Antioxidants and ROS scavenging ability in ten Darjeeling tea clones may serve as markers for selection of potentially adapted clones against abiotic stress

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Abstract Ten Darjeeling tea clones (BT15/263, RR17/144, B777, T253, B157, Sundaram, HV39, AV2, K1/1 and TTV1) were collected from the experimental garden of Darjeeling Tea Research and Development Centre, Kurseong. Total phenol, flavonoids and two antioxidating enzymes (peroxidase and superoxide dismutase) were estimated. The total phenol ranged between 241 and 28 GAE mg g⁻¹ of leaf dry weight. The highest amount obtained in four clones, B15/263 (241.47), RR17/144 (221.2), B777 (154.54) and B157 (140.23 mg g⁻¹). Flavonoids were estimated as Catechin equivalent (CE) and ranged between 56.88 and 20.81 CE mg g⁻¹ leaf dry weight. Higher amounts occurred in BT15/263 (56.88 mg g⁻¹), B777 (50.59) and RR17/144 (48.63). Antioxidant activities were measured following DPPH and ABTS free radical scavenging procedures and the results were well according to total polyphenol content among the clones. Total phenols, ranges of correlation in DPPH assay were $r^2=0.990-0.989$, $p\leq 0.05$; in flavonoids $r^2=0.954$, $p\leq 0.01-0.987$, $p\leq 0.05$). Similarly, ABTS percent scavenging results were quite significant. The IC₅₀ values were determined for both DPPH and ABTS

assay. PAGE expressions of isoforms in two antioxidative enzymes and quantification of them also varied much among the investigated clones. The incidence of total phenols, flavonoids, DPPH and SOD and ROS scavenging assay in in-situ condition, might be used as biochemical markers towards the superior adaptability against abiotic stress. In the present work, four clones (B15/263, B777, RR17/144 and B157) would be designated as comparatively better suited to the predicted abiotic stress.

Keywords ABTS · Darjeeling tea · DPPH · Flavonoids · Free radical scavenging · Peroxidase · Superoxide dismutase · Total phenols

Introduction

Tea is widely used beverage across the world. It properly refers to infusions prepared from leaves of the tea plant, *Camellia sinensis* (L.) Kuntze, an evergreen flowering tree belongs to the family Theaceae, native to the mountainous regions of south-western China and neighboring countries (Chang 1981; Chang and Bartholomew 1984; Ming and Zhang 1996). The main two commercial varieties are small-leaved *C. sinensis* var. *sinensis*, adapted to cool and high altitude, and large-leaved *C. sinensis* var. *assamica* (J. W. Mast.) Kitam., grown to tropical and sub-tropical environments. Wight (1962) mentioned that the genus *Camellia* consists of three species each with a specific plant types, viz. *Camellia sinensis* (China type), *Camellia assamica* (Assam type) and *Camellia assamica lasiocalyx* (Cambod type). Tender shoots of tea (consisting of two or three leaves and a bud) are harvested periodically to produce either 'black' (i.e. withered and fermented) or 'green' (i.e. withered but

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not fermented) tea. South-East Asia (i.e. India and Sri Lanka) and Eastern and Southern Africa (i.e. Kenya, Malaya, Tanzania, Uganda and Mozambique) are the major producers of black tea, while China and Japan are the major producers of green tea. Ecophysiology of the commercially grown tea plant is much climate specific. Tea growing regions of the above-mentioned countries have similar climate as cool, tropical conditions (Kingdon-Ward 1950). Tea is also grown across a range of altitudes from sea level up to about 2,200 m. above sea level (Carr 1972). However, though the tea plant is cultivated all over the world, it grows best in tropical and subtropical areas with adequate rainfalls, good drainage and a slightly acidic soil (Graham 1999) to include countries such as Russia and Georgia in the Northern hemisphere and Argentina and Australia in the Southern.

Camellia is a monogeneric perennial tree, a natural cross-breeding between these species and subspecies with considerable variations among the individual. The usual practice of tea cultivation involves the selection of natural or artificial crossing or somaclonal variation of desired combinations through vegetative propagation (clones). From the morphological, physiological and biochemical analysis, it reveals that the characteristics variation are much wide (Richards 1966; Bandyopadhyaya and Das 2008). Improved yield and quality depends on the better vegetative growths of the selected traits. As the well-defined clones are genetically uniform and stable, clonal selection is the deciding factor to attribute the improvement of tea production (Shanmuhgarajah 1994; Barua 2008). Bandyopadhyaya and Das (2008) pointed out the disadvantages of this practice over the seed propagation population, as the clones are less prone to both biotic and abiotic stress due to their high genetic plasticity and these variability considered to be more desired trait for its combat efficiency against natural hazards like disease incidence, pest infestation and wide environmental changes (Bandyopadhyaya et al. 2011).

