Differential Activity of Antioxidative Enzymes in Active and Temporarily Dormant Buds of Tea (*Camellia sinensis*)

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Abstract: The purpose of the study is to reveal the changes in the activity of different antioxidative enzymes in banjhi and active buds. Reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, are by-products of biological redoxreactions. Plant produces antioxidant enzymes, such as catalase, superoxide dismutase, ascorbate peroxidase, glutathione reductase and peroxidase to scavenge these reactive oxygen species. In the present study activity of polyphenol oxidase and antioxidative enzymes such as catalase, ascorbate peroxidase, superoxide dismutase, glutathione reductase and peroxidase were analyzed to find their variation in dormant (banjhi) and active buds in five clones of tea (*Camellia sinensis*) grown in the field. The activity of all antioxidative enzymes was higher in dormant bud than the actively growing bud in all the clones. But the activity of polyphenol oxidase was found to be reversal of the antioxidative enzymes. While considering the difference in enzyme activity between dormant and active buds the clone UPASI-10 showed lowest difference for catalase and ascorbate peroxidase. The same clone UPASI-10 showed higher difference for superoxide dismutase and peroxidase activity. The clone UPASI-3 showed highest and lowest difference for the activity of catalase and peroxidase and moderate difference in the activity of all of the enzymes were observed in the clone UPASI-9 and CR6017. A combined study about the changes in the activity of different antioxidative enzymes might help in determining the most suitable dormancy breaking agents.

Key words: Bud dormancy · catalase · *Camellia sinensis* · Peroxidase · superoxide dismutase

INTRODUCTION

Plants are subjected to several biotic and abiotic stresses that adversely affect growth, metabolism and yield [1]. Tea is a perennial woody plant commonly cultivated in subtropical regions. While the shoot elongates, its apex commonly known as the bud, is tightly wrapped in a foliage leaf which is normal in all respects that it is not expanded [2]. The rest or dormancy is the condition at which the leaves are not being unfolded and the apex is bounded by janams or bud scales (and the bud is called dormant bud). It is the temporary suspension of visible growth of any plant structure containing meristem [3]. In tea, under cultural conditions of south India, such dormant buds (which are locally called 'banjhi buds') develop throughout the year due to different abiotic stresses. Tea is generally manufactured from two leaves and a bud. 'Banjhi buds' are not favored since their presence has a detrimental effect upon the yield and quality of made tea.

The control of dormancy in perennial buds is not yet clear although many changes in growth regulators have been reported during its induction and release [4]. Dormancy in vegetative buds is divided into three

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categories namely para dormancy (correlative inhibition), ecodormancy and endodormancy (innate dormancy). Therefore, in case of tea, a clear definition on the term dormancy ("banjhiness") is needed. Banjhiness is the state in which new tea leaves temporarily stops expanding, due to the presence of "banjhi bud" [5-8].

Understanding the biochemistry of dormant and active buds is needed to serve as a basis for solving the problems of artificially breaking and inducing bud dormancy [9]. It has been noted that biochemicals such as phenols [10] and enzymes such as catalase [11], peroxidase [12], glutathione reductase [13] etc play key role in maintenance and release of dormancy in plants. Relationship between reactive oxygen species (ROS) metabolism and dormancy breakage in both plant seeds [14] and vegetative buds [15] has been reported. It was observed that different stimuli lead to dormancy release and are consistent with the hypothesis that temporary oxidative stress and respiratory stress might be a part of the mechanism that leads to bud break [16].

The present study describes the variation in activity of antioxidative enzymes in active and dormant ("banjhi") buds of field grown tea plants.

MATERIAL AND METHOD

Plant Material: Active and dormant buds with first leaf of five tea cultiars (UPASI-3, UPASI-9, UPASI-10, UPASI-17 and CR-6017) were collected from the experimental farm in UPASI-TRF, Valparai, Tamil Nadu and used in the experiment. Crude enzymes were extracted from these materials.

