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Research Article

DETERMINATION OF ANTIOXIDANT ENZYMES LEVEL IN HEALTHY AND TAPPING PANEL DRYNESS (TPD) SYNDROME AFFECTED BARK TISSUES OF RUBBER TREE (*HEVEA BRASILIENSIS* MUELL. ARG.)

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ABSTRACT

Oxidative stress may lead to overproduction of reactive oxygen species (ROS) which are highly toxic ultimately cause stimulation of antioxidant enzymes and damages to proteins, lipids, carbohydrates and DNA molecules in bark tissue of *Hevea brasiliensis* during onset of Tapping panel dryness syndrome (TPD). In this study, Antioxidant enzymes level in bark tissues of seven different healthy and TPD affected clones of *Hevea brasiliensis* namely RRII 105, PB 217, PB 235, PB 285, PB 5/51, PCK2 and GT1 was determined to monitor TPD occurrence. The results revealed that increased level of antioxidant enzymes activity was noticed in bark tissues of TPD affected clones while it was found to be low in healthy clones. Maximum level of superoxide dismutase (SOD) activity noticed was 0.634 U/mg FW in healthy PB 5/51 clone compared with its TPD affected clone. The CAT activity was also found to be high in healthy PB 5/51 clone (1.988 U/mg) than TPD affected clone. In the case of peroxidase (POX) activity, it was found to be low in all healthy clones while the activity was significantly increased in TPD affected clones and maximum activity was noticed in PCK2 followed by PB285. Antioxidant enzymes level was also determined by native PAGE analysis and its results showed that isoenzymes of SOD and POX banding pattern are directly correlated with biochemical assays activity. This research correlates the activities of antioxidant enzymes between healthy and TPD affected clones and paves the path to understand the role of SOD, CAT and POX during onset of TPD and leads to future genetic studies on antioxidant enzymes and its genes in *Hevea brasiliensis*.

Keywords: *Hevea brasiliensis*, Antioxidant enzymes, TPD, Reactive oxygen Species (ROS), Rubber tree.

INTRODUCTION

Severe environmental conditions especially abiotic stresses affect the rubber plants frequently, and showed adverse effects on plant growth development and latex production. The complex regulatory mechanisms of plants have been adapting to various environmental stresses, by increasing in the cellular of reactive oxygen species which is one of the consequences of stressed plants. Even under normal conditions, higher plants produce ROS during the metabolic process. The apoptotic death of cells or oxidative damage to cells occurred as a result of excess amount of ROS in plants. Development of antioxidant defense system in plants may protect them against oxidative stress induce cell damage. ROS and, more particularly, H_2O_2 play versatile roles in plant normal physiological processes and resistance to stresses. Recently, H_2O_2 has been functioned as a signaling molecule and regulate the expression of some genes in cells. Reactive oxygen species (ROS) occurred in biological systems as division of normal metabolism. Increased levels of ROS due to adverse environmental factors like drought stress leads detrimental to the plant. Plants have developed elaborate mechanisms to manage ROS at sustainable levels to escape from cellular damage caused by these excess ROS. Antioxidant enzymes play an important role in lowering the ROS levels to avoid severe oxidative stress. The antioxidant enzymes such as

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superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase play a major role in combating oxidative stress. ROS are implicated in the cell growth, differentiation, progression, and death. Low concentrations of ROS may be essential in processes such as intracellular signaling and defense against micro-organisms. Nevertheless, higher amounts of ROS play a role in the aging process. As a protection mechanism against the excess amount of ROS, several enzymatic and non enzymatic antioxidant activities may persist under stress conditions in plants. Therefore, a defence system promotes the regulation and enhanced expression of these enzymes level. The enzymatic and non-enzymatic antioxidant defence systems include, glutathione peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD), ascorbic acid (vitamin C), vitamin A, catalase (CAT), glutathione (GSH), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascobate reductase (MDHAR), glutathione synthase (GS), and glutamate-cysteine ligase (GCL), b-carotene, and a-tocopherol (vitamin E) in *Hevea brasiliensis*. (Nuringtyas *et al*., 2022; Zhang *et al*., 2017)

Antioxidants are defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions and they are also called as oxidation inhibitor (Pokorny & Korczak 2001). Antioxidants prevent cell and tissue damage as they act as scavenger. A variety of components act against free radicals to neutralize them from both endogenous and exogenous origin. The SOD catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. Peroxide is destroyed by CAT or GPX reaction (Fridovich, 1995; Sandalio *et al*., 1997; Teixeira *et al*., 1998). Catalase is a tetrameric enzyme and molecular mass is 240 kDa. The cells can be protected from hydrogen peroxide by catalase. CAT reacts very efficiently with H_2O_2 to form molecular oxygen and water; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity. GPX (80 kDa) catalyses the hydrogenperoxides using GSH, thereby protecting cells against oxidative stress induced damages. In fact, one of the most essential antioxidative defense mechanisms is glutathione metabolism (Grazioli *et al*., 1998; Sigalov & Stern, 1998). Monodehydroascorbate (MDHA) is produced when two molecules of APX are used to turn H_2O_2 to water. Monodehydroascobate reductase (MDHAR) catalyses the quick disproportionate conversion of MDHA into reduced ascorbate (AsA) and oxidised ascorbate (DHA) wchich is recycled by DHAR to AsA using reduced glutathione (GSH) as reducing substrate to protect cells and tissues from oxidative damage (Zhang *et al*., 2017).