Environmental factors, those impose adverse effects on tea plants and reduce the normal growth and yield of *Camellia sinensis* refer as stresses. Stresses may be (a) biotic stresses, which are pathogenic in nature, such as disease, insect/pest, weeds etc. (b) abiotic stresses (physical environment) such as salinity, heat, cold, drought etc. Loss by biotic stresses usually decrease crop yield less than 10 %, but abiotic stresses, are responsible up to 65 % reduction in yield (Ellis and Nyirenda 1995). Among the abiotic stresses drought is one of the major threat that adversely affect the plant growth and yield all over the world (Stephen and Carr 1989). The manufactured tea is potential source of readily available beverage worldwide but its production is much more prone to environmental changes (Stephen and Carr 1989; Wijeratne 1996; Ng'etich 2000). Under prolonged dry weather conditions, growth of tea is adversely affected by plant water deficits created by lack of soil moisture and

associated high saturation vapour pressure deficit of the air (Stephen and Carr 1989). On the basis of morphological characters, soil and leaves moisture contents, selection of draught tolerance clones were designed at National Tea Research Institute, Mansehra (Waheed et al. 2012).

Generation of ROS (reactive oxygen species) in plants is an inevitable outcome during normal metabolic processes. These unsaturated reduced oxygen species are toxic by-products, generated at low levels in non-stressed plant cells in chloroplasts and mitochondria, and also by cytoplasmic, membrane-bound or exocellular enzymes involved in redox reactions (especially photosynthetic electron transport processes and respiration). Extra amounts of ROS occur under stressful conditions such as pathogen attacks, wounding, herbivore feeding, UV light, heavy metals and others (Wojtaszke 1997; Díaz et al. 2001). Generally plant's cells try to keep the possible low level of ROS concentration because they are more reactive than molecular oxygen (O_2) (Wojtaszke 1997), leading to apoptosis. ROS are known to damage cellular membranes by inducing lipid peroxidation (Ramadani and Prasad 1998), damage DNA, proteins, lipids and chlorophyll (Mittova et al. 2000). The commonly known ROS are superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$) originating from one, two or three electron transfers to di-oxygen (O_2). Plants develop several enzyme and antioxidative systems as a defensive force against excess ROS, which can slow down or scavenge or disrupt the oxidative chain reactions (Sgherri et al. 2003). It was well established that non-enzymatic scavengers are essential in the protection of cellular components from most ROS, but they cannot cope with reducing radicals such as superoxide or metastable hydroperoxides (Chaudiere and Ferrari-Iliou 1999). Grudarzi and Pakniyal (2009) opined that the plants defend against ROS by inducing the activities of certain antioxidative enzymes like catalase, peroxidase, glutathione reductase and superoxide dismutase. Alscher et al. (2002) reported that the scavenging of ROS by increased activation of antioxidant enzymes can be attributed to efficient adaptability against abiotic stress. Apart from the enzymatic system, several epidemiological studies have suggested the importance of secondary plant metabolites, the phenolic compounds; those are consumed in reducing the incidence of ROS mediated cellular apoptosis in oxidative stress (Kinselia et al. 1993; Schlesier et al. 2002).

There are immense interests of biologists on antioxidative property of polyphenols in plants as a combat force against detrimental influence of environmental factors which leads to generate free radicals (Ostrowska et al. 2001; Zurina et al. 2010). The most commonly used antioxidant activity determination methods are ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (1, 1-

diphenyl-2-picrylhydrazyl) assay. Both of them have excellent reproducibility characteristics under certain assay conditions. The ABTS radical (cation, ABTS^+) must be generated by enzymes or chemical reactions (Arnao 2000). Another important matter is that ABTS^+ can be dissolved in aqueous and organic media, in which the antioxidant activity can be measured, due to the hydrophilic and lipophilic nature of the compounds, those existed in the plant cells. In contrast, DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle et al. 1996) and is a useful reagent for investigating the free radical scavenging activities of polyphenol compounds (Duan et al. 2006). Many researchers have reported positive correlation between free radical scavenging activity. DPPH can only be dissolved in organic media, especially in ethanol, this being an important limitation when interpreting the role of hydrophilic antioxidants. Both radicals show similar bi-phase kinetic reactions with many antioxidants. Hydrogen peroxide itself is not very reactive, but it would provide highly reactive species $\text{OH}^\cdot$ through Fenton reaction (Halliwell and Cross 1994). Earlier reports suggested that H_2O_2 could induce DNA breakage in the intact cell and purified DNA (Imlay and Linn 1988). Thus removal of H_2O_2 is crucial for medicinal importance. The reducing capacity of a compound may serve as indicator of its potential antioxidant capacity (Meir et al. 1995). The antioxidant capacity of compounds has been attributed to various factors such as prevention of chain reaction, chelating metals, reductive capacity and radical scavenging ability etc. (Diplock 1997; Yildirim et al. 2001). Phenolic compounds are commonly found in plants and have been reported to possess several biological activities including a strong antioxidant activity (Duh et al. 1999; Chandini et al. 2008). The antioxidant capacity of phenolic extracts is very often attributed to their radical scavenging ability mediated by hydroxyl groups (Hatano et al. 1989). Along with other component interactions, it was also affirmed that possible antiradical activity of tea extracts was conditioned by antioxidant production (Gramza et al. 2005).

