REDUCED MEMBRANE DAMAGE AND HIGHER LEA PROTEIN CONTENT UNDER LOW TEMPERAURE: PROBABLE CAUSES FOR DELAYED DEFOLIATION OF *HEVEA* IN NORTH EAST INDIA

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Two popular clones of *Hevea brasiliensis* were studied to understand their defoliation pattern in relation to some of the biochemical parameters during the winter season. The low temperature induced changes such as membrane injury, anthocyanin and late embryogenesis abundant (LEA) protein accumulation were examined. The early defoliating clone RRII 105 showed high membrane injury, anthocyanin accumulation and rapid chlorophyll degradation with low LEA protein, whereas the late defoliating clone RRIM 600, recorded the opposite trend. It can be inferred that the temporal difference in wintering and in the biochemical parameters studied are related to cold susceptibility.

Key words: Anthocyanin, Chlorophyll, Defoliation, *Hevea brasiliensis*, LEA protein, Membrane damage, Wintering.

INTRODUCTION

Low temperature is one of the environmental parameters that profoundly affect normal metabolic functioning of plants. *Hevea brasiliensis* is a deciduous tree that defoliates during the low temperature period in the northeastern region of India (Sethuraj *et al.*, 1989). This tree species exhibits genetic variation in defoliation pattern (known as wintering) (Webster and Paardekooper, 1989; Vinod *et al.*, 1996). The genetic variation in temporal behaviour of leaf abscission and its intensity in a given agroclimatic condition indicates the probable involvement of biological factors contributing to this. Chilling induced leaf abscission is reported to be triggered by oxidation processes under sub optimal temperatures (Kuo and Tsai, 1984; El Abd *et al.*, 1986). The biochemical parameters such as chlorophyll degradation during the course of leaf maturation (Maedema, 1982; Plazaola and Becerril, 2001) and membrane leakage show the extent of damage at cellular level when the plants experience low temperature below a threshold level (He and China, 1986). One of the protective mechanisms in plants against such cellular damage is the synthesis of proteins such as LEA proteins (Baker *et al.*, 1988; Close *et al.*, 1989; Xu *et al.*, 1996; Jayaprakash *et al.*, 1996) and anthocyanin like pigments (Rabino and Mancinelli, 1986).

The objective of the present study was

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to assess the role of some of the biochemical parameters like accumulation of anthocyanin, LEA proteins, chlorophyll degradation and membrane damage, involved in the senescence that precedes defoliation of *H. brasiliensis* and the clonal response to low temperature stress in Tripura. This may be useful for developing markers for cold tolerance in rubber.

MATERIALS AND METHODS

Study site and sampling

The experiment was conducted in 15 year old *Hevea* trees at Taranager Farm of Regional Research Station, Rubber Research Institute of India, at Agartala, Tripura (91° 15'E, 23° 25'N; 30 m above MSL). Two clones with contrasting wintering behaviour *viz.*, RRII 105 (early) and RRIM 600 (late) were selected for this study. Wintering pattern of twenty five trees from each clone was observed individually during the peak wintering period at weekly intervals for a period of two years. Visual scoring of wintering pattern was done using the scale previously employed by Vinod *et al.* (1996) and expressed as percentage of defoliation.

Analytical methods

Leaf samples were taken from a composite pool of physiologically mature leaves of five trees from each clone, during the defoliation period for two consecutive years. For *in vitro* studies on membrane leakage and anthocyanin estimation, intact branches were used.

Chlorophyll concentration

Chlorophyll concentration was estimated in healthy green leaves during the prewintering and wintering seasons. Five leaf discs of 1 cm diameter each were incubated in 80 per cent acetone:DMSO (1:1) mixture overnight in the dark. The absorbance of the acetone extract of chlorophyll was read at the wave length of 546 nm, 652 nm and 663 nm. Concentration of chlorophyll was calculated using the formula described by Arnon (1949).

Membrane leakage under *in vitro* low temperature condition

To study the effect of low temperature on membrane integrity, uniformly matured twigs (one each) were collected from five trees of each clone and brought to the laboratory within half an hour. These twigs were then exposed to 10°C for 48 h in presence of light in a BOD incubator (Caltan, DT 909). The cut ends of the twigs were dipped in water immediately after detaching from the trees. Simultaneously, another set of branches from same trees were maintained at laboratory temperature (32°C) as control. After 48 h of incubation five leaf discs were sampled from each composite pool of leaves from both the sets of branches. The leaf discs were incubated in a uniform volume of sterile water in test tubes overnight under normal conditions. Absorbance at 273 nm (A_1) was recorded both for treatment and control samples using the UV spectrophotometer. The samples were then autoclaved at 1.26 kg/cm^2 pressure for 20 minutes and again the absorbance was recorded at 273 nm (A_2). The injury to the membrane was calculated using the formula: $[A_2-A_1)/A_2] \times 100$ (Sullivan and Ross, 1979).