Small variations in the physiological activity of antioxidant enzymes may induce dramatic effect on the resistance of cells to ROS-induced damage to the genome and cell killing (Limoli *et al.*, 1998). Addition of H_2O_2 causes a dose-dependent increase in CAT mRNA in both exponentially growing and confluent cells. Excess level free radicals may cause harmful oxidation that can damage cell membrane and even cell death. Antioxidants may have the ability to scavenge free radicals in the system and neutralize them before they do any damage to body cells. Antioxidant enzymes have been demonstrated its protective mechanisms against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Naturally occurring antioxidants in plant cells include a) enzymatic and peptide defence mechanisms (catalases, peroxidases, superoxide dismutases, glutathione and other proteins), b) non-enzymatic mechanisms, phenolic defence compounds (vitamin E, flavonoids, phenolic acids and other phenols); nitrogen compounds (alkaloids, amino acids and amines); cartenoids and chlorophyll derivatives. Both the enzymatic and non-enzymatic antioxidants have been playing an important function as natural antioxidant. In rubber tree clones, chilling stress also causes defoliation, dead branches, damaged bark, and death of trunk (Huang & Pan, 1992). It is widely accepted that chilling stress implies the formation of radicals and other reactive oxygen species (ROS) (Miller *et al.*, 2008; Mittler *et al.*, 2004) and activation of antioxidant enzymes (Hodges *et al.*, 1997), in plants and rubber tree (Mai *et al.*, 2010).

MATERIALS AND METHODS

Plant material

This study was conducted using the bark tissues of seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg namely RRII 105, RRII 285, PB 217, PB 235, PB 5/51, PCK2 and GT1. The rubber plants/clones were grown in the field of M/s Harrisons Malayalam Pvt. Ltd, Punalur, Kerala. The bark tissue samples were collected from seven years old rubber clones separately, soaked in the phosphate buffer and stored in liquid nitrogen. The samples were then transported from Kerala to Periyar University, Salem.

Measurement of Antioxidative enzyme activities

Both healthy and TPD affected barks of *Hevea brasiliensis* were homogenized in a pre-chilled mortar and pestle under ice-cold conditions with 2 mL extraction buffer [50 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 1 mM EDTA]. The homogenate was centrifuged at 10,000 rpm for 15 min. The supernatant was collected and used for the quantification of POX, SOD, CAT and APX antioxidative enzyme activities. In addition, the protein content was also estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

SOD Enzyme Assay

SOD catalyses the dismutation of the highly reactive superoxide anion to O_2 and less reactive species to H_2O_2. The SOD activity was estimated by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp & Fridovich, 1971). The reaction mixture (3 mL) contained 100 mM potassium phosphate

buffer (pH 7.8), 0.1 mM EDTA, 13mM methionine, 2.25 mM NBT, 60 µM riboflavin, and enzyme extract. After mixing, the contents in the tubes were illuminated under light for 15 min. Enzyme extract kept in the dark was served as blank, while buffer with no enzyme extract which kept in the light was served as control. The absorbance was noticed at 560 nm against a blank using UV–visible spectrophotometer. NBT reduction in the light was recorded in the presence and absence of enzyme extract. SOD activity was expressed as absorbance of control minus absorbance of sample, giving the total inhibition. One unit of activity was the amount of enzyme required for 50 % reduction in color and was expressed in units of the enzyme (mg/protein/h).

APX Enzyme Assay

APX activity was determined using the method of Nakano & Asada (1987). The reaction mixture (3 mL) contains 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.3 mM H_2O_2, and enzyme extract and used for estimation of enzyme activity. The oxidation of ascorbic acid was detected by the decrease in absorbance at 290 nm for 3 min using UV–visible spectrophotometer. The enzyme activity was estimated using the extinction coefficient 2.8/mM/cm and expressed in units per milligram protein. One unit of enzyme was the amount necessary to decompose 1 µmol of substrate per minute at 25 °C.

CAT Enzyme Assay

Catalase activity was estimated by measuring the decomposition of hydrogen peroxide. About 100 µL of enzyme extract was added into the reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 20 mM H_2O_2. The decrease of the absorbance at 240 nm was recorded. Activity was calculated using an extinction coefficient of 39.04/mM/cm. One unit of CAT activity was defined as the amount required for degradation of 1 µmol of hydrogen peroxide per minute per milligram protein under assay conditions (Beers & Sizer, 1952).

POX Enzyme Assay

Peroxidase activity was determined according to the method of Zhang *et al*. (2005). The reaction mixture (3 mL) contained 100 mM potassium phosphate buffer (pH 6.1), 96 mM guaiacol, and 12 mM H_2O_2, and enzyme extract was used for measurement of enzyme activity. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm. The enzyme activity was calculated using the extinction coefficient 25.5/mM/cm and expressed in units (mg/protein).One unit of enzyme was the amount necessary to decompose 1 µmol of substrate per minute.

Estimation of Total protein content

Total protein content was estimated according to method of Bradford (1976) using bovine serum albumin (BSA) as standard and expressed as mg/g FW.

