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Full Length Research Paper

Variation in antioxidant and aroma compounds at different altitude: A study on tea (*Camellia sinensis* L. Kuntze) clones of Darjeeling and Assam, India

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Plants at high altitude are subject to enhanced oxidative stress due to high UV fluence (resulting from air rarefaction) compared to places at low altitude. For survival, plants have developed specific cell protective compounds viz. the flavonoids. Flavonoid derivatives (viz. flavonoid glycoside) may also serve as precursor of aroma producing compounds and are thus relevant in studies on plant quality under varying UV fluence. This study was conducted with an aim to understanding the effect of geographic location viz. altitude on aroma compounds in tea leaf, focusing on the internationally reputed Darjeeling tea. Darjeeling grown tea clones (compared with the same clones growing in Assam) are found to have higher; (a) accumulation of flavonoids and flavonoid glycosides that are known to function as UV screens and antioxidants with flavonoid glycosides, additionally serving as aroma precursors, (b) activity of phenylalanine ammonia lyase for flavonoid biosynthesis, (c) activity of β-D glucosidase that releases aroma conferring aglycons from flavonoid glycosides, (d) accumulation of aglycons viz. linalool that is known to cause aroma in tea. In all the cases, a varietal difference (manifested in respective clones) was observed. The understanding developed from the study should help not only for plant survival in the face of global climate change but also for aroma enhancement in tea plants cultivated at non-conventional sites.

Key words: UV radiation, reactive oxygen species, flavonoid glycoside, aroma, tea, high altitude.

INTRODUCTION

Plants at high altitude under naturally prevailing high UV fluence are subject to enhanced oxidative stress (Lesser, 1996; Balakrishnan et al., 2005). To combat such stress, plants have evolved effective cell protective mechanisms that retard cell damaging processes thus enabling plants to survive on earth. In this context, a group of effective antioxidants, viz. the flavonoids specifically present in plants (Stapleton and Walbot, 1994) are particularly relevant. These compounds maintain antioxidant potential in cells due to the phenol-quinone tautomerism in the side chain; the flavonoid group of compounds serves as electron acceptors from reactive oxygen species (ROS) mediated oxidized macromolecules, thereby protecting cells against UV caused ROS mediated damaging effect (Kirsch, 2001; Rebeiro et al., 2002). Accumulating in plant epidermis and showing absorption for UVA and UVB (Cockell and Knowland, 1999) by virtue of $\pi \rightarrow \pi^*$ transition in core nucleus make flavonoids good contenders for sun screening protection in plant cells. Robberecht and Caldwell (1978) demonstrated that flavonoids block up to 95 to 98% transmittance of incoming UV radiation into plant cells. Derivatives of flavonoids, viz. flavonoid glycosides are also involved in conferring aroma compounds to various extents under variation in environment (Carrao-Panizi and Bordington, 2000). In view of the on going, study of plants growing at high altitude and at low altitude (under natural UV radiation-high and low respectively) seems a worthwhile proposition for generating information of interest particularly in the face of a global climate change. In this context, tea clones cultivated under similar tea cultivation

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practices in Darjeeling and clones of the same varieties cultivated in Assam, both places at the same latitude (~27°N), ensures comparison in data between the two locations (Cabrera et al., 1995) on altitudinal variation in cell protection and quality of plants.

UV fluence is higher in the subtropical regions (Turunen and Latola, 2005); during the months of April to July, when the sun shifts to the Northern hemisphere, highest UV fluence is recorded within 15 to 35°N latitude (http//teanewsdarjeeling.blogspot.com/2010/04/rain-cheer for teaindustry-plantars-html), that encompasses many of the developing nations in northern Africa and southern parts of Asia including India. During this period, tea plants of Darjeeling and Assam produce the first flush (that is, first yearly burst) of leaves that produces best tea each year. Of the cultivation sites at these two places at different altitude, tea leaf of Darjeeling, India, is internationally reported for flavor/aroma quality (Lachman et al., 2003). For an in depth understanding towards developing strategies for value addition in tea plants cultivated at other sites, a comparison of experimental data between clones of different varieties of tea plants growing in tea cultivation sites of Darjeeling at ~4500 ft msl and of Assam at ~350 ft msl, where UV fluence presents single major variation in environmental factor, is presented here.

Plants growing in same location, that is, under equal UV fluence may exhibit variation in the degree of cell damage actually incurred; this is due to their variation in varietal/genetic make-up that determines the extent of genetically conferred flavonoid mediated defense against UVB radiation (Mazza et al., 2000). Reports that genetic block in the synthesis of flavonoids (that is, phenolic UV absorbing sunscreens) in phenylpropanoid mutants of *Arabidopsis* (Kliebenstein et al., 2002) and susceptibility of such plants to UV radiation (Li et al., 1993; Lois and Buchanan, 1994; Landry et al., 1995; Ryan et al., 2002) provide evidence to suggest a genetic effect as the basic cause for plant protection against natural UV radiation. Survival/occurrence of different plant groups at different altitudinal levels exemplifies such a phenomenon (Kreft et al., 2002). Constitutive levels the basic genetically controlled UV absorbing compounds viz. flavonoids and the readiness of plants to accumulate/enhance these in plants under enhanced UV radiation have been correlated with UV-B tolerance in several studies (Murali and Teramura, 1986). Such reports indicate epigenetic up-regulation of enzymes in the biosynthetic pathway for synthesis of flavonoids. Accumulation of flavonoids (in *Pisum sativum* leaf) only within hours of UV induction (Strid and Porra, 1992; He et al., 1994) provide further evidence for UV effect superimposed on the basic genetic attribute for flavonoid production and thereby establishes the existence of a relationship between increased UVB radiation and enhancement of genetically attributed flavonoid synthesis level in plant cells. Induction of enhanced flavonoid group of compounds in response to enhanced UV fluence may thus be seen as a prophylactic induction of reactionary biosynthesis of the flavonoid group of compounds (Strid and Porra, 1992). Phenylalanine ammonia lyase (PAL) and other enzymes involved in the biosynthetic pathway for phenolic compounds viz. flavonoids is also reported to be up-regulated by enhancement of UV (Logeman et al., 2000; Kubasek et al., 1992). This and other more direct studies (Strid and Porra, 1992; He et al., 1994) indicate an UV up-regulation effect on biosynthesis of flavonoids. Studies on effect of UV on the biosynthetic pathway involving the supply pathways from primary metabolism and also the final steps for flavonoid secondary product formation indicates an enhancing effect of UV on most of the enzymes involved in flavonoid production (Logeman et al., 2000).

