde in latex of ethephon treated trees compared to control trees in clone RRII 105.

Wholeness of lutoids is necessary for the proper functioning of the proton pumps operating in the lutoid membrane, which maintain the correct balance of ions and pH in the C- serum.

The results showed that even though there is production of toxic oxygen species due to tapping and stimulation,, the antioxidant system is efficient in removing

these molecules and protect the membrane of lutoids from damage for a long time in ethephon treated trees as evidenced by the increase in SOD activity (Figure-91).

Regeneration time of latex has led to envisaging and then proposing in practice a reduction in tapping frequency. There must be sufficiently long interval between two tapping to allow adequate regeneration of latex. Stimulation accelerates the regeneration phenomenon as evidenced by the increase in glutamine synthetase activity.

In the present study, associated changes after stimulation in low frequency tapped trees include a reduced BI of lutoids , high ATP and C-serum thiols, higher activities of C-serum glutamine synthetase , SOD and glutathione reductase . These are transitory changes or observed after the first tapping. Parameters not affected by stimulation are turgor pressure (Fig-41) and initial flow rate (Fig-44).

4.5. Stimulation and intensive tapping

It has been shown that excessive flow of latex due to over exploitation and over stimulation is a primary cause for Tapping Panel Dryness (Xu Wenxian *et al.*, 1983). The physiological damages caused by stimulation or intensive tapping give rise to physiological imbalance between latex production and latex flow.

In this experiment, when the low frequency tapped trees after stimulation are changed immediately to high frequency, latex yield was found to be maximum on third and fourth intensive tapping and then decreases (figure-39). A general decrease in PI, TSC and DRC (Fig-48, 57 &54) were observed. Bursting index

showed a decrease up to third tapping and then increases. TP and IFR did not show any variation after stimulation and intensive tapping (Fig-42 &45).

An increase in peroxidase activity was observed (Fig-89) with an increase in consecutive tapping after stimulation. Peroxidases produce quinones, a product of phenol in latex and would further form highly toxic semiquinone and superoxide radicals through non enzymatic reactions and damage the laticiferous cells and it is one of the factors responsible for the peroxidative degradation of the membrane of lutoids. The higher phenol (Fig-60) and peroxidase activity indicate that stimulation and intensive tapping lead to an increased peroxidative reaction. But no drop in thiol content was observed in latex or C-serum. Hence the trees possess some protection from the higher peroxidative reaction. Glutathione reductase, which converts oxidized glutathione formed if any, to its reduced form is very low in intensively tapped trees. This is because the requirement of the enzyme is less when the thiol levels are high.

SOD, the major enzyme involved in the protection of cells against reactive oxygen species, is reduced after stimulation and intensive tapping. The imbalance between the toxic peroxidative and scavenging activities is an important factor for membrane damage and reduction in yield.

Glutamine synthetase, which is the major enzyme, involved in protein renewal decreases after third tapping but higher than that before stimulation. This indicates draining of enzyme after every intensive tapping. The rate of synthesis of the enzyme is not balanced with the rate of removal. The variation in the

concentration of physiological regulators viz. latex ATP and pH may also influenced the activity of this enzyme.

A general increase in C- and B- serum proteins was observed after stimulation and intensive tapping. This indicates that ethylene triggers a general activation of protein synthesis or protein turn over in the latex.

4.6. Stimulation and tapping rest

When the low frequency tapped trees after stimulation were given tapping rest for different periods such as 2, 6,10 and 14 days, it was observed that maximum yield was obtained in trees with 6 day rest after stimulation and then decreases (figure-40). Minimum PI and BI were observed in trees with six-day rest (figures 49&52). A high PI and BI was observed in trees with 10 and 14 day rest after stimulation. TSC decreases in these trees. Latex phenol showed an increase up to 10 day rest and then decreases. Proline content in latex and thiols in C-serum reached to the initial level after 14-day rest. When tapping rest is given, removal of latex is stopped and these compounds take some time to reduce the rate of their biosynthesis.

C-and B-serum protein level did not show any change after stimulation and tapping rest. It may be due to the maintenance of steady state level by synthesis of the protein at a rate just sufficient to replenish the protein lost by degradation. SDS-PAGE profile of C-and B- serum proteins did not show any variation in trees with different periods of tapping rest (Fig-68&72).