Quantification of MDA content

Lipid peroxidation was determined by measuring the total amount of malondialdehyde (MDA) contents according to the method of Davenport et al. (2003). Briefly, fresh bark tissues (0.1 g) were homogenized using 2 mL of 5 % (w/v) trichloroacetic acid in an ice bath and centrifuged at 10,000 rpm for 10 min at 4 °C. About 1-mL supernatant was mixed with equal volume of 0.67 % (w/v) thiobarbituric acid, and the mixture was kept in boiling water bath for 30 min, then cooled and centrifuged. The absorbance of the supernatant was performed at 450, 532, and 600 nm. The MDA content was estimated using the formula given below:

Assay of in-gel enzyme

Bark tissues of both healthy and TPD affected rubber clones containing equal amounts of soluble protein were subjected to non-denaturing native discontinuous Polyacrylamide gel electrophoresis (PAGE) without sodium Dodecyl sulfate (SDS). In-gel assay for POX isoenzymes was performed using 7.5% separation and 4% stacking gels at 4◦C. After electrophoresis, the gels were incubated in phosphate buffer (pH 6.1) containing 96 mM guaiacol. The reaction was initiated by adding 12 mM H_2O_2 and incubated at room temperature till brown colour bands were appeared. It was then washed with distilled water and placed on 7.5% acetic acid for 1-2 min to destain the gel. In-gel assay for SOD isoenzymes was prefered using 12% separation gel and 4% stacking gel at 4◦C. After electrophoresis, the gels were pre-incubated in the solution consists of 0.2M EDTA and phosphate buffer (pH-7.8) for 30 min. The gels were incubated in 2.5 mM NBT, followed by 2.8 mM riboflavin for 45 min in the dark. The gels were then placed in distilled water and exposed to white light for 15-30 min. SOD isoenzymes were visualized as colorless bands on the purple background and washed with 10% acetic acid finally.

Statistical analysis

All the data were analysed with SPSS software and the differences between healthy and TPD affected trees were analysed by one-way ANOVA and Student-Newman Keul's Test at $P<0.05$%. The value of $P<0.05$ was considered to be significant difference. Each value was the average of two biological replicates tested in triplicate.

RESULTS AND DISCUSSION

The activities of antioxidant enzymes in bark tissues were examined. The level of POX, CAT, SOD, APX, Protein and MDA contents was compared between healthy and TPD affected clones of *Hevea brasiliensis*. The dismutation of the superoxide dismutase (SOD) generates ROS in plants, mainly in chloroplasts. SOD catalyzes the superoxide (O_2-) radical into oxygen (O_2) or Hydrogen peroxide (H_2O_2). Superoxide dismutase (SOD) are increased in the healthy clones of PB 235 and PB 5/51 (Figure 1C) whereas the level of SOD was also increased in the TPD affected clones of RRII 105, PB 285, PB 217,

PCK2, GT1 when compared to the healthy clones of the rubber tree (Figure 1A). The TPD affected clone PCK2 shows the increased activity of 7.342U/mg FW when compared to healthy clone which has a decreased activity of 3.356U/mg FW (Figure 1B). Catalase (CAT) is a key enzyme, which protects the cells from oxidative damage. It catalyzes the decomposition of H_2O_2 into water and oxygen. The activity of CAT was found to be high in TPD affected clones of RRII 105, PB 285, PB 217, PB 235 whereas the level of CAT was reduced in PB 5/51, PCK2 and GT1 TPD affected clones when matched to healthy clones (Figure 2A and 2B). In the case of PB 235, the activity was decreased in healthy clone as 1.107U/mg FW and significantly the activity was increased in TPD affected clone as 1.988U/mg FW respectively (Figure 2C).

Peroxidase (POX) plays a major role in decreasing the accumulation of H_2O_2 and maintains cell membrane integrity. The level of POX was decreased in the healthy clones (Figure 3A and 3C) but the activity was increased in the TPD affected clone of PCK2 as 0.782U/mg FW and the healthy clone show decreased activity as 0.596U/mg FW (Figure 3B). Ascorbate Peroxidase (APX) which detoxify the peroxides to H_2O_2 using ascorbate as a substrate. APX are labile in the absence of AsA (Ascorbate), to protect the plants from oxidative damages. The level of APX enzyme was increased in all TPD affected clones when compared to the healthy clones of *Hevea brasiliensis* (Figure 4A and 4C). The expression of the enzyme was high in TPD affected clone GT1 as 1.714U/mg FW when compared to its healthy clone activity (0.148U/mg FW) (Figure 4B). The level of total soluble protein contents was increased in the healthy clones of RRII 105, PB 285, PB 5/51, PCK2 and GT1 whereas the level of total soluble protein was decreased in healthy clones of PB 217 and PB 235 (Figure 5A and 5C). The PCK2 healthy clone shows maximum expression of protein (0.785U/mg FW) when compared to its TPD affected clone (0.345U/mg FW) (Figure 5B). The more amount of free radicals causes the over production of Malondialdehyde (MDA). The level of MDA content is found to be increased in the healthy clones of RRII 105, PB 217, PB 235, PB 5/51, GT1 whereas the content was decreased in the PB 285 healthy clone (Figure 6A). The expression of MDA in healthy and TPD affected clone of PCK2 were found to be as same (Figure 6B) and an increased activity of MDA was found to be 0.0125U/mg FW in the healthy clone of PB 235 (Figure 6C).