In leaf of a number of plant species, the major flavonoids are found as derivatives in the form of flavonoid glycosides. These compounds exist as flavonoids linked by 1-2 and/or 1-6 glycoside bonds with sugar rings and exhibit a number of biological activities particularly conferring antioxidant potential (Rebeiro et al., 2002). Antioxidant potential of flavonoid glycosides have been reported in black berry, blue berry and red wine grape (Cho et al., 2004) and several other plants. In tea leaf, flavonoid glycosides is added to antioxidant potential, such compounds also contribute towards better quality in tea (Goswami and Barbora, 1994); the author suggests that the tasters' score for strength could be due to presence of higher amounts of flavonoid glycosides in the tasted tea. Das et al. (1994) also demonstrated the presence of flavonoid glycosides in Indian tea clones. In Chinese tea clones, Li et al. (2005) have reported glycosides with monoterpene alcohols and aryl alcohols as aglycons that on enzymatic hydrolysis, release aglycons (Conesa et al., 2002) such as linalool and geraniol that confer aroma in black tea, oolong tea and green tea (Sakata et al., 1995; Ogawa et al., 1995). Other derivatives of flavonoids viz. methylated flavonoid di- and tri glycosides (Franski et al., 2002) acetylated flavonol glycosides (Shahat et al., 2003) have also been reported in different plant species.

In different plants, the aglycon moiety is known to confer typical flavour. In soybean, flavonoid glycosides on cleavage at the glycosidic bonds by the enzyme β-D glucosidase, releases non reducing β-D glucose, and the terminal aglycone that have been reported to confer flavour, and also antioxidant potential in plants (Carrao-Panizzi and Bordington, 2000). Expression of the gene for β-D-glucosidase, the enzyme reported to split flavonoid glycoside, has also been reported to be enhanced (up-regulated) under abiotic stress (Spano et al., 2005) including UV radiation.

MATERIALS AND METHODS

Plant materials

Sampling scheme selection of leaves from high and low altitude tea

gardens were randomly selected, each following the scheme simple random sampling without replacement (SRSWOR). Young leaves were collected from tea plants/clones of genotypes T78, AV2 and CP1 growing in conventional tea cultivation sites viz. Ging tea estate, Darjeeling, (at high altitude ~4500 ft msl) and Tocklai experimental station of tea research association (TRA), Tocklai, Assam (at low altitude ~ 350 ft msl). Both these places are at ~27°N latitude where variation in UV radiation at the respective altitudes represents the major variable environmental factor. From both these cultivation sites, leaves from clones of different varieties were collected for experimentation each year during the 1st flush (April to June) that provides the best tea of fresh and strong flavour (http//teanewsdarjeeling.blogspot.com/2010/04/rain-cheer for teaindustry-plantars-html).

Method

Flavonoid analysis

Total flavonoid in young leaf of clones originating from different varieties growing both at high and at low altitude was estimated by high performance liquid chromatography (Figure 1 and Table 1a) according to the method of Moriguchi et al. (2001). Experimental sample (young leaf) was extracted in 1 ml of a (1:1) (v/v) mixture of dimethyl sulphoxide (DMSO) and methanol; debris was removed by centrifugation at 6000 rpm for 3 min. The supernatant was diluted with distilled water and aliquots were injected into the chromate-graphic system consisting of waters 501 pump, an automatic sampler, a Hypersil ODS reverse phase column (Hewlett Packard, 125×ϕ 4 mm r.d.), and UV diode array detector (waters, detector 486). The elution schedule consisted of an initial 2 min of 20% methanol in 10 mM phosphoric acid followed by a gradient with increasing concentration of methanol from 20 to 100% in 55 min at the flow rate of 1 ml/min. The detector was set to measure spectra from 220 to 400 nm since the conjugated π systems of flavonoids afford good UV absorption; the eluent was monitored at 285 nm, that is, within the UV range of wavelength coinciding with absorption maxima (λ max) of flavonoids (that matches with UVB absorption). Rutin (quercetin-3-rutinoside) (Santa Cruz Biotechnology) was used as standard (0.01 mg/ml).

Flavonoid glycoside analysis

Flavonoid glucoside was specifically assayed (Poole and Poole, 1994) by thin layer chromatography (TLC). Methanol: water (1:1) extracts (in aliquots) were spotted with micro-capillary tube on silica gel coated plates (Figure 2). The silica plate (stationary phase) was placed in chloroform: acetone: methanol (20:6:5) that provided the mobile phase for the TLC run of the chemical components of the leaf extracts. Constituents of leaf were observed by specific (for flavonoid glycosides) color development using aqueous solution of 1% $FeCl_3$ and K_3 Fe (CN) $_6$.

Assay of rutin (specific flavanoid glycoside)

Rutin related assay of tea clones were done according to the modified method of Atanassova and Bagdassarian (2009). 500 mg of tea leaves are extracted in 50 ml of 80% methanol. With that, 2 ml distilled water and 5 ml ammonium molybdate were added. The mixture was diluted to 50 ml by adding distilled water. The standard rutin solution was prepared by dissolving 0.02 g of rutin in 50 ml of 80% methanol. Then, 2 ml of this standard rutin solution was considered comparable with sample material (tea clones) extract as mentioned. The absorbance of the sample extract and also the rutin preparation were determined at 360 nm. The percent rutin content (%) of the samples was determined according to the following calculations and as shown in Table 1b.