The flavonoids have been recognized as the main chemical components in plant leaves. In the leaf flavonoid complex, 14 compounds were characterized (isolated and structurally determined and/or chromatographically identified by use of authentic standards): quercetin, kaempferol, and isorhamnetinmonosides and disides containing the D-galactopyranosyl, D-glucopyranosyl, L-arabinofuranosyl, L-arabinopyranosyl, L-rhamnopyranosyl, and/or D-xylopyranosyl units as the sugar residues. Over the past decade evidence has accumulated that plant polyphenols and, especially, flavonoids are a most important class of defense anti-oxidants. With several endogenous antioxidants they play a role in optimum protection from oxidative stress caused by the increase in the level of reactive oxygen species (ROS). The documented mechanism of

flavonoid antioxidant action includes suppressing ROS formation (by inhibition of enzymes or chelating trace elements involved in ROS generation), direct ROS scavenging and up-regulating or protecting antioxidant defenses (Middleton and Kandaswami 1993; Pietta 2000).

In view of the above, the present work, in a limited scope, out of 30 recognised clones of Darjeeling tea released by Tea Research Association, India, available ten clones were taken into consideration for biochemical characterization towards a preliminary selection of comparatively resistant clones against changed climatic factors. Total phenolic compounds, flavonoids were assayed as well as the scavenging abilities of unwanted free radicals generated within the plant body were measured following ABTS and DPPH assay. Qualitative and quantitative analyses of two antioxidative enzymes PRX and SOD were also estimated for a comparative documentation among the clones investigated towards their stress tolerance ability.

Materials and methods

Ten available Darjeeling tea clones, out of 30 clones (released by Tea Research Association, India), namely B15/263, RR17/144, Sundaram, HV39, T253, B157, AV2, K101, TT-V1 and B777 were collected from the experimental garden of Darjeeling Tea Research and Development Centre (DTR and DC), Kurseong—a high altitude area of Darjeeling district, West Bengal, India. In all cases, 4th–6th leaves from the apex were selected for maintaining the equal maturity level. Rudimentary leaf buds were collected in ice for enzyme analysis. The geographical location of the collection site includes $26^\circ 55' \text{N}$ $88^\circ 12' \text{E}$; altitude 1,527 m above mean of sea level. Average rainfall is 344.5 mm/year and temperature ranges between 22 and 4°C . Relative humidity prevails maximum 90 % and minimum 80 %.

Methanolic extracts were prepared by maceration of tea leaves (10 g) with 250 ml of 95 % ethanol, at room temperature for 24 h and then filtered. The procedure was repeated thrice. The extracts were filtered and centrifuged (4,500 g, 15 min). The solvent was evaporated on rotary evaporator (Neocool Circulator, CF 300, Yamato). The concentrated methanolic leaves extracts were kept frozen (-20°C) until further use (Huda-Faujan et al. 2009).

Determination of total phenol Total phenol contents (TPC) in the extracts were determined following modified Folin–Ciocalteu method (Singleton and Rossi 1965). Aliquots ($0.05\text{--}0.3 \text{ mg ml}^{-1}$) of the extractants were prepared for all the samples. To 1 ml of the methanolic solution of the extractants, 2 ml of Folin–Ciocalteu reagent (previously diluted with water at 1:10 v/v) and 1.6 ml (7.5 %) of sodium carbonate (Na_2CO_3) were added. The mixture was allowed

to react at room temperature for 30 min for colour development. Spectrophotometric absorbance (Helios γ , Thermo Electron Corporation) was measured at 765 nm. Gallic acid was used as a positive control in the same concentrations. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg g^{-1} of dry material based on the calibration curve, where X was the absorbance and Y was the gallic acid equivalent (mg g^{-1}). Each experiment was carried out in triplicates.

