Indian Journal of Natural Rubber Research, **16** (1 & 2) : 93-101, 2003

ANTIOXIDANT DEFENCE SYSTEMS AND DROUGHT TOLERANCE IN *HEVEA BRASILIENSIS*

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Sreelatha, S., Jacob, J., Prakash, G., Simon, S.P., Annamalainathan, K. and Chandrasekhar, T.R. (2003). Antioxidant defence systems and drought tolerance in *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, **16** (1 & 2) : 93-101.

The dry rubber yield, girth of the trees, biochemical composition (phenols, aminoacids, sugars, protein and glutathione) and the activities of antioxidant enzymes peroxidase (PER), ascorbate peroxidase (APOX), superoxide dismutase (SOD) and polyphenol oxidase (PPO) in the leaf and bark tissues of ten rubber (*Hevea brasiliensis*) trees each belonging to low and high yield and low and high girth categories were determined during summer (peak drought) and post-monsoon (drought free) seasons during 1996-1998. During this period, the mean dry rubber yield of low yield category trees ranged from14.4 to 34.9 g/tree/tap and for the high yield category it was 32.3 to 107.7 g/tree/tap, depending upon the seasons. The mean girth ranged from 27.5 to 30.2 cm and 76.5 to 82.9 cm for the low and high girth category trees, respectively. The biochemical composition and enzyme activities of the leaf and bark tissues of the four categories showed wide variations. The glutathione content in the bark was higher in the high yielding than in the low yielding trees, irrespective of the seasons. The high yielding trees showed greater PER and APOX activities in the leaves and lower PPO activity in the bark than the low yielding trees. High girth trees consistently showed increased leaf PER activity compared to low girth trees during both the seasons indicating their intrinsic drought tolerance capacity. The possibility of using the above parameters as markers for drought tolerance is discussed.

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Key words: Active oxygen species, Antioxidants, Drought tolerance, Environmental stress, *Hevea brasiliensis*.

INTRODUCTION

Expanding natural rubber (NR) cultivation to newer areas and increasing the productivity of the existing plantations are the two ways to bridge the gap between the demand and supply of NR. But, adverse environmental conditions such as drought, high and low temperatures, high solar radiation, low atmospheric humidity, poor soils *etc.* limit the expansion of cultivation to newer areas in several rubber producing countries (Pushparajah, 1983; Sethuraj *et al.*, 1989; Jacob *et al.*, 1999). Stressful environment caused by conditions such as drought is a productivity limiting factor even in the traditional rubber growing areas. Under conditions of abiotic stress like drought or low temperature and biotic stress such as over exploitation, the plants experience oxidative stress (Krishnakumar *et al.*, 1996). Oxidative stress is defined as the cumulative and accumulated effects of the potentially lethal reactions initiated by various forms of active oxygen species (AOS). Evidences suggest that many environmental stresses have their effects directly or indirectly through the production of active oxygen species (AOS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) following impairment of electron transport systems (Elstner, 1982; Smirnoff, 1993; Mc Kersie and Lesham, 1994; Jacob and Nataraja, 2000). In many plants drought and associated high intensity of light leads to diversion of photosynthetic electrons for the production of AOS which cause inactivation of enzymes,

chlorophyll, lipid peroxidation or protein degradation which damage cellular membrane systems and trigger early senescence. A highly efficient antioxidative defence system, consisting of both enzymatic and non-enzymatic antioxidants are present in all plant cells to detoxify the harmful AOS (Foyer *et al.*, 1994). The O_2^- are mainly scavenged by the enzyme superoxide dismutase (SOD), which catalyses the dismutation of two molecules of superoxide into oxygen and hydrogen peroxide. Hydrogen peroxide is then disposed of enzymatically by non-specific peroxidases (PER) or ascorbate peroxidase (APOX) and by producing low molecular mass antioxidants mainly glutathione, α-tocopherol and phenolic compounds (Winston, 1990; Scandalios, 1994).