Calculations:

Calculations are based on averaging results from analyses of triplicate samples.

The rutin content (R) % in the sample is calculated as follows:

$$(R)\ \% = \frac{A_{sample} \times C \times 50 \times 100}{A_{standard} \times W \times 2}$$

Where A_{sample} = absorbance of the sample at 360 nm, $A_{standard}$ = absorbance of the standard solution at 360 nm, C= concentration of standard solution of rutin in gm/ml, W = weight of the sample in gm, 2 ml = volume of the sample taken.

Assay for phenylalanine ammonia lyase (PAL)) (EC number 4.3.1.24)

200 mg tissue were homogenized at 4°C in a pre-cooled mortar and pestle with sea sand and 150 mg of poly vinyl pyrrolidone (PVP) in 3 ml of 0.1 M borate buffer (pH 8.8) containing 50 mM β-mercapto ethanol according to Saunders and McClure (1974). The homogenate was centrifuged at 18,200 g for 30 min at 4°C. Supernatant (1 ml) containing the enzyme phenylalanine ammonia lyase (PAL) was added to 0.5 ml of 50 mM L-phenylalanine (substrate) for enzyme assay. PAL activity was assessed by monitoring the increase in A_{290} against a control without phenylalanine over a period of 4 h at 1 h intervals. The rate of appearance of trans-cinnamic acid was taken as a measure of enzyme activity using an increase of 0.01 $A_{290} \equiv 3.09$ nmol of trans-cinnamic acid formed. The PAL activity is expressed in P_{kat} (pmol trans- cinnamic acid) formed per second per mg total protein (Figure 3) (total protein in leaf extract was assessed according to the method of Bradford (1976).

Total UV absorbing compounds analysis

200 mg of each sample were placed in 1.4 ml of 99:1 methanol: HCl and allowed to extract for 48h at -20°C. Absorbance of extract was read spectrophotometrically at 305 nm for determination of total UV absorbing compounds (Mazza et al., 2000) (Figure 4).

Antioxidant assay

The antioxidant potential of leaf extracts was determined as Ribeiro et al. (2002) on the basis of scavenging activity of the stable 2, 2' – diphenyl-1 picrylhydrazyl (DPPH) free radical (Figure 5). Aqueous extract (0.1 ml) of experimental leaf was added to 3 ml of a 0.004% methanolic solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated as [(Ao-Ae) / Ao] x 100 (where Ao = absorption without extract, Ae= absorption with extract).

β-D glucosidase (EC number 3.2.1.21) enzyme assay

A method, modified over that of Matsuura et al. (1989) and Jiang and Li (1999) was used. Experimental leaf tissue was extracted in buffer (50 mM sodium acetate buffer pH 5.0 containing 1 mM ethylene diamine tetra acetic acid (EDTA), NaCl, $MgSO_4$, 1 mM