Peroxidase (POD): Peroxidase activity (EC 1.11.1.7) was determined as described by Chance and Maehly [17]. Bud tissue (one gram) was homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 7.0), centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant was used as enzyme source for further assay. The assay mixture consisted of 3 mL of 0.5M pyrogallol, 0.1 mL of enzyme extract and 0.5 mL of 1% (v/v) hydrogen peroxide. The activity of peroxidase was estimated spectrophotometrically at 430 nm and expressed as U mg^{-1} protein. One unit of peroxidase will form 1mg of purpurogallin from pyrogallol in 20 s.

Catalase (CAT): Catalase (EC 1.11.1.6) activity was determined as described by Luck [18]. Bud tissue (0.5 g) was homogenized in 25 mM sodium phosphate buffer (pH 7.0) and made up to 25 mL with the same buffer.

The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant was used for assay. The assay mixture consisted of 5 mL of distilled water, 2.5 mL of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mL supernatant and 0.1 mL of 25 mM hydrogen peroxide. The activity of catalase was determined spectrophotometrically at 240 nm and expressed as µmol H_2O_2 reduced min^{-1} mg^{-1} protein.

Ascorbate Peroxidase (APX): Ascorbate peroxidase (EC 1.11.1.11) activity was assayed by following the method of Nakano and Asada [19]. Two hundred and fifty mg of bud tissue was homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) polyvinyl pyrrolidone (PVP) and 2 mM ascorbate. The homogenate was centrifuged at 12,000 rpm for 10 minutes at 2°C and the supernatant was used as crude enzyme extract for assay. The reaction mixture contained 1.14 mL of 50 mM potassium phosphate buffer (pH 7.0), 1.5 mL of 2 mM ascorbate, 300 µl of 1 mM ethylenediamine tetra acetic acid (EDTA), 100 µl of crude enzyme extract and 24 µl of 12.3 mM hydrogen peroxide. The activity of ascorbate peroxidase was estimated at 290 nm and expressed as µmol ascorbate oxidized min^{-1} mg^{-1} protein.

Glutathione Reductase (GR): Glutathione reductase (EC 1.6.4.2) activity was estimated by following the method of Carlberg and Mannervik [20]. Bud tissue (0.5 g) was homogenized in 1 mL of prechilled 0.1 M potassium phosphate buffer (pH 7.0) containing 20 µl of 25 mM phenylmethaneslufonyl fluoride (PMSF) and 25 µl of 200 mM dithiothreitol (DTT) and centrifuged at 10,000 rpm for 40 minutes at 4°C. The reaction mixture contained 0.75 mL of 0.1 M potassium phosphate buffer (pH 7, containing 2mM ethylenediamine tetra acetic acid), 75 µl of nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), 75 µl of 20 mM oxidized glutathione, 100 µl of supernatant and made upto 1.5 mL with distilled water. The decrease in absorbance at 340 nm was recorded for two minutes. GR activity was as µmol NADPH oxidized min^{-1} mg^{-1} protein.

Superoxide Dismutase (SOD): The SOD (EC 1.15.1.1) activity was determined by following the method of Van Rossun [21]. Bud tissue (0.5 g) was homogenized in 5 mL of phosphate buffer (pH 7.8). The homogenate was centrifuged at 20,000 rpm at 4°C for 10 min. Three mL of reaction mixture consisted of 50 µl of the supernatant, 13 mM L-methionine, 75 µM p-nitroblue tetrazolium chloride (NBT), 100 µl ethylenediamine tetra acetic acid (EDTA)

and 2 µl riboflavin in a 50 mM potassium phosphate buffer (pH-7.8). The assay reaction was carried out at 25°C and illuminated by 40W fluorescent tubes. The blue formazane produced by NBT photo reduction was measured by increase in absorbance at 560 nm. The reaction mixtures without tissue supernatant under light and dark conditions were used as respective reaction control. The SOD activity was expressed as U mg^{-1} protein. One unit of SOD was defined as the amount of protein causing 50% decrease of the SOD-inhibitable NBT reduction.