Under normal situations, the AOS are safely and effectively detoxified by various enzymatic and nonenzymatic antioxidant defence mechanisms. However, when the antioxidant capacity becomes inadequate to manage the AOS completely, oxidative stress could gradually set in. Green leaves produce large quantities of AOS in their chloroplasts, because thylakoid membranes are potential sites of their generation (Asada, 1992). When the photosynthetic carbon assimilation rates are inhibited more than the photochemical reactions, molecular oxygen becomes an alternative sink for photosynthetic electrons and thus produces large quantities of AOS (Jacob and Nataraja, 2000). Obviously, in a green leaf experiencing an abiotic stress, high light intensities will aggravate the AOS production (Barber and Anderson, 1992; Fryer *et al.*, 1998; Jacob and Nataraja, 2000).

In non-photosynthetic tissues, mitochondrial electron transport system is a very powerful source of AOS production (Winston, 1990). In laticiferous cells of *Hevea brasiliensis*, the lutoid membranes are also a source of AOS (Jacob *et al.*, 1988).

In India, the poor agroclimatic conditions prevailing in the non-traditional areas of rubber cultivation obviously limit the productivity of this crop (Jacob *et al.*, 1999b; Vijayakumar *et al.*, 2000). Several studies relating to the constraints for *H. brasiliensis* cultivation in agroclimatically stressful regions and the physiology of drought tolerance have been conducted in traditional and in the North Konkan regions (Vijayakumar *et al.*, 1988, Chandrasekhar *et al.*, 1990; Premakumari *et al.*, 1993). However, none of these have addressed the issues related to oxidative stress and antioxidant activity in *H. brasiliensis* plants. The present study taken up at Regional Research Station, Dapchari, located in a highly drought-prone area in the North Konkan region of the country was aimed at addressing this with an objective to identify markers, if any, for drought tolerance.

MATERIALS AND METHODS

The present experiment was conducted during the period 1996-98 in a polyclonal plantation at Regional Research Station of Rubber Research Institute of India, Dapchari, in the North Konkan, Maharashtra. The trees were 12 year old, when the experiments began. The climate in Dapchari is characterized by extremely severe drought concomitant with high temperature and solar radiation and low atmospheric humidity for six to seven (January to June) months every year (Jacob *et al.*, 1999). During the monsoon season that extends four to five months from mid-June, this region gets an average rainfall of 2400 mm but for the rest of the year there is practically no rain. The experimental trees were grown without irrigation in summer except for minimal life saving irrigation in the first year of planting. Drought tolerant and susceptible trees were identified on the basis of yield data for two years and girth. The trees were classified into high/low yield and high/low girth types. From a population of 300 trees, ten trees per type were selected for this study. Leaf and bark

samples were collected from each of these trees during peak-summer and post-monsoon seasons for two consecutive years (1996-1998). Biochemical composition (aminoacids, phenols, sugars, glutathione and protein) and activities of enzymes such as peroxidase (PER, EC 1.11.1.7), ascorbate peroxidase (APOX, EC 1.11.1.11), polyphenol oxidase (PPO, EC 1.14.18.1) and superoxide dismutase (SOD, EC 1.15.1.1) were assayed in the leaf and bark tissues. Extracts for the assay of PER, SOD and PPO activities in the leaf and bark samples were prepared by homogenising 0.5 g tissue in 3.5 ml of extraction buffer containing 50 mM potassium phosphate buffer (pH 7.4) and 200 mg polyvinyl pyrrolidone (PVP). The homogenates were centrifuged at 20,000 rpm for 20 min at 4°C and the supernatants were used for the assays; PER and PPO activities were measured according to the methods described by Amako *et al.* (1994) and Karr and Mishra (1976) and expressed in relative units per milligram fresh weight of tissue (One enzyme unit is defined as the change in absorbance per hour caused by the enzyme). Total SOD activity was assayed by the inhibition of the photochemical reduction of Nitro blue tetrazolium (NBT). One unit of SOD was defined as the amount of enzyme (mg protein) that produced 50 per cent reduction of NBT under the assay conditions as described by Giannopolitis and Ries (1977).