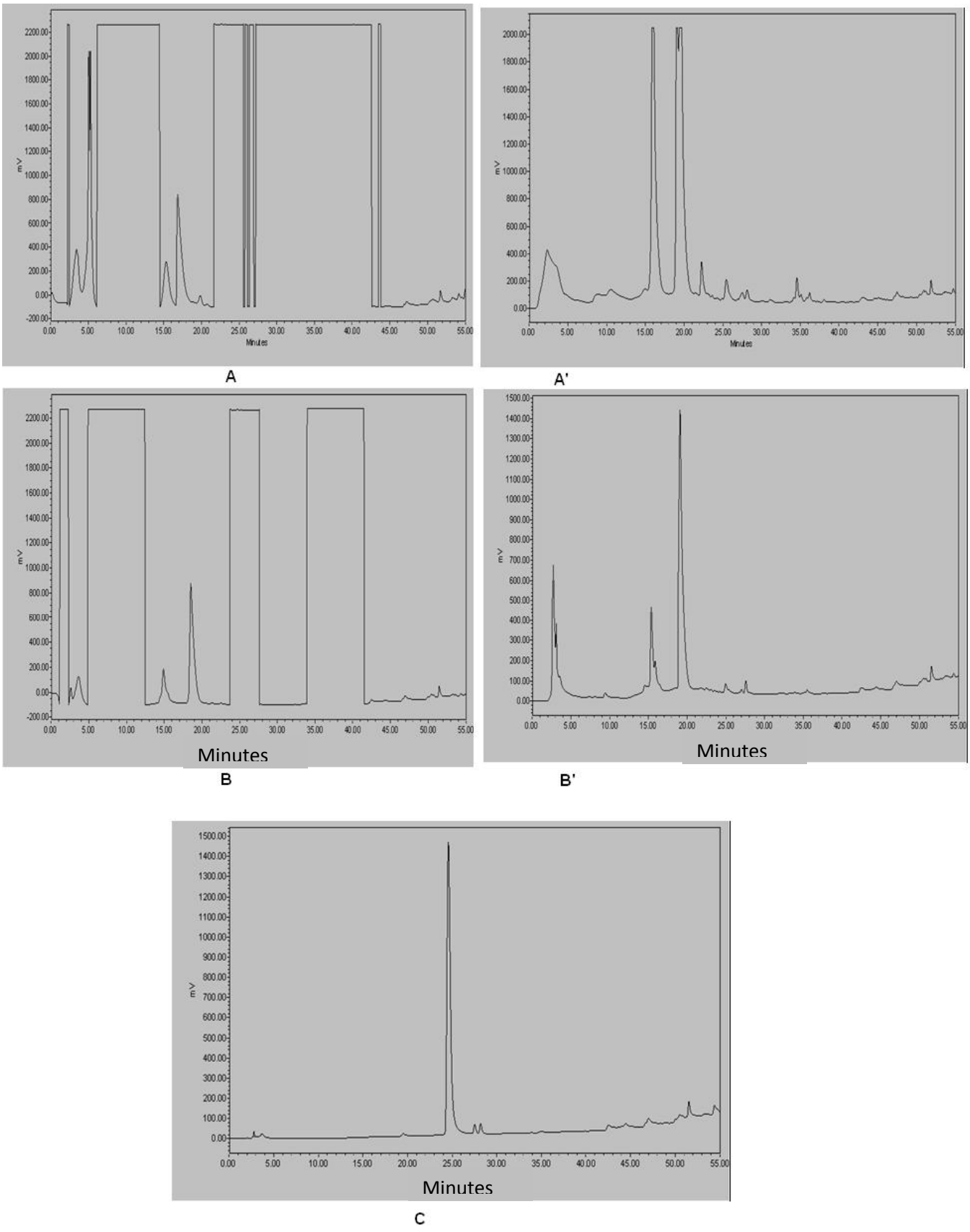


Figure 1. Flavonoid analysis by HPLC scan recorded at 285nm using DMSO: MeOH solvent system at 0-55min retention time in hypersil ODS2 column A = T78(D), A' = T78(A), B = CP1(D), B' = CP1(A), C = standard rutin.

Table 1a. Quantitative estimation for total flavonoids and flavonoid glycosides taken from HPLC scan, eluant taken at 285nm by UV diode array detector (waters 486) (Figure 1).

Tea clones	Peak area within 0-55 min retention time (mV-min)	Peak area at 25 min retention time (mV-min)	Concentration Of rutin (Mg/Ml)
T78 (D)	123700.11	11700.00	0.05
T78 (A)	1095.00	9.90	0.000044
CP1 (D)	95611.00	10000.90	0.04
CP1 (A)	832.75	8.0	0.000035

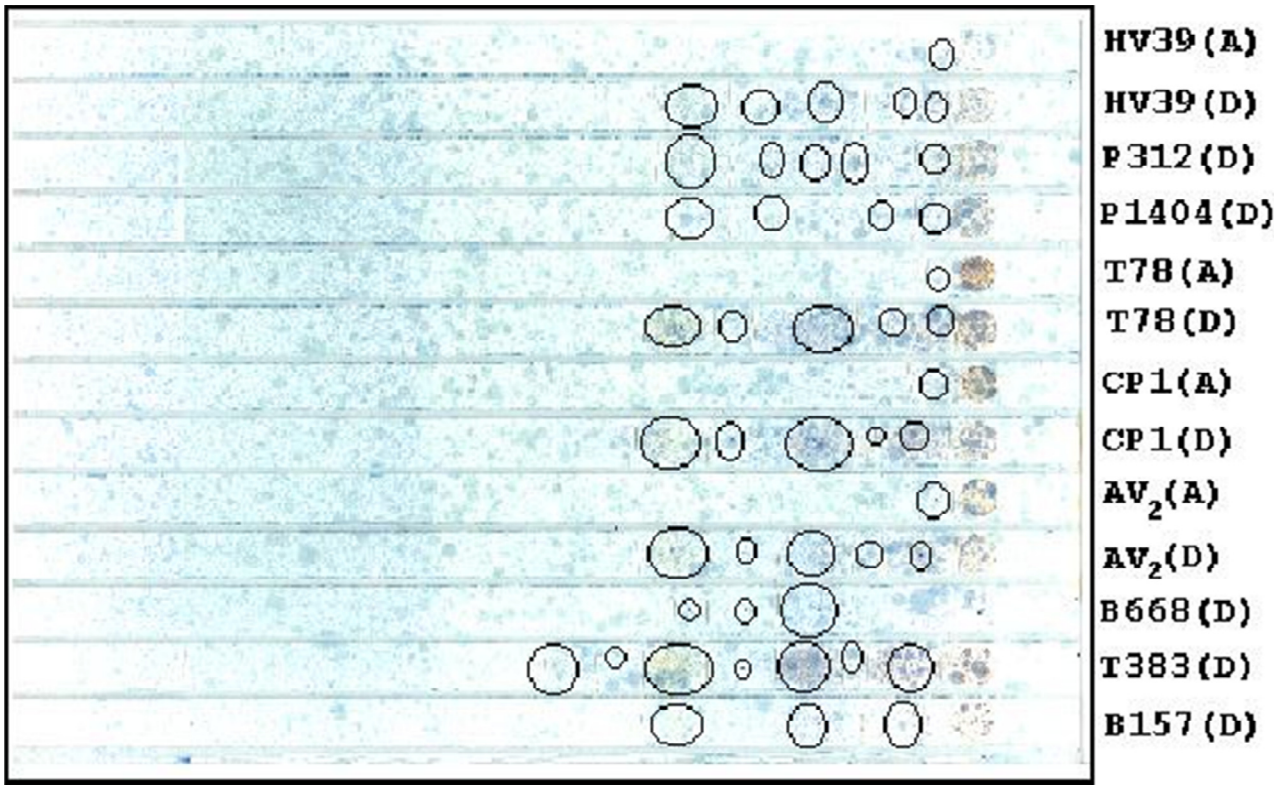


Figure 2. Thin layer chromatographic separation on plate of flavonoid glycoside in tea clones growing in high and low altitude. 1 = B157(D),2 = T383(D),3 = B668(D),4 = AV2(D),5 = AV2(A),6 = CP1(D),7=CP1(A), 8 = T78 (D),9 = T78(A),10 = P1404(D),11= P312(D),12 = HV39(D),13 = HV39(A).

Table 1b. Rutin content (%) in different tea clones.