Polyphenol oxidase (PPO): The activity of PPO (EC 1.10.3.1) (soluble and bound form) was determined as described by Singh and Ravindranath [22]. Leaf tissue (five gram) was homogenized in mortar and pestle using liquid nitrogen and the fine powder was incubated in ice cold acetone for 30 min at 4°C. The powder was filtered in Whatman filter paper No. 1 using vacuum pump and subsequently washed with acetone till it changed to white colour. 0.5 g of white acetone powder from active and dormant shoot were dissolved in 10 mL of distilled water. The suspension was centrifuged at 5000 rpm for 30 minutes at 4°C. The supernatant was used for estimation of soluble PPO activity and the residue was extracted using 0.2 M Na_2SO_4 and used for estimation of bound PPO activity. The activity was determined spectrophotometrically at 380 nm with (+) catechin as substrate and expressed as U mg^{-1} protein. One unit of enzyme activity was defined as that amount which caused a rate of change of 0.0001 absorption units per minute at 380 nm.

Assay Condition and Protein Estimation: All assays were done at 25°C. Soluble protein content was determined from the crude enzyme extracts [23] with BSA (Bovine Serum Albumin) as standard. All spectrophotometric analyses were conducted in a UV/visible spectrophotometer (Ultrospec 2100 Pro, Amersham Bioscience, USA). In all the experiments, there were five replications and each experiment was repeated thrice. Data analysis was done with the software AGRES version 7.01.

RESULT AND DISCUSSION

Activity of antioxidative enzymes such as CAT, APX, SOD, GR and POD were analyzed to find their variation in dormant (banjhi) and active (normal) buds in the field grown tea plants. In addition to the above enzymes, polyphenol oxidase (PPO) activity was also
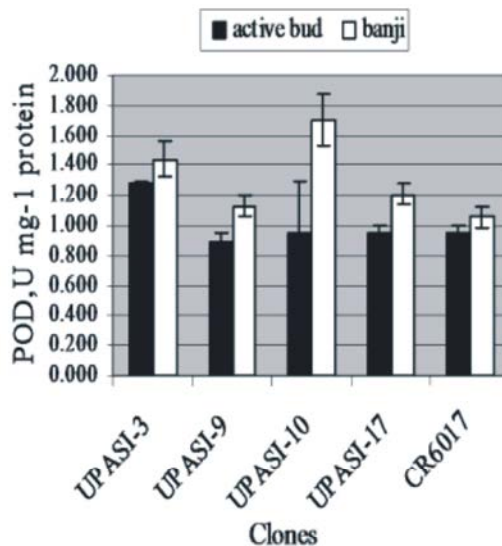

Fig. 1: Variation in peroxidase (POD) activity in dormant and active buds of different tea cultivar. Data presented are mean of five replicates with ±S.E.

analyzed, since it plays a key role in regulating the quality of made tea. To find whether the trend of the enzyme activity is same in both active and dormant (banjhi) buds, five different tea cultivar were selected from the field maintained by UPASI-TRF. Activity of all antioxidative enzymes was found to be higher in 'banjhi' bud. The above results are supported by the earlier findings on dormancy release with oxidative stress and respiratory stress [15]. But in case of PPO the result was reversal to the trend of antioxidative enzymes. The activity of PPO was found to be lower in 'banjhi" bud.

Peroxidase (POD): In all cultivar used in this study, 'banjhi' buds recorded higher POD activity than the normal buds (Fig. 1).