For APOX assay, 5 mM ascorbate was included in the extraction buffer. The activity was measured immediately in fresh extracts as described by Nakano and Asada (1981) in 3 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide, 0.5 mM ascorbate and 0.1 mM ethylene diamine tetra acetic acid (EDTA). The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm and expressed in units per hour per milligram protein. Protein content in the extract was measured following the procedure of Lowry *et al.* (1951). Total glutathione from the tissues was extracted in 5 per cent (w/v) tricholoacetic acid (TCA), centrifuged at 10,000 rpm for 20 min at room temperature, the supernatant extracted with diethyl ether to remove TCA and estimated according to Boyne and Ellman (1972). Phenols, sugars and aminoacids were extracted in 80 per cent alcohol and measured as described by Swain and Hillis (1959), Scott and Melvin (1953) and Moore and Stein (1948), respectively, and expressed in milligram per gram fresh weight of the tissue.

The mean values (± SE) of data on yield, girth and biochemical composition calculated for the leaf and bark of high and low yielding trees were for ten trees. Differences between treatments were compared using independent t-test.

RESULTS AND DISCUSSION

Yield and girth categories

The mean rubber yield of trees, which showed seasonal variations on expected lines, remained significantly different between the high and low yield categories throughout the experiment period (Table 1A). In the high yield category, dry rubber yield was 107.7 g per tree per tap in October 1997 and 32.3 g per tree per tap in the summer of 1998. Similarly, the mean girth ranged from 76.2 to 82.9 cm in the high girth group compared to 27.5 to 30.2 cm in the low girth group during the experimental period (Table 2A). The low girth trees did not reach the prescribed tappable girth even after 13 years.

Biochemical composition

In general, the levels of biochemical components such as proteins, amino acids, phenols, glutathione and sugars of the leaves of high and low yielding trees did not show any significant differences during the stress and stress-free seasons (Table 1A). However,

Table 1. **Yield and biochemical composition (mg/g fresh weight) of the leaf and bark of high and low yielding trees**

Parameter	October '96 (stress free)		April '97 (under stress)		October '97 (stress free)		April '98 (under stress)	
	HY	LY	HY	LY	HY	LY	HY	LY
A. Leaf								
Yield (g/t/t)	81.9 ± 9.20	31.5 ± 3.60 **	51.9 ± 6.50	14.4 ± 1.70**	107.7 ± 13.80	34.9 ± 10.30**	32.3 ± 6.10	14.5 ± 2.60**
Amino acids	3.1 ± 0.10	2.8 ± 0.09 NS	1.9 ± 0.11	2.1 ± 0.28 NS	4.3 ± 0.33	3.9 ± 0.31 NS	3.8 ± 0.39	3.5 ± 0.33 NS
Phenol	7.4 ± 0.22	8.0 ± 0.63 NS	3.0 ± 0.11	3.2 ± 0.19 NS	8.8 ± 1.04	5.2 ± 0.55**	2.2± 0.22	2.9 ± 0.27 NS
Sugars	63.2 ± 2.1	59.0 ± 2.6 NS	39.6 ± 1.1	37.7 ± 1.8 NS	42.8 ± 3.1	31.3 ± 2.26*	44.6 ± 4.3	41.6 ± 2.6 NS
Glutathione	3.2 ± 0.42	3.4 ± 0.29 NS	3.4 ± 0.28	3.3 ± 0.19 NS	4.8 ± 0.18	2.9 ± 0.37 **	2.7 ± 0.39	3.3 ± 0.39 NS
Protein	19.1 ± 1.90	17.6 ± 1.4 NS	17.4 ± 1.0	16.1 ± 1.3 NS	19.8 ± 1.1	13.7 ± 0.95 *	24.9 ± 2.9	21.5 ± 1.3 NS
B. Bark								
Amino acids	1.76 ± 0.09	1.54 ± 0.10 NS	0.88 ± 0.09	0.49 ± 0.03**	1.72 ± 0.105	1.61 ± 0.11 NS	2.60 ± 0.19	2.71 ± 0.16 NS
Phenol	4.56 ± 0.18	4.50 ± 0.16 NS	4.56 ± 0.29	4.1 ± 0.25 NS	4.48 ± 0.13	4.39 ± 0.15 NS	2.09 ± 0.17	1.97 ± 0.18 NS
Glutathione	0.67 ± 0.07	0.46 ± 0.049 *	0.47 ± 0.007	0.34 ± 0.01**	2.18 ± 0.29	1.72 ± 0.19 **	0.88 ± 0.06	0.79 ± 0.08*
Protein	12.60 ± 1.20	13.35 ± 0.95 NS	9.17 ± 0.75	8.45 ± 0.42 NS	11.99 ± 0.55	9.08 ± 0.38 **	12.66 ± 0.63	11.07 ± 1.0 NS
Sugars	42.20 ± 2.70	40.87 ± 1.34 NS	25.49 ± 1.40	29.76 ± 1.70 NS	40.38 ± 2.28	41.35 ± 1.28 NS	27.11 ± 1.91	31.20 ± 2.93 NS