Tea clones	Rutin content (%)
T78 (D)	0.65±0.001
T78 (A)	0.005±0.001
CP1 (D)	0.55±0.001
CP1 (A)	0.001±0.001

Estimation of total rutin (flavonoid glycoside) content (%) determined spectrophotometrically at 360 nm.

phenyl methane sulphonyl fluoride (PMSF), 1 M sucrose, 2% PVP, 5.6 mM β-mercaptoethanol, and centrifuged at 20,000 g for 20 min. Supernatant was taken for enzyme assay. One ml assay mixture contained 4 mM paranitrophenyl-β-D-glucopyranoside (PNPG), in sodium citrate buffer (pH 4.0) and 60 µl leaf extract. After incubating for 10 min at 40°C, the reaction was stopped by adding 3 ml of aqueous sodium carbonate (2% w/v) and the color, developed as a result of p-nitrophenol liberation, was measured

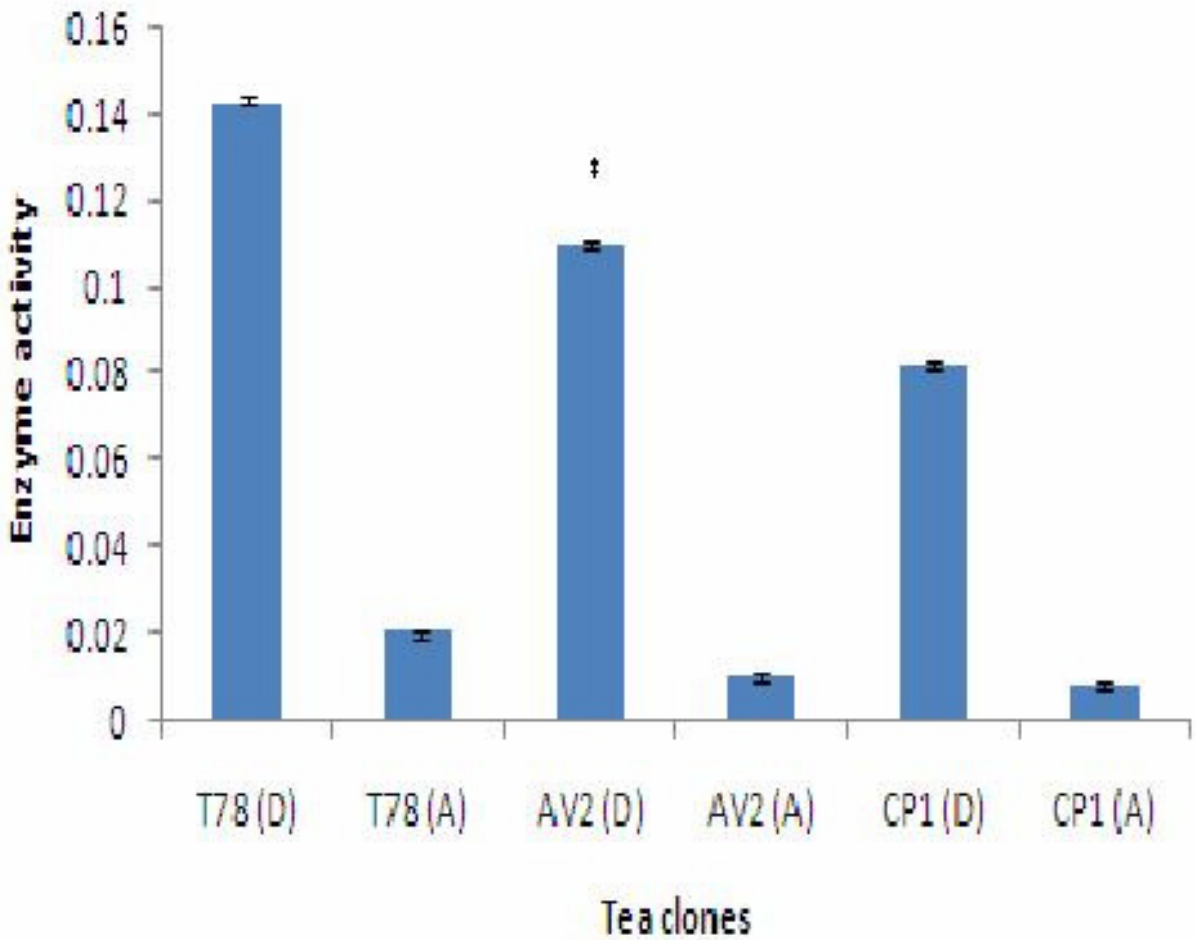


Figure 3. Spectrophotometric assay of enzyme phenylalanine ammonia lyase of tea clones viz. T78, AV2, CP1. D = Darjeeling, A = Assam. ($P < 0.001$, for S. D n = 6).

spectrophotometrically at 410 nm. The change in absorbance at 410 nm gives assessment of the enzyme activity. The data expressed as activity / mg protein is presented in Figure 6 (total protein in leaf extract was assessed according to the method of Bradford, 1976).

Assay for linalool

Percent content of linalool was measured according to reports in http://www.ffcr.or.jp/zaidan. Ten milliliters of leaf extract (200 mg leaf extracted in 10 ml of Tris-Cl buffer pH 7.5) was transferred into a flask and allowed to stand in ice water for 10 min; after this, 20 ml of dimethylaniline was added and shaken well. This was followed by addition of 10 ml acetyl chloride and 5 ml of anhydrous acetic acid and left to stand for 5 min in ice cold condition, allowed 30 min at room temperature, followed by heating in a water bath at 50°C for 4 h. After cooling, the contents were transferred to a separating funnel, and washed 3 times with 75 ml ice cold water. The oily layer was then washed with 25 ml of dilute sulfuric acid. Sodium hydroxide solution was added to alkalinize the washings until turbidity subsided. The extract was then washed with 10 ml of sodium carbonate solution until it become alkaline; then again, it was washed with 25 ml of sodium chloride solution until the solution become neutral. Oily phase was transferred into a dried flask. 2 g of anhydrous sodium sulfate was added, shaken and allowed to stand for 30 min; the extract was then filtered. The filtrate was weighed and taken for assay. Using a blank and a standard linalool (Sigma, Aldrich), the percent content of linalool of the sample (Figure 7) was done.