When considering the 'banjhi' buds cultivar UPASI-10 and CR6017 showed higher and lower POD activity respectively. In case of active bud cultivar UPASI-3 and UPASI-9 were found to be with higher and lower POD activity respectively. Except UPASI-3 active buds of all cultivar were recorded with uniform activity, but variation was observed in 'banjhi' buds of all cultivar. Highest difference in POD activity was recorded in the clone UPASI-10, clone CR6017 showed marginal difference in POD activity. 'Banjhi' and active buds of cultivar UPASI-9, UPASI-17showed uniform difference in POD. From the present study it was clear that the POD activity was higher in dormant buds. Phenolic compounds and POD are known to play an important role in the
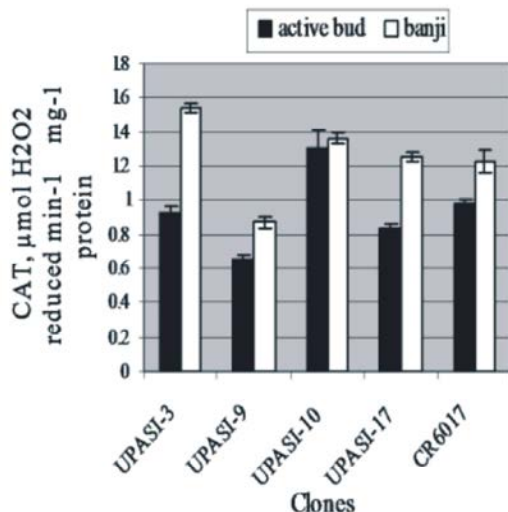
Fig. 2: Variation in catalase (CAT) activity in dormant and active buds of different tea cultivar. Data presented are mean of five replicates with ±S.E.
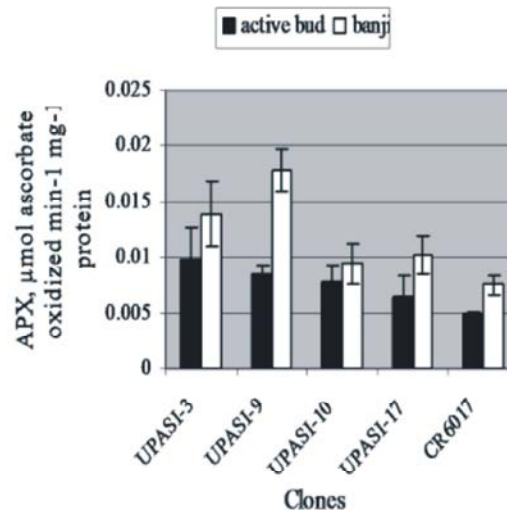

Fig. 3: Variation in ascorbate peroxidase (APX) activity in dormant and active buds of different tea cultivar. Data presented are mean of five replicates with ±S.E.

ripening and senescence of plants [24]. The actual function of POD in senescence is very ambiguous because of their ability to produce lignin and to reduce growth by cross linking cell wall materials [25]. The presence of POD activity with the ability to oxidize NADH generating H_2O_2 could be an alternative way to regulate the levels of H_2O_2 [15]. In grapevine it was observed that the activity of catalase and POD was greatest during dormancy and then decreased during the end of dormancy [26].

Catalase (CAT): The present study revealed higher CAT activity in 'banjhi' buds of all the cultivar compared to normal buds (Fig. 2).

Highest and lowest CAT activity of 'banjhi' buds were recorded in UPASI-3and UPASI-9. While considering the active buds, it was observed that clone UPASI-10 and UPASI-9 showed highest and lowest CAT activity respectively. Highest difference of CAT between the 'banjhi' and active bud was observed in the clone UPASI-3, which was followed by UPASI-17 and CR6017 respectively. Marginal difference in CAT activity of 'banjhi' and active bud was observed in the clone UPASI-10. In peach flower buds, the activity of CAT was lowest at the end of the dormancy period [11]. Positive correlation of intensity of dormancy and CAT activity was observed earlier in grapevine buds [27]. The observation in the trend of CAT activity revealed that reduction in CAT activity in active bud preceded the maximum accumulation of H_2O_2 [28]. Decreased

activity of catalase and other antioxidative enzymes may result in higher H_2O_2 content, which may be one of the earlier events leading to the termination of dormancy. Studies in other plants using CAT inhibitor and H_2O_2 treatments on dormancy, showed that similar responses may take place in both seeds and buds of fruit tree, which provides additional evidence that common regulatory mechanism of dormancy could occurs in different plant tissues [29]. In our study it was found that activity of catalase was higher in dormant buds of all cultivar and our result was supported by the earlier reports [28]. It has been suggested that catalase inhibitor or exogenous application of H_2O_2 induce dormancy breakage by favoring the oxidative pentose phosphate pathway [14]. Decreased activity of catalase and other antioxidative enzymes in normal bud tissue results in increased level of endogenous H_2O_2 and this increased level of H_2O_2 might activate the oxidative pentose phosphate pathway (PPP), which may lead to overcome dormancy.