Determination of total flavonoids Estimation of total flavonoids in the plant extracts were carried out following Jia et al. 1999 and Lakshman 2012. Aliquots ($0.2\text{--}1.2 \text{ mg ml}^{-1}$) of the extracts were prepared for all the samples. To 1 ml of methanolic solution of the extracts, 0.3 ml of 5 % sodium nitrite (NaNO_2) were added and mixed. The mixture was kept at 25 °C temperature for 5 mins. After which, 0.3 ml of 10 % aluminium chloride (AlCl_3) was added. The tubes were then allowed to stand at room temperature for 5 min. Following which, 1 mM sodium hydroxide (NaOH) solution was added and the absorbances of the solutions were measured at 510 nm. Catechin was used in the same concentration as a positive control. Total flavonoid content was calculated as Catechin equivalent (mg g^{-1}) on the calibration curve, where X was the absorbance and Y was the Catechin equivalent (mg g^{-1}). Each experiment was carried out in triplicates.

DPPH radical scavenging assay The free radical scavenging activity of different plant extracts were determined using the stable radical DPPH (1, 1-diphenyl-2-picrylhydrazyl) following Ribeiro et al. (2002). Freshly prepared DPPH solution (25 mg L^{-1}) in methanol was prepared and 3.9 ml from this was mixed with 0.1 ml of extract in methanol containing $0.05\text{--}0.3 \text{ mg ml}^{-1}$ conc. of the extract. The absorbance was measured at 517 nm after 30 min. Butylated Hydroxy Toluene (BHT) in the same concentration as the sample was used as positive control. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_c - A_t)}{A_c} \times 100$$

Where A_c is the absorbance of the blank reaction and A_t is the absorbance in presence of the sample of the extracts. Each experiment was carried out in triplicates.

ABTS radical scavenging assay To determine ABTS radical scavenging assay, the method of Re et al. (1999) was adopted. The working solution was prepared by mixing two stock solutions 7 mM ABTS solution and 2.4 mM potassium per-sulfate solution, in equal quantities and allowing them to react for 12 h at room temperature in the

dark. This solution was diluted 60 times while working. Fresh ABTS solution was prepared for each assay. Plant extracts (0.1 ml) in methanol containing $0.005\text{--}0.03 \text{ mg ml}^{-1}$ conc. of the extract were allowed to react with 3 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min. Butylated Hydroxy Toluene (BHT) in the same concentration as the sample was used as positive control. The ABTS scavenging capacity of the extract was calculated as:

$$\text{ABTS radical scavenging activity (\%)} = \frac{(A_c - A_t)}{A_c} \times 100$$

Where A_c is the absorbance of the blank reaction and A_t is the absorbance in presence of the sample of the extracts. Each experiment was carried out in triplicates.

Enzymes: qualitative estimation by gel electrophoresis

Extraction of enzyme Two grams of young leaf buds were macerated in powder with liquid nitrogen with a mortar-pestle and then 0.1 g PVP and 5 ml of extraction buffer was added. The extraction buffer consists of 1 M Sucrose, 0.2 M Tris-HCl and 0.056 M β -Mercaptoethanol; pH was adjusted at 8.5 after volume made up by double distilled water. The macerates were centrifuged at 1,500 g for 20 min at 4°C to collect the supernatants.

PAGE analysis Equimolar amounts of protein were loaded in each well. Different isoforms of enzymes were separated by native gel electrophoresis. Isozymes were stained for definite enzymes following Das and Mukherjee (1997). The gel electrophoresis experiment was repeated five times with the same molecular weight marker. In each case, the number of isoforms and similar OD values obtained at same R_{mf} . Gel was documented with a Gel-Doc System (Biostep GmbH—Germany) and densitometry analysis for band intensity and Relative Mobility Factor (R_{mf}) were estimated with Kodak-MI software.

Quantitative estimation: enzyme assay

Peroxidase (PRX, E.C.1.11.1.7) Two hundred milligram fresh leaf sample extracted in 1–1.5 ml 0.9 % KCl and centrifuged at 12,000 g for 15 min at 4 °C; the supernatants used as enzyme sample. Absorbance were taken spectrophotometrically (Helios γ , Thermo electron Corporation, USA) at 460 nm with respect to the standard curve prepared following Shannon et al. (1966) with minute modification.

Superoxide dismutase (SOD, E.C.1.15.1.1) Cell sap extracted from 200 mg of leaf and 1–1.5 ml 50 mM Phosphate buffer, pH adjusted to 7.0; centrifuged at 12,000 g for 15 min at 4 °C. Supernatants were used for enzyme samples. Standard curves were prepared of standard enzyme samples at different aliquots (50, 100, 150, 200, 250 $\mu\text{g ml}^{-1}$). Absorbances measured at 550 nm following the protocol described by Keith et al. (1983) with slight modification.

A two-tailed bivariate correlation coefficient (Pearson coefficient) was calculated with SPSS-12 for incidence of correlation values of polyphenol, flavonoids, two enzymes (PRX and SOD) with per cent scavenging by both DPPH and ABTS assay and their IC_{50} values (Table 1).