Anthocyanin concentration

Leaf tissue (1 g) from the same composite sample used for membrane damage

studies was homogenized in 5 ml of methanol containing 1.0 N HCl and maintained at 4°C for four hours (Christe *et al.*, 1994). The particulates were removed by centrifugation of the homogenate at 10,000 g for 3 min. The absorbance of the clear supernatant read at 530 nm (per gram of tissue) was recorded. This served as a measure of anthocyanin content (Kho *et al.*, 1977).

Expression of LEA protein

The expression of LEA proteins in these two clones was studied by using dot blot analysis employing LEA-1 and LEA-2 polyclonal antibodies. Lyophilised leaf samples of each clone were homogenized in 100 nM Tris-HCl buffer (pH 8.2) containing 20 nM $MgCl_2$, 10 mM KCl, 1mM EDTA, 1 mM PMSF to obtain a cell free extract. The protein content of the sample was quantified by the procedure of Bradford (1976) using BSA as standard. Two micrograms of soluble protein was smeared on a piece of PVDF membrane and allowed to dry. The membrane was incubated for 3 h in 5 per cent casein solution for blocking. It was then washed with phosphate buffer saline (PBS) thrice for 5 min each and incubated in the primary antibody solution (1:3000) prepared in casein solution for 2 to 3 h with gentle shaking. The membrane was then washed three times for 5 min each with PBS before incubating with secondary antibody prepared with casein solution (1:1000). After two hours incubation, the membrane was washed with PBS and PBST thrice each as mentioned above. The substrate BCIP/NBT was then added with gentle shaking. With the appearance of a clear spot, the reaction was terminated by washing the membrane with water, and subsequent air-drying. The data were analysed for statistical significance.

RESULTS AND DISCUSSION

Temperature during winter period

The maximum and minimum temperatures during the experimental period are shown in Fig. 1. The maximum temperature fluctuated between 30.8°C and 24.6°C whereas the minimum temperature was below 12°C from the first week of December till the end of the experiment. Though a steep decline in minimum temperature was noted from the first week of November, the lowest temperature was recorded to be in the second week of January.

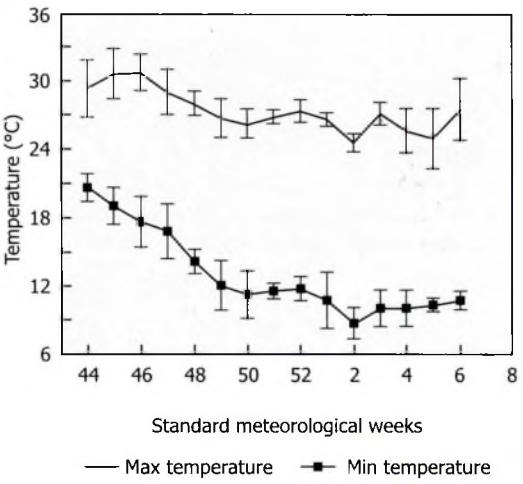

Fig. 1. Mean temperature recorded in Taranagar farm, Agartala, Tripura, from first week of November to first week of February, 2000-2001

Defoliation

It was observed that defoliation in RRII 105 was initiated earlier than that in RRIM 600. During the second week of December (2000 and 2001), clones RRII 105 and RRIM 600 showed around 50 per cent and 25 per cent defoliation respectively (Fig. 2). Wintering pattern of

Hevea clones over the weeks and between the years is not random (Vinod *et al.*, 1996). Therefore, it was assumed that the early defoliation in clone RRII 105 might be triggered by low temperature stress. RRIM 600 seems to be less susceptible to the low temperature as indicated by late defoliation.

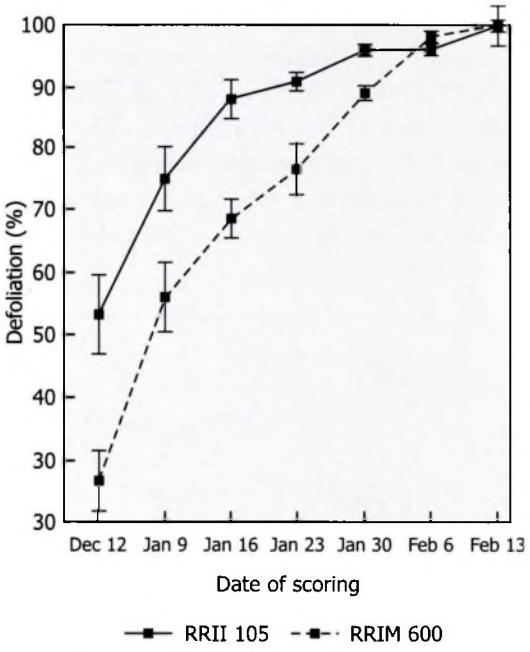

Fig. 2. Defoliation pattern of two *Hevea* clones during peak winter period in Tripura