HY – High yielding trees; LY – Low yielding trees; n = 10; ** Significant at $p \leq 0.01$; * at $p \leq 0.05$; NS – Non-significant

Table 2. **Girth and biochemical composition (mg/g fresh weight) of the leaf and bark of high and low girth trees**

Parameter	October '96 (stress free)		April '97 (under stress)		October '97 (stress free)		April '98 (under stress)	
	HG	LG	HG	LG	HG	LG	HG	LG
A. Leaf								
Girth (cm)	76.2 ± 0.78	27.5 ± 0.6**	76.5 ± 0.66	27.8 ± 0.9**	80.2 ± 0.7	30.2 ± 0.93**	82.9 ± 2.2	30.1 ± 0.9**
Amino acids	3.3 ± 0.12	3.1 ± 0.1 NS	2.0 ± 0.2	2.6 ± 0.18 NS	3.8 ± 0.11	3.6 ± 0.16 NS	5.5 ± 0.33	3.9 ± 0.19**
Phenol	7.3 ± 0.33	10.36 ± 0.8*	2.8 ± 0.08	5.5 ± 0.56**	7.7 ± 0.83	10.9 ± 0.84*	2.8± 0.26	3.8 ± 0.49 NS
Sugars	61.9 ± 2.4	64.9 ± 4.2 NS	37.5 ± 2.28	32.3 ± 1.5 NS	43.4 ± 1.9	42.9 ± 3.2 NS	47.2 ± 5.2	36.6 ± 5.2 NS
Glutathione	2.8 ± 0.32	3.9 ± 0.29 *	3.58 ± 0.27	4.9 ± 0.26*	2.98 ± 0.52	5.09 ± 0.43 **	3.1 ± 0.49	4.1 ±0.43 NS
Protein	23.3 ± 2.9	14.1 ± 0.63**	14.5 ± 0.72	17.8 ± 0.69 *	15.8 ± 1.04	19.8 ± 0.8 *	25.7 ± 2.5	22.9 ± 1.4 NS
B. Bark								
Amino acids	1.68 ± 0.13	1.57 ± 0.10 NS	0.805 ± 0.07	0.567 ± 0.05**	1.73 ± 0.08	1.47 ± 0.096 NS	2.45 ± 0.13	3.70 ± 0.296**
Phenol	4.55 ± 0.19	4.26 ± 0.15 NS	4.49 ± 0.297	3.51 ± 0.311*	4.53 ± 0.12	4.16 ± 0.16	2.01 ± 0.16	2.311 ± 0.23 NS
Glutathione	0.57 ± 0.07	0.47 ± 0.03 NS	0.476 ± 0.01	0.32 ± 0.009**	1.94 ± 0.18	1.64 ± 0.22 NS	0.869 ± 0.04	0.967 ± 0.05 NS
Protein	12.02 ± 1.40	11.63 ± 1.25 NS	9.73 ± 0.62	9.43 ± 0.298 NS	12.34 ± 0.44	12.06 ± 0.52 NS	11.39 ± 0.71	10.9 ± 0.804 NS
Sugars	45.07 ± 2.80	50.16 ± 3.31 NS	32.84 ± 2.27	32.91 ± 1.04 NS	46.47 ± 2.99	52.53 ± 2.2 NS	34.92 ± 2.26	47.55 ± 2.74**