Results

The total polyphenol content (TPC) was determined spectrophotometry, using gallic acid as standard, the TPC was expressed as gallic acid equivalents (GAE) in g/100 g material. The Folin–Ciocalteu assay is one of the oldest methods developed to determine the content of total phenols present within the plant body. In this work, the total phenol content of ten tea clones from Darjeeling was taken in to consideration. Among the investigated clones, the ranges of incidence of total phenols were varied widely as 241–28 mg g^{-1} GAE. The highest amount of total phenol occurred in the clones namely BT15/263 (241.47 mg g^{-1} GAE), RR17/144 (221.2), B777 (154.54) and B157 (140.23) and the lower amount occurred in HV39 (28.62), K1/1 (51.82) and AV2 (53.88 mg g^{-1} GAE). The average amounts of total phenols were represented in the figure (Fig. 1a) along with their standard errors.

Flavonoids were estimated in the investigated clones as catechin equivalents (CE) and expressed as per g dry weight. Among the investigated clones, high amount of flavonoid occurred in BT15/263 (87.65), B777 (83.58) and RR17/144 (75.45). The lower ranges of flavonoid recorded in Sundaram (42.57), HV39 (45.57) and AV2 (51.11). Flavonoids content in the rest clones ranged between 54.55 (T253) and 61.5 CE (K1/1). The graphical representation of average amount was provided with respective standard errors (Fig. 1b).

The antioxidant potential of leaf extracts was determined on the basis of scavenging activity of the stable 2, 2'-diphenyl-1-picryl hydrazyl (DPPH) free radical. Scavenging activities of the extracts on the stable free radical DPPH were assayed in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence of the sample. The dependence curves of remaining DPPH• radical in the reaction system of plant extract's concentration in precise time was presented in Fig. 1c, d. The analysis of

curves permitted affirmation that studied extracts showed the DPPH• radical scavenging ability, dependent on their concentration in the system. Moreover, per cent inhibition inversely varies with the required IC_{50} values.

Scavenging activities of the leaf extracts on the stable free radical DPPH were assayed, in which the bleaching rate of DPPH is monitored after a fixed time period at a characteristic wavelength in presence of the sample. Among the clones investigated, DPPH mediated per cent inhibition of free radical estimated in highest percentage occurred in the clones BT15/263 (26.51 %), B777 (24.82 %), RR17/144 (23.41 %) and B153 (18.13 %) and comparatively lower values occurred in K1/1 (9.22 %), HV39 (9.28 %) and Sundaram (11.72 %). The per cent inhibition value inversely varied with the 50 % free radical scavenging value of the extractants (IC_{50}). Hence, the samples required for 50 % scavenging were high in K1/1 and HV39 (0.948 and 0.922 respectively) (Fig. 1c, d).

The ABTS assay was calibrated with the Butylated Hydroxy Toluene (BHT). All tea leaf extracts displayed antioxidant activities as they were able to scavenge the $\text{ABTS}^{\bullet+}$ radical cation. The ABTS+ study revealed that the per cent scavenging of free radicals occurred highest among the ten clones were in BT15/263 (46.63 %), RR17/144 (38.95 %) and B777 (31.98 %); lower per cent inhibition found in HV39 (19.87 %), TTV1 (22.24 %) and K1/1 (23.79 %). The clones which showed the high amount of per cent inhibition, IC_{50} (required for 50 % scavenging of free radical) values were lower (Fig. 1e, f).

Antioxidative enzyme estimation revealed that in BT15/263, the highest numbers of isoforms of peroxide (PRX) were obtained (six bands), whereas, in others, mostly four and in some cases three bands were recorded. The densitometry analysis showed, in BT15/263, the highest OD was 155.27 (at 0.51 R_{mf}) and lowest OD observed as 68.58 (at 0.69 R_{mf}). In all the cases of K1/1 and HV39, the PRX isoforms were three in different R_{mf} . Rest of the cases, four isoforms of PRX were recorded. The OD values were much less in all three R_{mf} values occurred in Sundaram, K1/1 and HV39, in comparison to the other clones investigated (Figs. 2 and 3a).