Biostatistical analysis

In every assay, student's t-test was performed. P-values were tested in each case and in all experiments, standard deviation (S.D) values were varied according to the value of n (number of times experiments are done).

RESULTS AND DISCUSSION

Total flavonoid by high-performance liquid chromatography (HPLC) method

Spectrophotometric scans at 285 nm of the HPLC eluants reveal a varietal difference in total flavonoid (including flavonoid glycosides) content among the tea clones representing different varieties (Figure 1). The scans, covering a period of 0 to 55 min retention time in all the

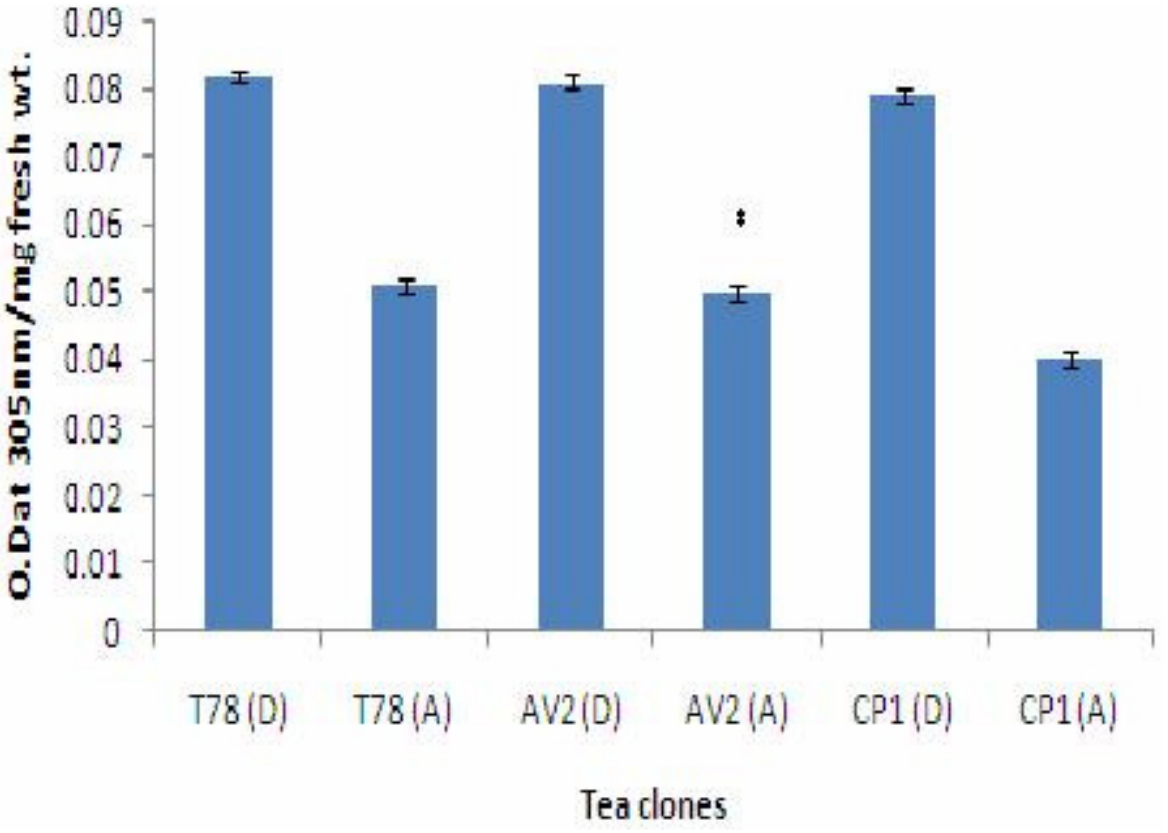


Figure 4. Spectrophotometric assay of total UV absorbing compounds of different tea clones viz. T78, AV2, CP1. D = Darjeeling, A = Assam. ($P < 0.001$, for S. D n = 6).

samples, show a near base-line separation, revealing clear varietal difference in flavonoids between the tea clones grown at each altitude viz. in Darjeeling (~4500 ft msl) and in clones of the same varieties in Assam (~350 ft msl). An enhancing effect of high altitude related high UV (that is the major environmental factor differing in these two cultivation sites) is further evident in the Darjeeling clones. Hofmann et al. (2000) have similarly reported that in *Trifolium repens*, where leaf quercetin glycoside, that is, rutin is a varietal expression, varying in amount in different cultivars, a pronounced UV-B induced increase in the flavonol glycosides is also evident. A quantitative estimate of peak area determined according to Schierle et al. (2004) within 0 to 55 min retention time is given in Table 1a. This data shows higher values in high altitude grown tea clones (A and B in Figure 1) superimposed on the varietal difference observed in each altitude. Studies on nine *T. repens* populations have also revealed UVB related variation in accumulation of flavonol glycoside; under enhanced UV radiation the content of quercetin glycoside (viz. rutin) was found to be about three-times more than in the controls (Hoffman et al., 2000) presumably due to UV light induced up-regulation of flavonoid glycoside synthesis (Logeman et al. 2000). For such synthesis, a UV photoreceptor mediated signal transduction pathway has also been implicated (Pratt and Butler, 1970). HPLC scan of the standard rutin (Santa Cruz Biotechnology) sample in 25 min eluant (0.01 mg/ml, peak area 2227.5 mV-min at 25 min retention time), equivalent with concentration 0.01 mg/ml is given in Figure 1(marked as C). Comparison of these scans with that obtained for the authentic flavonoid glucoside viz. rutin sample shows clearly that amongst other types of flavonoids and flavonoid glycosides shown in the scans, rutin is a major component in the Darjeeling grown tea clones (Figure 1, marked A and B); in the Assam grown clones presence of rutin appears comparatively insignificant (Figure 1, marked A' and B').