Chlorophyll concentration

The total chlorophyll concentration in leaves was monitored during the pre-wintering and wintering periods. Both the clones showed comparable chlorophyll concentration in the pre-defoliation period. Subsequently, the early defoliating clone RRII 105 had a declining trend in the concentration of chlorophyll till the second week of February. During the corresponding period, chlorophyll concentration of the late defoliating clone, RRIM 600 was higher (Fig. 3). It has been reported that low temperature susceptible plants show reduced pigmentation or pale green appearance (Maedema, 1982; Stamp, 1984; Stamp *et al.*, 1983; Baker and Nie, 1994). Chlorophyll is more liable to get photo-damaged under conditions of low temperature and high irradiance (MacWilliam and Naylor, 1967). Chlorophyll degradation during peak winter period was higher in the clone RRII 105 than in RRIM 600, implying a higher susceptibility of the former to low temperature.

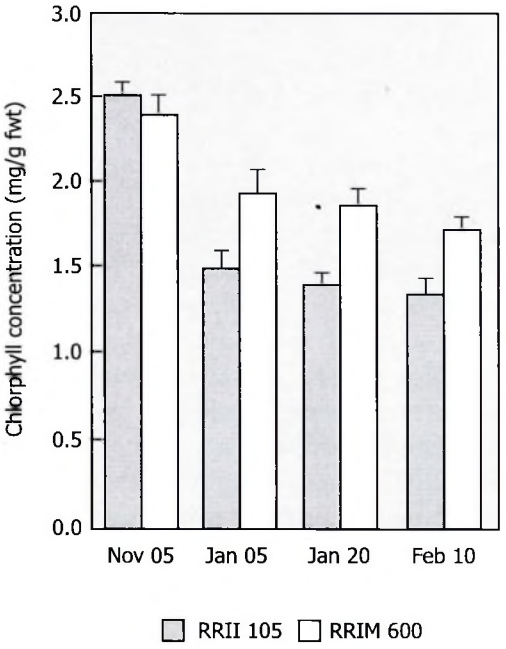

Fig. 3. Total chlorophyll content during pre-winter and winter periods in Tripura

Membrane injury

The membrane injury caused by exposure to low temperature (10°C for 47 h) was significantly high for the clone RRII 105, where the leaves experienced a greater membrane damage than for

RRIM 600 (Table 1). The increased membrane permeability and electrolyte exudation could be due to membrane injury caused by low temperature (He and China, 1986). It appeared that the structural stability of the plasma membrane was maintained in the resistant clone whereas it was affected in the susceptible (Collins *et al.*, 1993; Collins *et al.*, 1995; Sathik *et al.*, 1998). Results of this experiment revealed that in clone RRIM 600, the cell membrane is less injured by low temperature compared to RRII 105. The results further indicated that RRII 105 is susceptible to low temperature stress.

Table 1. **Effect of low temperature on membrane injury and anthocyanin content in the leaves of *H. brasiliensis* clones**

Clone	Membrane injury (%)	Anthocyanin content (A_{530}/g FW tissue)
RRII 105	18.67	0.314
RRIM 600	4.07	0.205
CD (P≤0.05)	3.20	NS

FW: Fresh weight; NS: Not significant

Anthocyanin content

The early defoliating clone, RRII 105, had more leaf anthocyanin concentration compared to RRIM 600, but the difference was non-significant (Table 1). Anthocyanin is a flavonoid, generally induced by low temperature (Rabino and Mancinelli, 1986; Shichijo *et al.*, 1993; Shvarts *et al.*, 1997). In Japanese parsley, there was differential accumulation of anthocyanin induced by low temperature in three genotypes (Hajime *et al.*, 2001). Several studies have shown that flavonoids inhibit the polar transport of auxin from the site of synthesis to other tissues (Jacob and Rubery, 1988; Faulker and Rubery, 1992; Bernasconi, 1996) and promotes the retention of auxin (Murphy *et al.*, 2000). The higher level of anthocyanin in RRII 105 seems to regulate auxin transport in the leaf leading to the accumulation of ethylene resulting in early defoliation at the onset of cold season. The possibility of an alteration in auxin : cytokinin ratio in adaxial and abaxial portion of leaf petiole leading to formation of abscission layer also cannot be ruled out.

LEA protein

The assay of LEA proteins revealed that both LEA 1 and LEA 2 were more in RRIM 600 compared to RRII 105. LEA proteins are synthesized in plant organs when they are exposed to high or low temperature (Houde *et al.*, 1995; Van Zee *et al.*, 1995). Synthesis of more LEA protein in the late defoliating clone RRIM 600 implies a better intrinsic mechanism to protect the macromolecules and membranes under cold stress.

In this study, both the *Hevea* clones with contrasting wintering pattern showed distinct characteristics of cold susceptibility whereas RRIM 600 appeared to be a less susceptible. This study indicates the possibility of using these biochemical parameters as primary indices in selection for cold tolerance of rubber clones.

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