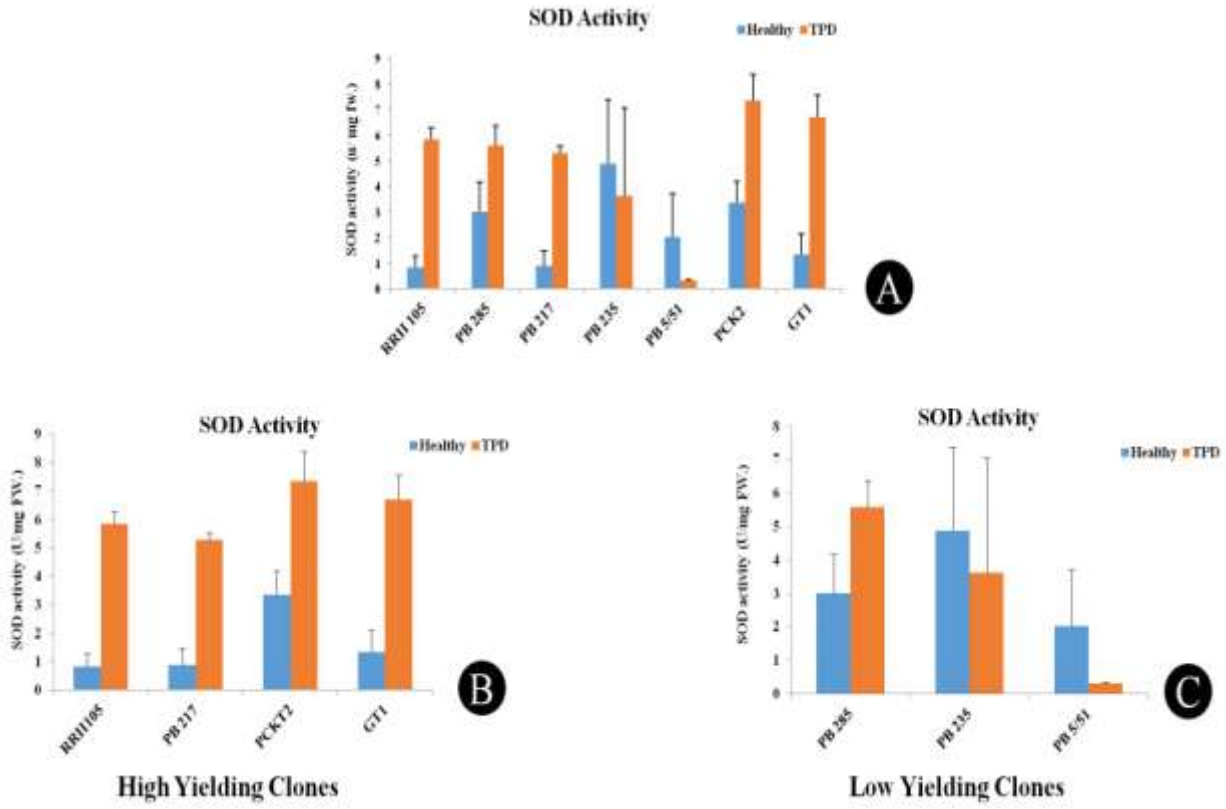

Figure 1. Antioxidative enzyme super oxide dismutase activity, between seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg. (A); in high yielding clones (B) and low yielding clones (C). The values are the means of three independent experiments ± SE.

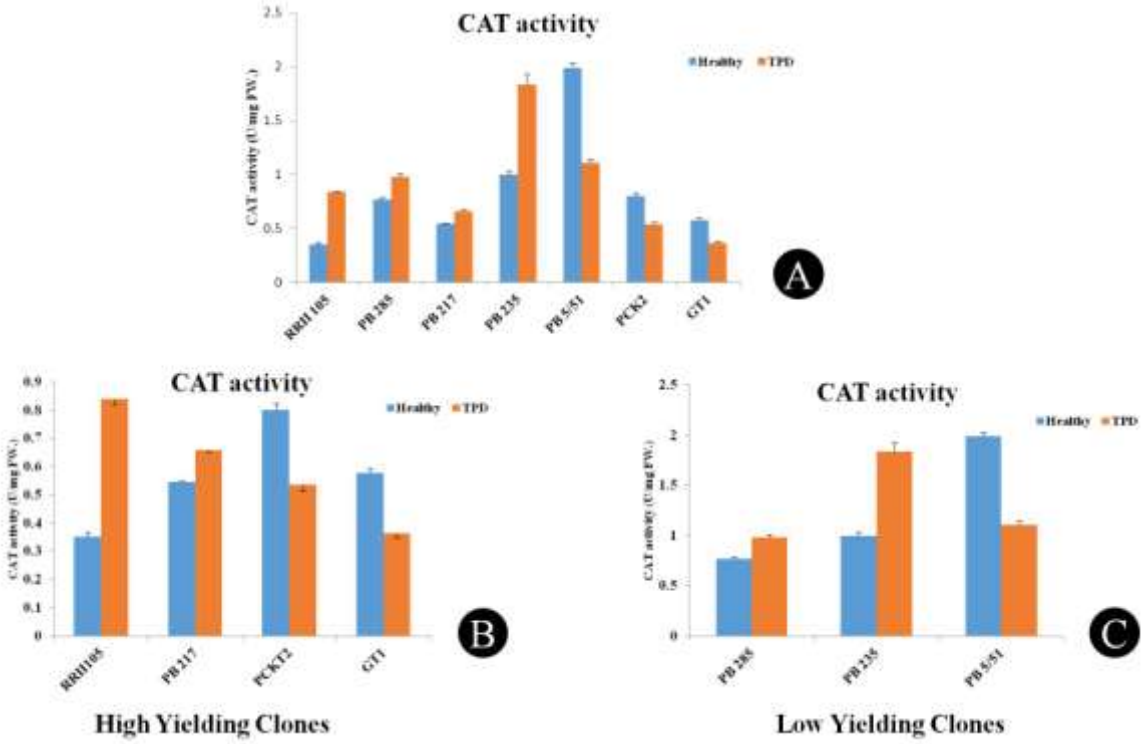

Figure 2. Antioxidative enzyme catalase activity, between seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg. (A); in high yielding clones (B) and low yielding clones (C). The values are the means of three independent experiments ± SE.