HG – High girth trees; LG – Low girth trees; n = 10; ** Significant at $p \leq 0.01$; * at $p \leq 0.05$; NS – Non-significant

significantly higher levels of glutathione was noticed in the bark of the high yielding trees under both stress and stress-free seasons (Table 1B). On the contrary, phenol and glutathione contents in the leaf showed a decrease in the high girth trees compared to the low girth trees both during stress and stress-free seasons (Table 2A) though no such differences were observed in the corresponding bark tissues (Table 2B).

Activities of antioxidant enzymes

In all the seasons the leaf PER (Fig. 1A) and APOX (Fig. 1C) activities were significantly higher in the leaves of the high yielding trees compared to the low yielding. There were no significant differences in the activities of PPO (Fig. 1B) and SOD (Fig. 1D) in the leaves of the high and low yielders. The high yielding trees showed significantly less PPO activity in all the seasons in the bark

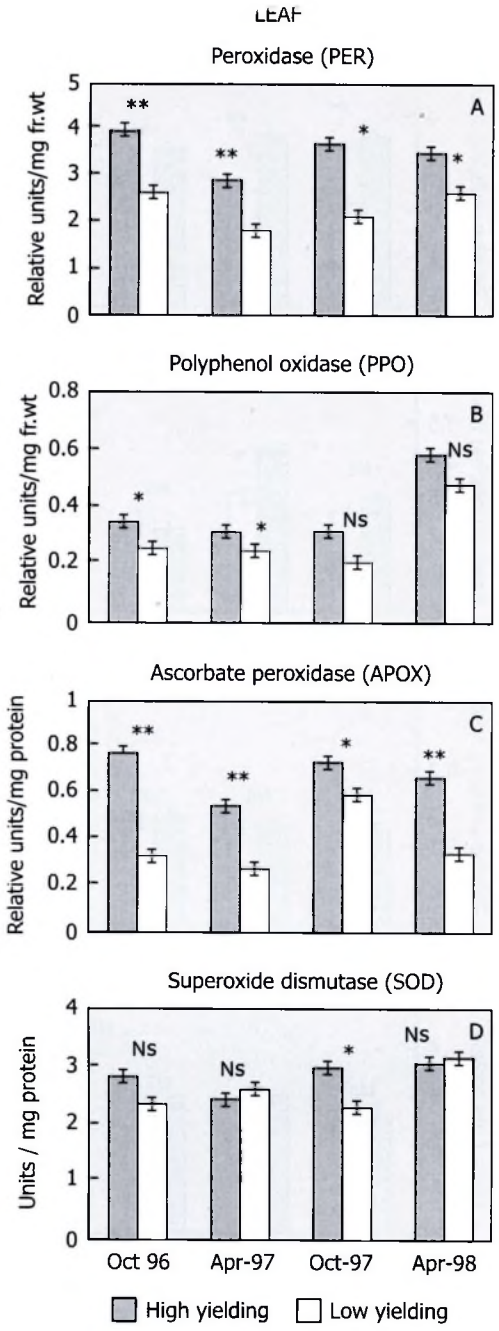

Fig. 1. Enzyme activity in the leaf of high and low yielding trees grown at Dapchari during stress and stress free seasons (n=10±SE)
** Significant at p≤0.01; * Significant at p≤0.05; NS: Not significant