Ascorbate Peroxidase (APX): APX activity was substantially higher in 'banjhi' buds than normal one. CR6017 showed the lowest APX activity in both normal and 'banjhi' buds while the highest activity was noticed in UPASI-3 and UPASI-9 in normal and 'banjhi' buds respectively (Fig. 3).

UPASI-9 and UPASI-10 showed the highest and lowest difference of APX activity between 'banjhi' and normal buds respectively. Interflush dormancy or phasic
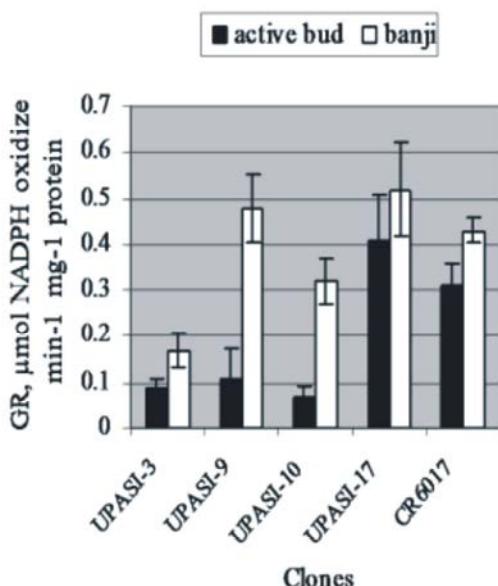
Fig. 4: Variation in glutathione reductase (RG) activity in dormant and active buds of different tea cultivar. Data presented are mean of five replicates with ±S.E.
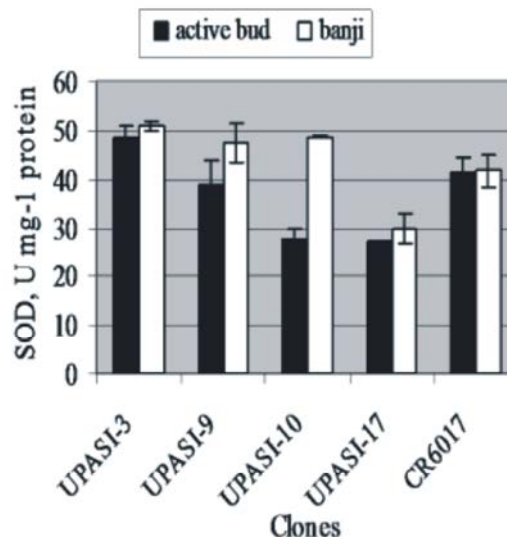

Fig. 5: Variation in superoxide dismutase (SOD) activity in dormant and active buds of different tea cultivar. Data presented are mean of five replicates with ±S.E.

growth of tea is a universal phenomenon [30]. The first attempt to use microarrays for a large scale characterization of changes in gene expression of woody bud during dormancy release has been recently reported [31]. Endodormancy release of raspberry lateral buds, which was stimulated by controlled chilling, was accompanied by induction of APX and GR genes. It was also reported that GR and APX do not plead in favour of the oxidative PPP activation [28]. Catalase might be responsible for the removal of excess ROS during stress, whereas APX might be responsible for the fine modulation of ROS for signaling [32].

Glutathione Reductase (GR): When comparing the 'banjhi' and actively growing buds, the trend of glutathione reductase was found to be higher in dormant buds of all cultivar studied (Fig. 4).