GS activity decreases after six-day rest (Fig- 75). In trees with ten and 14-day rest after stimulation, the GS activity was same as that of unstimulated trees. This indicates that the level of this enzyme may be under rapid control. If not needed they rapidly disappear but they can be synthesized quickly when needed. When supply of glutamine is high, the synthesis of GS may shut down.

Glutathine reductase activity is also less in trees with 10 and 14-day rest. The requirement of the enzyme is less when latex is not removed. Peroxidase activity decreased because of less intensive tapping. SOD also reached to the initial level after 10 and 14-day rest. The parameters not affected by tapping rest after stimulation were TP,.C- and B- serum protein and latex ATP.

4.7.Clonal variations in yield and associated biochemical changes

In this study, a significantly high latex yield was observed in high yielding clones compared to low yielding clones. Plugging index was low in high yielders compared to low yielders. Dry rubber content did not show any variation between high and low yielders (Table-13). PI indicates the intensity of flow restriction mechanism operating in the latex vessel after tapping. Lutoid stability is the predominant factor influencing PI. Clonal variations in PI have been well established (Milford *et al.*, 1969; Saraswathyamma and Sethuraj, 1975, Dey *et al.*, 1995; Usha Nair *et al.*, 1990). A higher PI in low yielding clones indicates difficult latex flow and lower yield.

A significantly high thiols and glutathione reductase activity in clone RRII 105 (Table-14) indicate active an active metabolism and protection of lutoid

membrane from peroxidation phenomenon induced by toxic oxygen species. In clone RRIM 600, which is also a high yielder showed an increase but statistically non significant.

No significant variation was observed in C- serum protein between high and low yielding clones. Latex from low yielding clones contain significantly higher amount of B-serum proteins. Also the destabilizing enzyme β -1,3 glucanase activity was significantly high in these clones (Table-15).

Glutamine synthetase activity was significantly high in clone RRII 105. But it was low in clone RRIM 600 which is also a high yielder and the activity was comparable with low yielding clones (Table-15). This indicates that efficient regeneration of latex takes place in clone RRII 105. The physiological activator of this enzyme ATP is also high in clones RRII 105 and RRIM 600. The other regulatory factors such as pH or substrate may be involved in these differences in activities.

No consistent pattern was observed in c-serum peroxidase between high and low yielders. Significantly high C and B- serum SOD was observed in high yielding compared to low yielders (Table-16). Chrestin *et al.*, 1984 reported that the activity of NADH quinone reductase, which produces toxic oxygen and leads to membrane damage, was high in low yielding trees and trees affected with tapping panel dryness. The activity of SOD is not enough to remove these species in low yielding clones and this leads to membrane damage and release of destabilizing factors. In high yielding clones there is active removal of these toxic species takes

place because of high SOD and the formed hydrogen peroxide is removed using B- serum peroxidase (Table-16).

A significantly high ATP content in high yielding clones indicates active metabolism. (Table-17). Adenylate energy charge of the system, which is a measure of the energy status, was high in high yielders. In the low yielding clone HP 20 it was significantly low because of high AMP but in clone RRII 38, a higher value was observed and AMP is low.

CONCLUSIONS

1.Eventhough thiol metabolism is activated during stress, decline in cytosolic scavengers such as SOD and catalase leads to peroxidative degradation of membranes, in situ coagulation and reduction in yield.

2. Tapping frequency has considerable influence on latex flow characteristics and nd biochemical parameters. High plugging and bursting index in unstimulated low frequency tapped trees were observed. Associated changes include low thiols, SOD and high β -1,3 glucanase activities.

3. A reduction in thiol and protein in the bark and increased level of proline and proteins in C- serum was observed in trees which developed partial dryness naturally when these trees were subjected to a normal tapping system without stimulation.

4. The present study shows that regulation of enzymes such as SOD, Glutathione reductase and glutamine synthetase is more important in latex flow particularly during stimulation. In intensively tapped trees draining of enzymes are more and rate of synthesis is not balanced with rate of their removal.

5. In conditions where tapping rest is given after stimulation, the synthesis of these enzymes may shut off since latex is not removed and resynthesised quickly when needed. Regulation of enzymes is a key way of physiological balance.
6. Significant clonal variations were observed in the case of SOD in C- and B-serum, thiols and ATP content in latex.

Results from all these studies bring out the importance of SOD, glutathione reductase, ATP and thiols as important parameters in regulating latex flow. SOD and thiols are probably very important in lutoid stability and hence latex flow. The role of peroxidase is different; it does not act as an antioxidant in these situations.