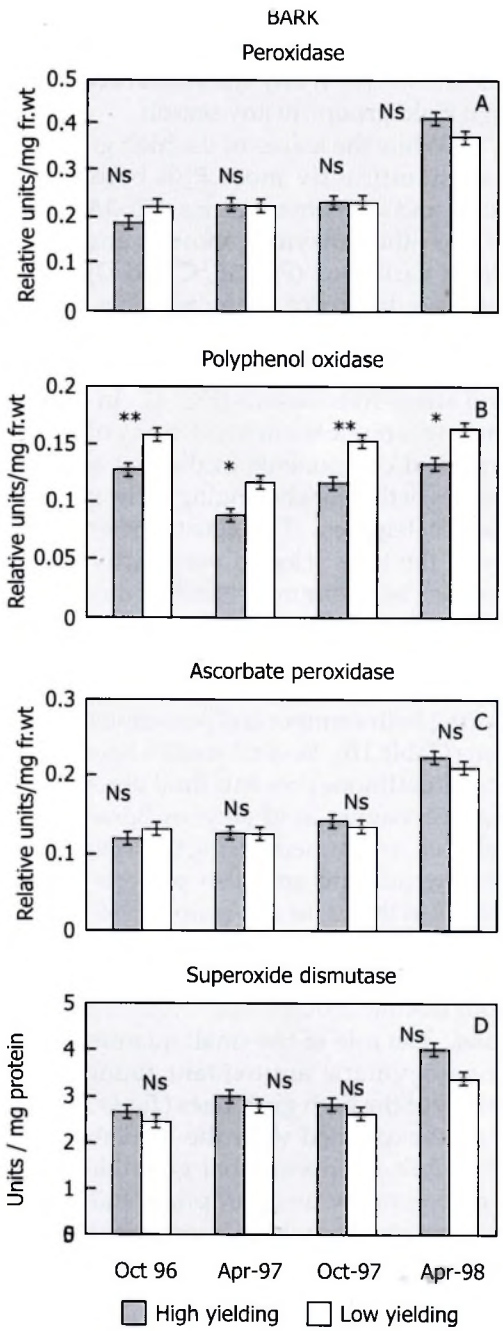

Fig. 2. Enzyme activity in the bark of high and low yielding trees grown at Dapchari during stress and stress free seasons. (n=10±SE)
** Significant at p≤0.01; * Significant at p≤0.05; NS: Not significant

tissues (Fig. 2B). However, the activities of PER (Fig. 2A), ASPX (Fig. 2C) and SOD (Fig. 2D) did not show any variations between the two yield groups in any season.

While the leaves of the high girth trees had significantly more PER both during stress and stress-free seasons (Fig. 3A). None of the other enzymes showed any appreciable variations (Fig. 3B, C and D). There were no difference in the activities of PER, PPO, ASPX and SOD in the bark tissues in both high and low girth trees during stress and stress-free seasons (Fig. 4). In general, there was no clear-cut trend in any of the biochemical components in the leaf and bark tissues of the trees belonging to the girth and yield categories. The genetic heterozygosity of the trees selected may partly explain the lack of a common trend in most of the biochemical parameters. However, the glutathione content in the bark tissues of the high yielding trees was significantly higher during both summer and post-monsoon seasons (Table 1B). Several studies have shown that glutathione prevents lipid peroxidation *via.* scavenging lipid alkyl or lipoxyl- radicals that are formed during the initial stages of peroxidation and also protects the enzymes in the tissues (Winston, 1990). While a high glutathione content in the bark appears to be associated with high latex yield both during drought and drought-free seasons. The role of the small quantity of this non-enzymatic antioxidant found in the leaves of the high girth trees (Table 2A) cannot be explained with the available data. Therefore, it appears that glutathione content may not be an appropriate and reliable marker for drought tolerance in *Hevea* in terms of girthing.