Similarly, superoxide dismutase (SOD) were analysed among the ten clones and revealed that in some cases the isoforms were four, in some cases three and only in HV39, it was two. The four isoforms were obtained in BT15/263, B157, B 777, RR17/144 and T253. The highest OD recorded in B157 (133.42 at 0.08 R_{mf}) and in BT15/263 (119.57 at 0.06 R_{mf}). In B777, the highest and lowest OD values were 97.95 (at 0.06 R_{mf}) and 76.85 (at 0.52 R_{mf}). Three isoforms were found in AV2 (with higher OD value of 76.19 at 0.083 R_{mf}), Sundaram (with higher OD value of 78.01 at 0.15 R_{mf}), TTV1 (with higher OD value of 76.41 at 0.41 R_{mf}) and K1/1 (with higher OD value of 64.41 at 0.23

Table 1 Bivariate (2 tailed) correlation among polyphenols, flavonoids, enzymes (PRX & SOD) and percent radicle scavenging activity

Parameter	BT15/263	RR17/144	B 157	Sundaram	T 253	B 157	AV2	K1/1	TTV1	HV39	Parameter
Phenol (GAE)	0.990**	0.964**	0.989**	0.718	0.687	0.602	0.685	0.734	0.312	0.397	DPPH% inhibition
Phenol (GAE)	-0.979**	-0.915*	-0.915**	-0.711	-0.663	-0.565	-0.597	-0.147	-0.577	-0.067	DPPH (IC ₅₀)
Flavonoids (Catechin)	0.998**	0.947*	0.966**	0.831	0.765	0.534	0.732	0.676	0.687	0.517	DPPH% inhibition
Flavonoids (Catechin)	-0.984**	-0.963*	-0.701	-0.682	-0.683	-0.456	-0.726	-0.784	-0.574	-0.517	DPPH (IC ₅₀)
Phenol (GAE)	0.997**	0.972*	0.983*	0.900	0.813	0.792	0.983*	0.688	0.511	0.421	ABTS% inhibition
Phenol (GAE)	-0.978**	-0.955*	-0.833	-0.624	-0.234	-0.658	-0.499	-0.455	-0.337	-0.469	ABTS (IC ₅₀)
Flavonoids (Catechin)	0.964*	0.974*	0.934*	0.900	0.833	0.856	0.982*	0.876	0.891	0.787	ABTS% inhibition
Flavonoids (Catechin)	-0.963*	-0.752	-0.843	-0.819	-0.625	-0.514	-0.905	-0.936*	-0.852	-0.682	ABTS (IC ₅₀)
PRX	0.987**	0.954*	0.939*	0.900*	0.896	0.739	0.672	0.719	0.795	0.853	DPPH% inhibition
PRX	-0.997**	-0.973*	-0.971*	-0.870	-0.795	-0.708	-0.835	-0.696	-0.881	-0.630	DPPH (IC ₅₀)
SOD	0.985**	0.870	0.769	0.868	0.795	0.814	0.699	0.726	0.697	0.087	DPPH% inhibition
SOD	-0.951*	-0.904	-0.936	-0.966*	-0.900	-0.840	-0.757	-0.633	-0.728	-0.415	DPPH (IC ₅₀)
PRX	0.973**	0.795	0.960*	0.951	0.840	0.640	0.887	0.207	0.780	0.559	ABTS% inhibition
PRX	-0.999**	-0.993*	-0.910*	-0.891	-0.872	-0.711	-0.765	-0.351	-0.967*	-0.492	ABTS (IC ₅₀)
SOD	0.953*	0.951*	0.995**	0.998*	0.981*	0.883	0.72	0.732	0.682	0.493	ABTS% inhibition
SOD	-0.976*	-0.946	-0.735	-0.729	-0.713	-0.770	-0.684	-0.622	-0.594	-0.007	ABTS (IC ₅₀)

*Correlation is significant, $p \leq 0.05$ (2-tailed)**Correlation is significant, $p \leq 0.01$ (2-tailed)

Fig. 1 Graphical representation of estimated **a** total phenols; **b** flavonoids; **c** percentage of free radicle scavenging with DPPH and **e** ABTS, **d** and **f** their IC₅₀ values of extracts of tea plants

R_{mf}). Only two isoforms of SOD obtained in HV39 (with highest OD value of 86.46 at 0.58 R_{mf}) (Figs. 2 and 3).

Quantitative estimation revealed that BT15/263, RR17/144, B157 and B777 were the high PRX contained clones (591.34, 581.35, 543.98 and 519.74 $\mu\text{g g}^{-1}$ of leaf respectively) and AV2, TTV1 and T253 were in group of moderate PRX activity (496.6, 486.7 and 476.87 $\mu\text{g g}^{-1}$ of leaf respectively). Sundaram, K1/1 and HV 39 showed comparatively less amount of PRX activity. B777, RR17/144, BT15/263 and B157 were found to be more SOD activity (378.98, 361.84, 342.76 and

326.87 $\mu\text{g g}^{-1}$ of leaf respectively). Moderate SOD activity were found in T253, TTV1 and K1/1 (313.15, 304.33 and 286.51 $\mu\text{g g}^{-1}$ of leaf respectively) and rest were below 300 $\mu\text{g g}^{-1}$ of leaf. Among all AV2 and HV39 were to be the lowest SOD activity (265.9 and 237.6 $\mu\text{g g}^{-1}$ of leaf) (Fig. 3c).