The yield in *Hevea* is closely related to the resistance of the laticiferous system to oxidative stress. Under stress situations, free radical production leads to lutoid damage, rubber particle aggregation and plugging. The role of SOD has always been reported as a protective enzyme and its high level is always considered to be a desired trait. However the present study reports down regulation of SOD might be useful for laticifer system to reduce the drain of latex after stimulation and intensive tapping. This is achieved by increasing the free radical damage of the lutoids leading to high rate of plug formation. However under extreme conditions of stress, such down regulation of SOD might lead to damage of lutoids and other membrane structures within the latex vessels and leads to Tapping Panel Dryness.

SUMMARY

Hevea brasiliensis is the major commercial source of Natural Rubber (cis, 1,4 polyisoprene), which is obtained from the latex of this plant. Latex is the cytoplasm of specialised cells called laticifers. Upon tapping laticifers are severed and expels latex, which contains 30-60 % rubber. Besides rubber the latex contains various organelles including lutoids (single membrane bound microvacuoles with lysosomal characteristics). The production of rubber by the laticiferous system of *Hevea* depends on two major limiting factors. Latex flow after tapping and regeneration of the cell materials lost during tapping. Ecoclimatic conditions, water availability of the soil, defoliation, frequency of tapping and stimulant application in the bark of the tree to increase the rubber yield control or limit latex flow. Studies on physiological and biochemical mechanisms involved in the control and stopping of flow after tapping and latex regeneration are essential for optimisation of yield and to propose the most suitable tapping systems to growers. In view of the above, experiments were carried out to study (1) the seasonal variations in latex flow characters (2) variations in the physiological and biochemical parameters related to latex flow under high and low frequency tapping (3) Immediate effect of stimulation in the

latex flow characters and (4) Clonal variations in parameters associated with latex flow.

Under normal conditions, latex yield variation of rubber tree is a clonal character particularly during peak season. But during stress situations different factors contribute to yield variations. In the present study even though thiol metabolism is activated during stress, decline in cytosolic scavengers such as SOD and catalase leads to peroxidative degradation of membranes and lower yield. A decrease in latex ATP and ADP observed during stress indicate a lower activation of metabolism. Higher hevein, beta 1,3 glucanase and low N- acetyl glucosaminidase activities observed during stress indicate that under normal conditions hevein does not interact with rubber particles but during stress, due to high lutoid instability, the contents are released earlier into the cytosol and destabilize latex.

Tapping frequency has considerable influence on latex flow characteristics and biochemical parameters. In the present study, high yield was observed in high frequency tapped trees compared to unstimulated low frequency tapped trees. Different factors were limiting in high and low frequency-tapped trees. Under d/2 the limitation is the short interval between tappings and in d/4, the physiological and biochemical factors that cause high PI and BI. These factors include low thiol, SOD in C- and B- serum and high beta 1,3 glucanase activity in B-serum. Proteins removed during tapping are efficiently regenerated in d/4-tapped trees as evidenced by the increase in Glutamine synthetase activity.

Significant clonal variations were observed in the activities of SOD in C- and B- serum and glutathione reductase activity, which indicate active removal of toxic oxygen species and protection of membranes from peroxidative degradation. A high ATP content in high yielding clones indicate active metabolism.

In the present study associated changes after stimulation in low frequency tapped trees include a reduced BI, high ATP, thiol, Glutamine synthetase activity in C-serum, SOD in C- and B- serum and glutathione reductase. These are immediate changes and in the long term the effects may be different.

The present study also shows that regulation of enzymes such as SOD, glutathione reductase and glutamine synthetase is more important in latex flow characteristics particularly during stimulation. In intensively tapped trees, the draining of these enzymes are more and the rate of synthesis is not balanced with the rate of their removal. Excessive latex flow could produce physiological damages and trigger excessive loss of active materials from the latex vessel system.

In conditions where tapping rest is given after stimulation, the synthesis of these enzymes shut off after a period of six days since latex is not removed for a long time. These may be resynthesised quickly when it is needed. Regulation of enzymes is a key way of physiological balance regulation.

Results from all these studies bring out the importance of SOD, Glutathione reductase and thiols as important parameters in regulating latex flow. SOD is probably very important in lutoid stability and hence latex flow. The role of peroxidase is different; it does not act as an antioxidant in these situations.

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