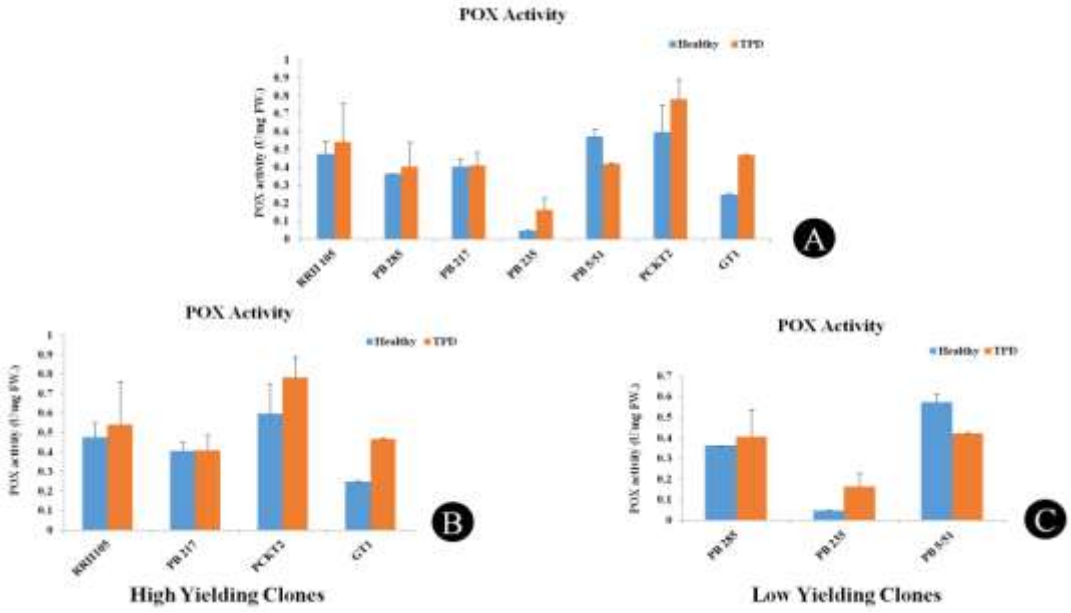

Figure 3. Antioxidative enzyme peroxidase activity, between seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg. (A); in high yielding clones (B) and low yielding clones (C). The values are the means of three independent experiments ± SE.

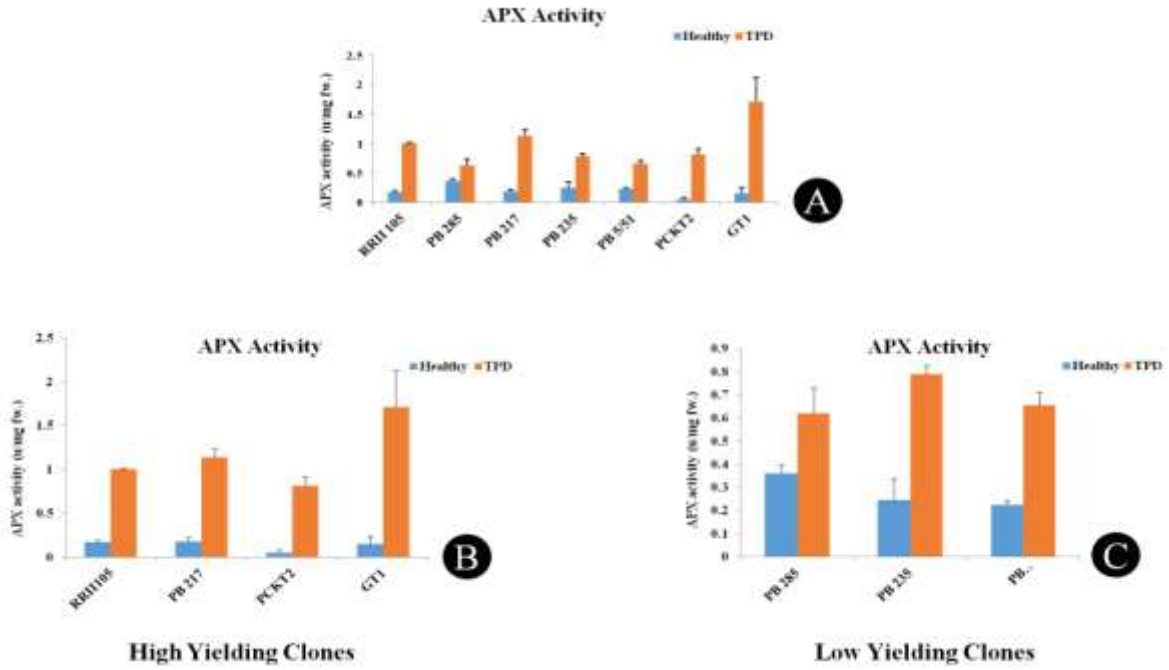

Figure 4. Antioxidative enzyme ascorbic peroxidase activity, between seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg. (A); in high yielding clones (B) and low yielding clones (C). The values are the means of three independent experiments ± SE.