Flavonoid glycosides visualized by TLC

For a specific study of flavonoid glycoside, a TLC method in methanolic leaf extracts with ferric salts for colour development is presented in Figure 2. Although qualitative, a clear difference could be observed with respect to flavonoid glycosides between the low altitude (Assam) and high altitude (Darjeeling) growing clones. Whereas the plants growing at high altitude showed about 5 to 6 spots, the same clones grown in low altitude showed only 1 spot. A comparison of the data on flavonoid glycosides obtained by different techniques viz.

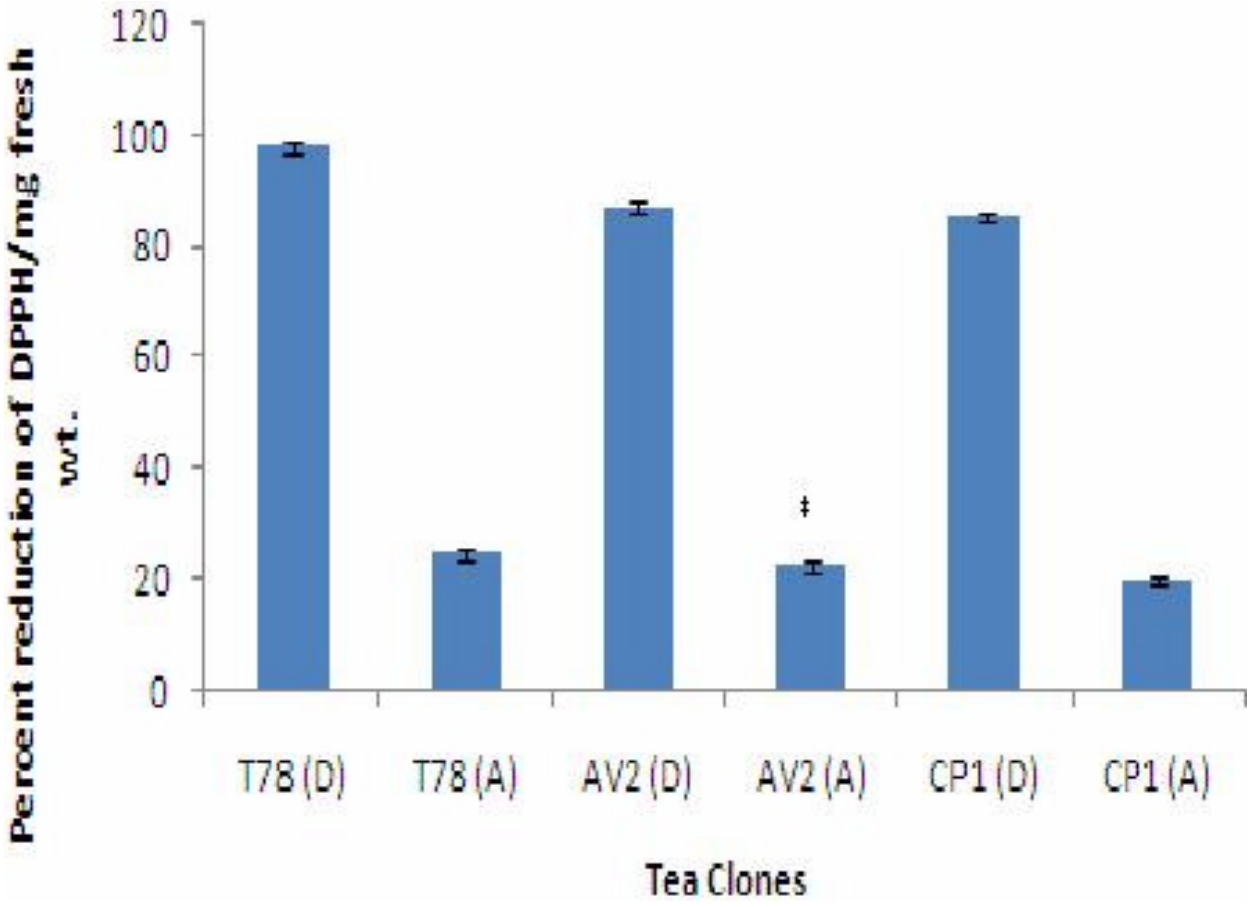


Figure 5. Spectrophotomatric assay of antioxidant potential in tea clones viz. T78, AV2, CP1 (D = Darjeeling, A = Assam) by percent reduction of DPPH recorded at 517 nm. ($P < 0.001$, for S. D, n = 6).

HPLC (Table 1a) and TLC (Figure 2) reveals that Darjeeling grown tea clones have greater accumulation of flavonoids and flavonoid glycosides that are known to serve as antioxidants and also as aroma precursors. It is pertinent to mention here that the market value of Darjeeling tea is internationally known to possess high antioxidant and aroma values (Lachman et al., 2003).

Spectrophtometric values (at 360 nm) for total rutin - a flavonoid glycoside, functioning as antioxidants show varietal difference in the clones growing at each altitude. For all the varieties, a superimposed enhancement due to UV over the genetic attribute in each variety was evident between clones at high and low altitude (Table 1b). The proportionately high value in clones of each variety at high altitude is presumably due to high altitude related high UV fluence. Studies on maize (Stapleton and Walbot, 1994) and on parsley (Logemann et al., 2000) have also shown higher flavonoid glycoside accumulation in plants growing under high (artificial) UV radiation than in the untreated control plants. Carrao-Panizzi and Bordingnon (2000) have also reported varietal difference in (iso) flavone glycosides is further altered by environment (in soybean cultivars).