The activities of four major antioxidant enzyme systems examined in the leaf and bark tissues, exhibited varying trends in the different yield and girth categories. For instance, leaves showed greater levels of PER and ASPX (Fig. 1A and 1C) while bark ex-

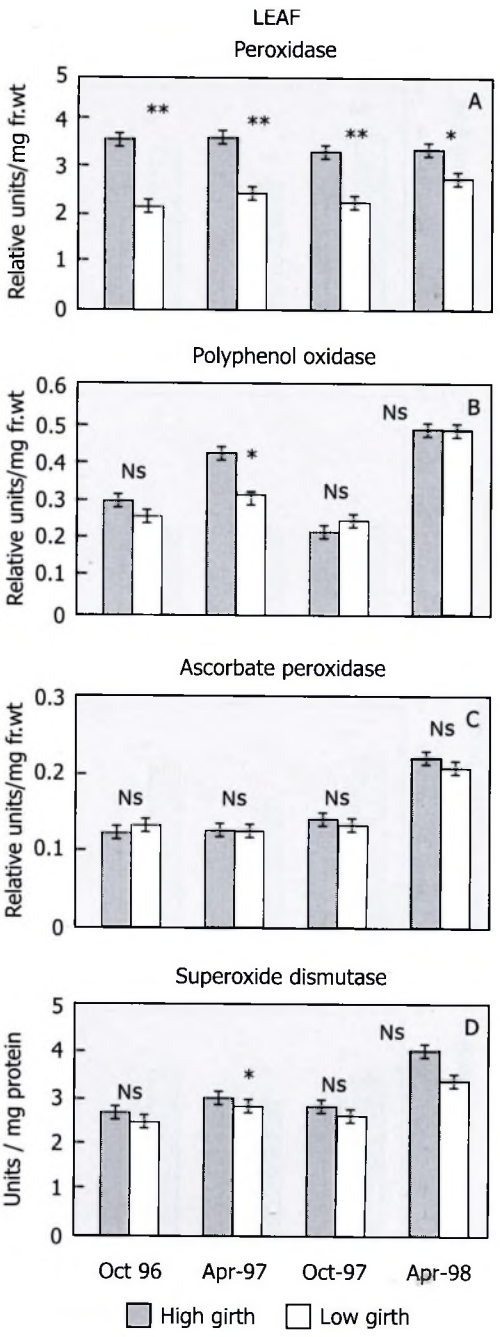

Fig. 3. Enzyme activity in the leaf of high and low girth trees grown at Dapchari during stress and stress free seasons. (n=10±SE)
** Significant at $p \leq 0.01$; * Significant at $p \leq 0.05$; NS: Not significant