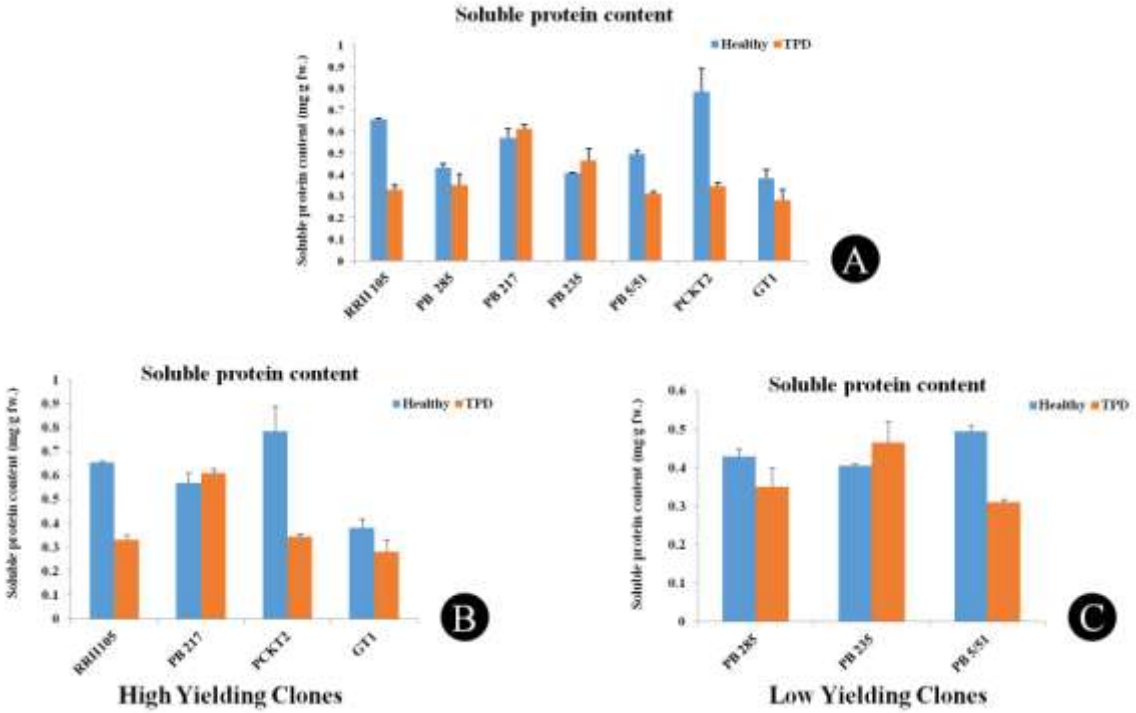

Figure 5. Soluble protein content, in seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg. (A); in high yielding clones (B) and low yielding clones (C). The values are the means of three independent experiments ± SE.

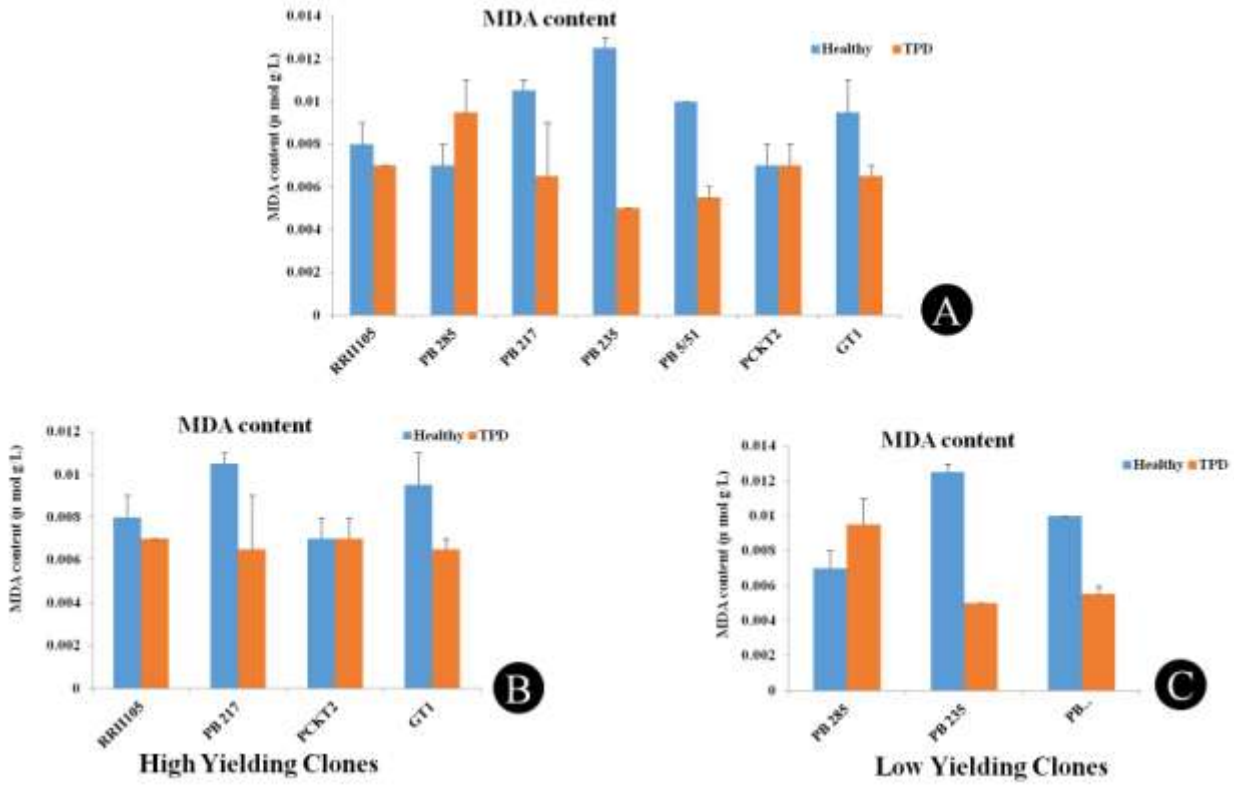

Figure 6. Malondialdehyde content, in seven different healthy and TPD affected clones of *Hevea brasiliensis* Muell. Arg. (A); in high yielding clones (B) and low yielding clones (C). The values are the means of three independent experiments ± SE.