The statistical analysis revealed that incidence of total phenols, flavonoids and antioxidative enzymes were directly correlated with per cent scavenging by DPPH and ABTS and inversely with IC₅₀ values. Phenolic compound and per cent scavenging by DPPH has significant correlation in case of BT15/263, RR17/144 and B777

Fig. 2 Graphical representation of OD vs. R_{mf} of two enzymes, PRX and SOD in ten tea clones

Fig. 3 **a** PAGE expression for peroxidase; **b** PAGE expression for superoxide dismutase; **c** graphical representation of PRX and SOD activity

($r^2=0.990$, 0.964 and 0.989 , $p \leq 0.05$ respectively). Similarly, ABTS also showed significant correlation in these clones ($r^2=0.987$ $p \leq 0.05$; 0.954 $p \leq 0.01$ and 0.976 , $p \leq 0.05$ respectively). Occurrence of flavonoids also

significantly correlated with per cent scavenging activity by DPPH and ABTS (Table 1). Incidence of PRX and SOD also showed better correlation values with free radicle scavenging activities.

Discussion

Premature cell destruction or wilting due to any stress in plant occurs when ROS production is beyond the capacity of antioxidant processes and detoxification mechanisms of its own. Aerobic organisms have developed complex systems protecting them from ROS, consisting of several enzymes and antioxidants. All plants produce a wide diversity of secondary metabolites. The most important groups of these metabolites are phenolic compounds. They are mainly synthesized from cinnamic acid, produced from phenylalanine triggered by L phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Dixon and Paiva 1995). Among the diverse functions of Phenolics in plants, it has been established earlier that enhancement of phenylpropanoid metabolism and the amount of phenolic compounds can be influenced under different environmental factors and stress conditions (Grace and Logan 2000; Lovala et al. 2000; Sakihama and Yamasaki 2002; Cheruiyot et al. 2007). The induction of phenolic compound biosynthesis was observed in wheat and maize in response to metal toxicity (Díaz et al. 2001; Winkel-Shirley 2002). Parida et al. (2004) reported that high salt concentration is a consequence of enhanced accumulation of free amino acids and total phenols. Content of flavonoids and phenolic compounds in root extract of *Rhizophora apiculata* and *Acanthus ilicifolius* was tested under oxidative stress and remarkable positive correlation was reported by Asha et al. (2012). In earlier work, it was evident that the increase in flavonoid concentration is mainly the result of conjugate hydrolysis and not due to de novo biosynthesis (Parry et al. 1994). Hence, increase in soluble phenolic compounds enhances lignin biosynthesis and attribute to the typical anatomical changes induced by stress and increase in cell wall endurance and the creation of physical barriers preventing cells against stress (Díaz et al. 2001).

DPPH is a stable free radical that can accept an electron of hydrogen radical to become a diamagnetic molecule. The reduction in DPPH radical was determined by the decrease of its absorbance at 517 nm (in ethanol) induced by antioxidants (Blois 1958). DPPH reaction has been widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate the antioxidant activity of plant extracts. Higher DPPH-scavenging activity of phenolic extracts were related to the total phenol and flavonoid contents (Ziying et al. 2007). In the present study, the per cent scavenging activity was determined in ten tea clones and found comparatively higher free radical scavenging mechanism occurred in BT15/263, RR17/144, B777 and B157 than those of the other clones investigated. The IC_{50} value for each extract, defined as the concentration of extract causing 50 % inhibition of absorbance, was determined

from the curves plotted and bar diagram was provided. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value would reflect greater antioxidant activity of the sample. Banerjee et al. (2008) experimentally substantiated that high phenolic compound, strong radical reducing ability leading to less IC_{50} values ($<2.9 \text{ mg ml}^{-1}$) in 11 mangroves.

The two antioxidative enzymes, peroxidase and superoxide dismutase were estimated both qualitative and quantitatively. It was evident that, though polymorphic incidence of PRX among the studied taxa was not prominent, only BT15/263 tea clone showed the highest number of isoform (six) and in other, it was mostly four or three isoforms. This result might be attributed to the relatively high suitability of this clone in the stressful environment. Rai et al. (2004) stated peroxidase which participated in lignin biosynthesis and might built up a physical barrier against stress response hazards. Soluble, apoenzymic PRX can scavenge H_2O_2 cooperating with phenolics (Takahama and Oniki 2000). Earlier works pointed out that the first step of antioxidant action is catalyzed by peroxidases where Phytophenolics can be regenerated from phenoxyl radicals by non-enzymatic reaction with ascorbate (reaction 2) inhibiting the formation of degraded products (Yamasaki et al. 1997; Sekihama et al. 2002; Sgherri et al. 2003).