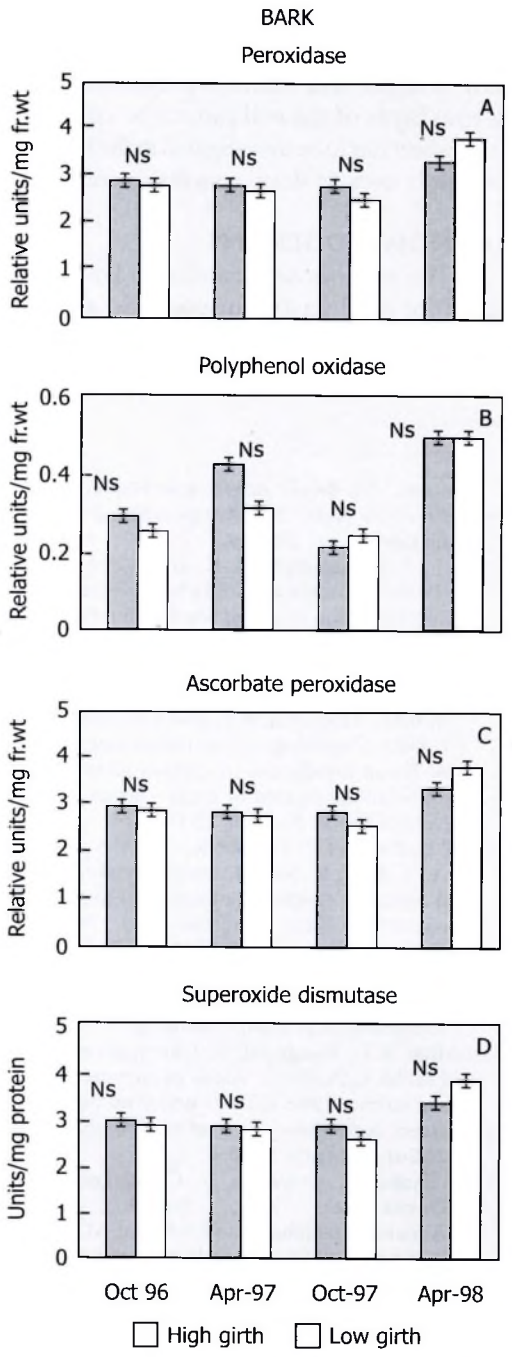

Fig. 4. Enzyme activity in the bark of high and low girth trees grown at Dapchari during stress and stress free seasons. (n=10±SE)
NS: Not significant

hibited decreased activity of PPO in the high yielding compared to low yielding trees in all the seasons (Fig. 2B). Similarly, a higher PER activity was also observed in the leaf tissues of high girth trees (Fig. 3A). These enzymes play crucial roles in protecting the tissues from acute oxidative damage. Peroxidases are a large family of ubiquitous enzymes, which are responsible for both scavenging of H_2O_2 by the oxidation of phenols and its regeneration through the oxidation of NADH (Siegal, 1993). The elevated activities of PER in the leaves of both the high yielding and high girth trees (Fig. 1A and 3A) indicate the formation of large amount of H_2O_2. Elevated H_2O_2 concentration could release PER from membrane structures with which it is normally associated (Zhang and Kirkham, 1994). APOX, which specifically uses ascorbate as a physiological reductant, is considered a crucial component in the metabolic defence against oxidative stress in green tissues (Asada, 1992). From the relatively higher activities of APOX it appears that the green leaf tissues were experiencing oxidative stress to a greater extent than the non-green bark tissues during summer. It is likely that the leaves that were exposed to sun may produce more AOS and FR as observed by Jacob and Nataraja (2000) than the bark tissues, which are under shade in the field.

Polyphenol oxidase enzyme catalyses the oxidation of phenols to quinones and its activity varies in response to biotic and abiotic stresses (Thipyapong *et al.*, 1995). Quinones are known to be highly toxic and responsible for the production of AOS (Pillinger *et al.*, 1994). Kasturibai *et al.* (1996) studied the biochemical basis for the ranking of the coconut cultivars/hybrids based on enzyme assay during non-stress and stress periods and reported that drought tolerant cultivars/hybrids had higher activities of SOD and PER and lower activities of PPO, APH (acid phosphatase) and AOAT (aspartate 2: oxaloglutarate amino transferase). The re-

sults of the present study suggest that the high activities of PER and APOX in the leaf and low PPO activity in the bark may be associated with drought tolerance in *H. brasiliensis*. Attempts are being made to use these as markers for screening drought tolerance. Obviously, the success of the use of a marker for drought tolerance depends on the reliability of the association between the marker and drought tolerance. The present observations were reproducible with a fair degree of accuracy. However, the likelihood of some of the trees belonging to the high girth or high yield categories having deep root systems and obtaining moisture from deeper layer of the soil cannot be ruled out. This aspect has to be investigated in the high and low girth trees to draw conclusive inferences.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Meena Singh for her logistic support and all those who directly or indirectly involved in the planting and maintaining the plantation.

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