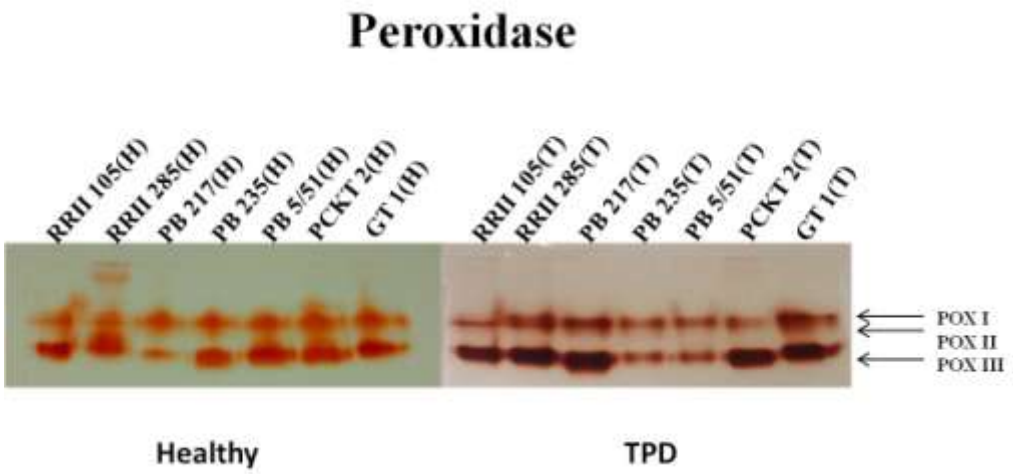

Figure 7. Isoenzyme pattern of Peroxidase (POX) antioxidant enzyme in bark tissue of *Hevea brasiliensis* among seven healthy (H) and TPD (T) affected rubber clones on native PAGE.

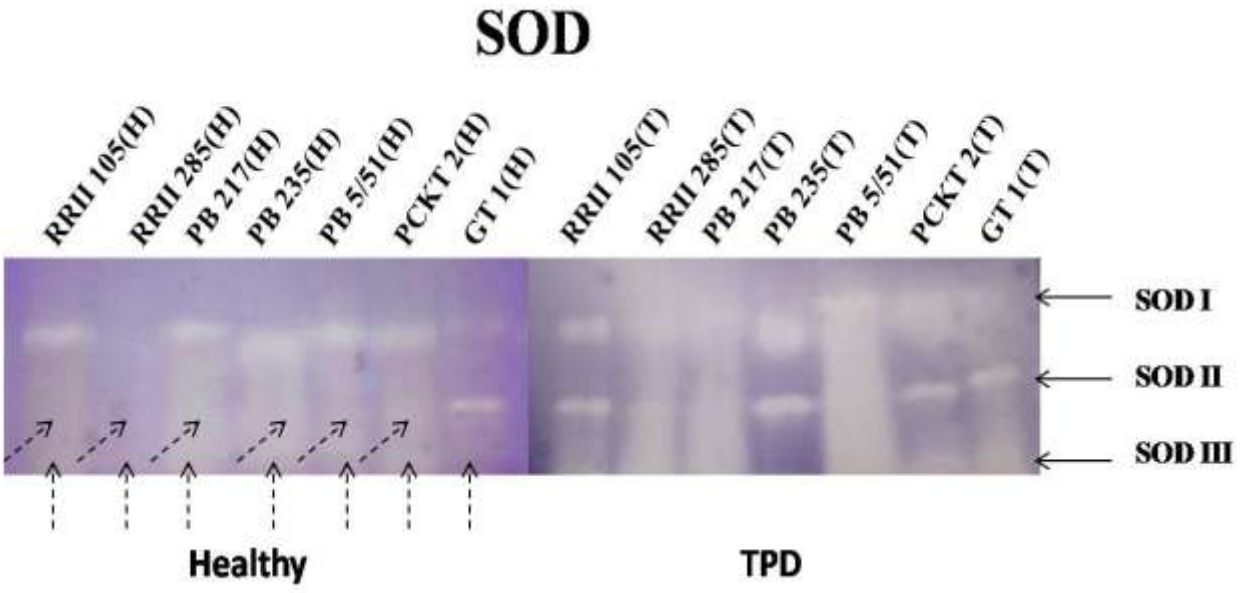

Figure 8. Isoenzyme pattern of superoxide dismutase (SOD) antioxidant enzyme in bark tissue of *Hevea brasiliensis* among seven healthy (H) and TPD (T) affected rubber clones on native PAGE. Dash arrows indicate the disappearance of SOD II and SOD III isoforms in healthy rubber clones.

A total of three isoforms of POX was found to be highly expressed in TPD affected clones when compared to the healthy clones and there were two intense isoforms of POX in TPD affected clones and faint isoforms were recorded in healthy clones respectively (Figure 7). The level of SOD activity on native PAGE was varied among different clones of the rubber tree and maximum three isoforms of SOD appeared in TPD clones (Figure 8). In this study, as suggested by earlier authors, protein degradation in TPD affected clones might be the result of increased activity of protease or other catabolic enzymes which were activated under stress condition or due to fragmentation of proteins due to toxic effects of reactive oxygen species resulting in reduced protein content (Davies, 1987). In TPD affected clones, bark tissues have been mainly affected, ultimately this condition elevates ROS in the cells that lead to oxidative damage which reduces the protein content than in the healthy clones. As a consequence of excessive ROS production, site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge and increased susceptibility of proteins to proteolysis occur. A decrease in the protein content would be a case in symptom of oxidative stress and has frequently been observed in stressed plants (Moran *et al*., 1994).