In banjhi buds, the highest and lowest GR activity was found in UPASI-17 and UPASI-3 respectively. While considering the difference in enzyme activity between dormant and active buds the higher glutathione reductase activity in dormant shoots was observed in UPASI- 9 followed by UPASI- 10 and the clone UPASI-3 showed lowest difference in GR activity. Like other antioxidative enzymes, GR was also found to be higher in dormant bud. Our result was supported by increase in

activity of GR with acquisition of frost tolerance and the onset of dormancy [13]. GR reduces oxidized glutathione (GSSH) to reduced form (GSH) in Halliwell-Asada Enzyme Pathway [33].

Superoxide Dismutase (SOD): Higher activity of SOD was noticed in 'banjhi' buds than normal buds (Fig. 5).

Among the cultivar, highest difference of SOD activity between 'banjhi' and active bud was observed in the clone UPASI-10, which was followed by UPASI-9. All other cultivar used in the study showed marginal difference in SOD activity. Highest and lowest activity of SOD in banjhi bud was recorded in the cultivar UPASI-3 and UPASI-17 respectively. Active buds of cultivar UPASI-3 and UPASI-17 showed highest and lowest SOD activity respectively. It was reported that SOD activity was inhibited in H_2O_2 treated plants, whereas, the cytosolic Ca^{2+} level remained to be elevated [34]. The GSH/GSSH ratio determines the level of protein thiols and can modify cytosolic calcium homeostasis via oxidative changes in sensitivity of Ca^{2+}-ATPase [35].

Polyphenol Oxidase (PPO): Among two types of buds (active and 'banjhi') used in the experiment, active buds showed significantly higher PPO activity than the 'banjhi' buds (Fig. 6).
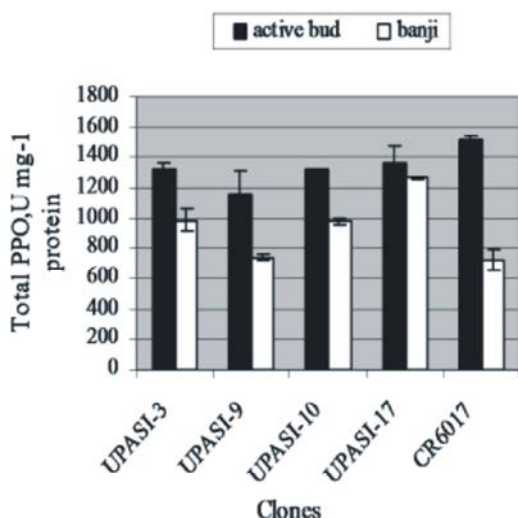
Fig. 6: Variation in total polyphenol oxidase (PPO) activity in dormant and active buds of different tea cultivar. Data presented are mean of five replicates with ±S.E.

The highest PPO activity was noticed in CR6017 and lowest in UPASI-9 in case of active buds, while UPASI-17 showed the highest and CR6017 showed the lowest PPO activity in case of 'banjhi' buds. Highest and lowest difference of PPO activity between 'banjhi' and active bud was in cultivar CR6017 and UPASI-17 respectively. PPO activity was higher in growing buds when compared to the dormant buds. This increase in PPO activity may be responsible in removing some growth inhibiting phenols [36] and the phenolic substance can modify the activity of these enzymes, as both inhibitors and stimulators.

Role of ROS in controlling the various plant processes is already known, but it is still not clear that how H_2O_2 interacts with hormones during dormancy induction and release. Recently it was reported that control of dormancy by hormones such as ABA and ethylene could be connected to H_2O_2 signaling [37].

CONCLUSION

Activity of antioxidative enzymes (CAT, APX, SOD, GR and POD) and polyphenol oxidase were analyzed to find their variation in dormant (banjhi) and active (normal) buds in the field grown tea plants. It was observed that activity all antioxidative enzymes were found to be higher in 'banjhi' bud. Based on the results of present study, it can be concluded that antioxidative enzymes plays major role in tea dormancy. These findings will help for further research in this area.

ACKNOWLEDGEMENT

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