With respect to incidence of isoforms in superoxide dismutase (SOD) among the investigated ten clones, in most of the cases 4 bands occurred (in BT15/263, B157, B777, RR17/144 and T253), the lowest number of isoform noted in HV39 (two bands) and the rest of the clones showed three isoforms (Sundaram, TTV1 and K1/1). Increased number of isoforms and enzyme activity might be related to the more sustainability of the plants. Therefore, it is evident that the clones producing increased amount of isoforms and enzyme activity of antioxidative enzymes like PRX and SOD may increase their ROS scavenging ability. It has been experimentally proved that significant enhancement of superoxide dismutase activities in green tea may have an antiprogenic activity due to its radical scavenging activity mechanism (Midori and Hasegawa 2003). An obvious consequence of salt stress in plants is the production of excess ROS in the form of O_2^- (Superoxide anion) H_2O_2 and the hydroxyl radicals ($OH^\bullet$) particularly in chloroplast and mitochondria (Mittler 2002; Masood et al. 2006). It was established that plants possess a number of antioxidative enzymes like Superoxide dismutase, ascorbate peroxidase, glutathione reductase etc. for protection against damaging effect of ROS (Asada 1992; Procházková and Wilhelmová 2007). Foyer et al. (1994) also opined that plants possess efficient system of scavenging ROS, thus protecting them from destructive oxidative reaction and antioxidant enzymes play a pivotal role in defense mechanisms in stressful environment. Successful stress management can be attributed to a plant to withstand the imposed stress and this probably happens by

either tolerance or efficient ROS management systems which permit to cope up with the imposed stress (Dasgupta et al. 2011). It is also postulated that the loss of ability to scavenge free radicals in stress is correlated to the decrease of antioxidant enzymes (Mittova et al. 2002). It is well established that environmental variability like temperature, soil moisture, relative humidity, rainfall, wind velocity etc. have much impact on yield, both quality and quantity (Humphries and Roberts 1964; Tanton 1982). Water stress affects biochemical constituents that determine the tea quality. Stress due to soil moisture causes a reduction in PAL (Phenylalanine ammonia lyase) activity. Lower PAL activity correlated with lower synthesis of flavones such as epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), (which are important precursors of theaflavin-3, 3'-digallate) that determines final tea quality (Jeyaramraja et al. 2003). The antioxidant activities were well correlated with the total polyphenol content in tea was experimentally reinforced by Anesini et al. (2008).

The essential processes leading to stress tolerance in plants are mainly the control of water loss through stomata and metabolic shifting through toxic ion homeostasis and osmotic adjustment. On the other hand, it has been established that stress tolerance can be correlated with enhanced ROS scavenging ability (Hasegawa et al. 2000; Kavitha et al. 2008). Superoxide dismutase (SOD) and several antioxidant enzymes are potentially involved in H_2O_2 metabolism leading to photoprotection. Antioxidative enzymes in relation to stress tolerance in different plants have been evaluated much, but remain focused because enhanced accumulation of these not only associated with stress tolerance, but also with stress sensitivity (Abogadalla 2010). Singh (1992) published a report where he described all 26 tea clones for Darjeeling tea garden released by TRA, India and classified their desired traits based on morphological parameters. He considered some clones as draught resistant among the 30 clones in four categories, viz. poor, fair, good and very good. The present biochemical characterization on the basis of total phenol, flavonoid, antioxidative enzyme production and efficient scavenging ability (% scavenging of ROS through DPPH and ABTS) is as a whole in conformity with earlier classification.

The present work provides a base line information towards the assessment of comparatively sustainable tea clones among the studied ten clones (out of about 30 clones for Darjeeling area, released by TRA) so far for better adaptability against probable environmental stress. In each clones, total phenols and flavonoids, their free radical scavenging ability, qualitative and quantitative analyses of two antioxidant enzymes (PRX and SOD) were addressed. Relatively higher accumulation of polyphenol, flavonoids, antioxidative enzymes and increased

free radical scavenging efficiency in-situ condition might be used as a biochemical marker towards the selection of comparatively sustainable clones against predicted stressful condition. In the present work, among the tea clones addressed, B15/263, B777, RR17/144 and B157 are to be more resistant clone, as these four clones were revealed to produce relatively higher amount of both phenolic and flavon compounds. The clones namely AV2, K1/1 and HV39 showed comparatively lower amount of phenol and flavonoid, which may be indicative to their inferior adaptability to environmental stress. However, this remains to be confirmed in future long-term studies.

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