Faridah *et al.* (1996) reported that an uncompensated oxidative stress might be involved in the onset of TPD. In order to protect the plant from oxidative stress, plants initiate the expression of antioxidative enzymes like SOD, CAT, POX, and APX in specific subcellular organelles of plants, against toxic ROS or free radicals which induces oxidative stress to plants. It causes cell damage, tissue damage and even tissue death. As a result of tissue damage, the dried tapping panel and damaged bark might be formed in TPD affected clones. As oxidative stress increased inside the plants, the level of antioxidant enzyme could be proportionally increased to lower the oxidative stress. Hence, the level of these antioxidative enzymes might have increased in TPD affected clones than healthy clones. POX, CAT, and SOD are major antioxidant enzymes associated with scavenging ROS (Foyer & Noctor, 2005). SOD is regarded as the first line of defense against oxidative stress in plants. It catalyzes the dismutation of O_{2^-} into H_2O_2, which is further removed by CAT and POX and other antioxidant enzymes (Hrishikesh *et al.*, 2008). In this study, biochemical assay results showed that an increased level of SOD, POX, and APX activity in TPD affected clones during onset of TPD. The level of antioxidative enzymes activities in different healthy and TPD affected clones were varied depends upon their TPD condition, genetic variation among clones themselves and physiological tolerance against stress. Another reason for an increased level of CAT and POX is may be the location of these antioxidant enzymes; CAT is located in the peroxisomes of cell where much of H_2O_2 is concentrated. Peroxisomes are found in tissue which is mainly affected in the case of TPD affected clones and POX is present in the cytosol and cell wall and is also involved in the removal of H_2O_2 (Racchi, 2013). Thus, an antioxidative enzyme level could be increased in TPD clones while compared with healthy clones of rubber tree. It is clearly showed that as level of oxidative stress increased, the level of primary antioxidative enzymes also increased proportionally in TPD affected clone than a

healthy one. Native PAGE results for isoenzymes of SOD and POX are directly correlated with biochemical assays activity. In this work, the presence of additional SOD isoforms SOD – II and SOD – III as well as changes in the intensity of the three POX isoforms in TPD affected clones could support the relationship between TPD and enhanced antioxidative enzymes activity. These findings clearly revealed that there was an increased level of antioxidative enzymes activity in TPD affected clones to rescue the plant from TPD. In all TPD affected clones, enhanced APX activity was obtained to may scavenge the H_2O_2 to protect the plant from oxidative stress during onset of TPD occurrence. The enzyme APX has a high affinity for H_2O_2 and is able to detoxify low concentrations of H_2O_2. It catalyzes the reduction of H_2O_2 to water by using ascorbate (Foyer & Halliwell, 1976). Many researchers have reported enhanced activity of APX in response to abiotic stresses in plants (Han *et al*., 2009; Maheshwari & Dubey, 2009). In TPD tree, the contents of protein, nucleic acid, thiols and ascorbic acid decreased, whereas the activities of RNase and proteinase increased generally. TPD affected rubber clones showed a lower level MDA content and increased activities of SOD, CAT and APX during onset of TPD. Since MDA, a cytotoxic product of lipid peroxidation is usually taken as an index of ROS level. The antioxidative enzymes and MDA content present in plants play a vital role leading to adaptation and ultimate survival of plants under stress condition to resist oxidative damage. Therefore, the change of MDA content in the bark tissues of the rubber tree was determined to reveal the level of ROS during onset of TPD occurrence. The harmful effect of H_2O_2 and over production of ROS owing to reduction of MDA content in TPD affected clones than that of healthy clones.

CONCLUSION

Results concluded that the SOD activity was found to be low in TPD clones indicating the presence of excess ROS species due to wound induced stress. It is interesting to note that the level of CAT and POX activity was found to be high in TPD clones suggesting the removal of toxic H_2O_2 level. The present study clearly showed that the production of excess ROS was noticed in TPD affected trees due to wound stress (tapping). The detoxification mechanism was activated by regulating antioxidative defense genes which ultimately enhanced the level of CAT and POX activities in TPD clones to overcome the oxidative stress caused by wound. The level of antioxidative enzymes was varied depends upon the types of clones and this may be due to the genetic nature of the rubber clone. The plants were gone under some stress and that may leads to the production of antioxidative enzymes to tolerate the plants against the excessive ROS production. Thus the levels of antioxidant enzymes was found to be increased in the TPD affected clones when compared to the healthy clones of rubber tree. The environmental stress, wound stress by continuous tapping may also leads to the causing of TPD in *Hevea brasiliensis* by stimulating or inhibiting some metabolic pathways; results in decreased contents of protein and nucleic acids and increased level of antioxidative enzymes SOD, CAT, POX and other enzymes. The regulation of antioxidants enzymes in bark tissue of *Hevea brasiliensis* in response to tapping, ethephon stimulation, and TPD incidence were also newly reported by this study. This research also covered the determination of antioxidant enzymes level in bark tissue of rubber tree as well as it was found that seven rubber clones varied significantly in their antioxidant enzyme levels.

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