Phenylalanine ammonia lyase activity (PAL) (EC number 4.3.1.24) activity

Phenylalanine ammonia lyase (PAL) activity exhibited a varietal difference in values at both altitudes - the values being higher (with respect to the value for the different varieties at low altitude) in the high altitude grown tea clones (Figure 3). The data suggest that phenylalanine ammonia lyase activity (PAL) (Figure 3), a key enzyme in the biosynthetic pathway for flavonoid production, although basically under genetic control, is further enhanced/up regulated at high altitude locations presumably due to altitude related high UV radiation. From studies on pea, Pluskota et al. (2005) have demonstrated UV stress induced enhancement in flavonoid biosynthesis through signal-transduced transcription (up-regulation) of genes for phenylalanine ammonia lyase, in the phenylpropanoid pathway/anthocyanin pathway. It may be noted this pattern of difference is also reflected in the value of accumulation of the flavonoids and its derivatives viz. flavonoid glycosides (such as, products of the anthocyanin pathway preceded by the phenylpropanoid

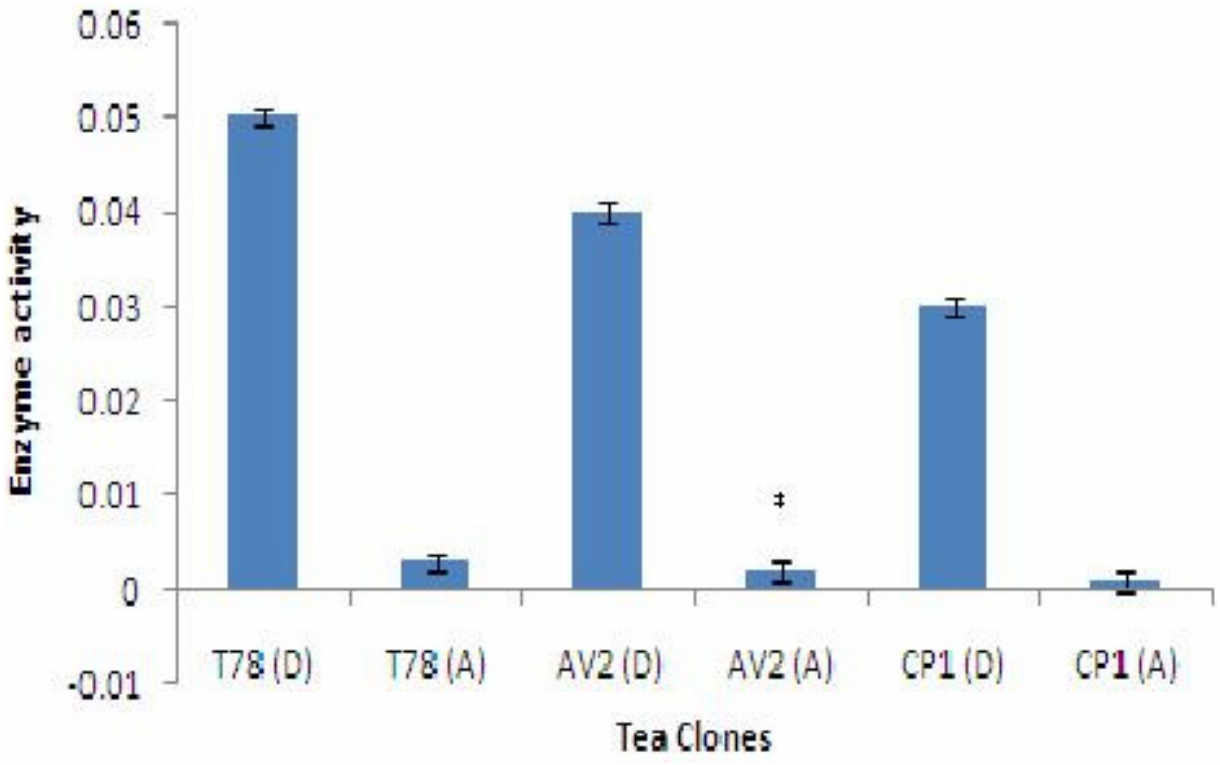


Figure 6. Spectrophotometric assay of β -D Glucosidase enzyme activity of tea clones viz. T78, AV2, CP1. D = Darjeeling, A = Assam. ($P < 0.001$, for S. D n = 6).

pathways involving PAL) as is recorded in Figures 1, 2 and 3 and Table 1.

Assay of UV absorbing compounds

Spectrophotometric assay (Mazza et al., 2000) at 305 nm of UV absorbing compounds (Figure 4) that represent an important role of the flavonoid group of compounds shows higher values in tea leaf of plants at high altitude (D) compared to plants of the same genotypes growing in low altitude (A) - a varietal difference between the clones still remain clearly evident at each altitude, appearing enhanced at high altitude. The higher values of UV absorbing was found to correlate with the higher content of flavonoids (Figure 1 and Table 1) that are known to function as UV absorbing compounds. Variation between genotypes with respect to UV absorbing compounds has also been reported in other field grown plants (Quaite et al., 1994). Caldwell (1968) has reviewed the possible roles of flavonoids in absorbing solar UV radiation, especially at high elevation.

Assay of total antioxidant potential

A varietal (genetic) difference in antioxidant potential between the varieties was evident at both the altitudes - the values for each variety was found to be further enhanced at high altitude, showing higher values in the Darjeeling grown clones compared to that of the low altitude (Assam) grown clones of the same varieties (Figure 5). Antioxidant potential data (Figure 5) shows clearly that in addition to a varietal difference, the altitudinal difference apparently (due to UV fluence) plays a major role in determining antioxidant potential in tea leaf. This explains why Darjeeling tea exhibits higher (than Assam clones) antioxidant potential as is conventionally known (Lachman et al., 2003). This data may be compared with Figure 1 that shows high value for flavonoids (that are polyphenolic compounds) that, in addition to serving as UV screens, may also serve as antioxidants. It is pertinent to mention here that flavonoids, flavonoid glycosides and several intermediates in the phenylpropanoid pathway that serve as antioxidants are reported to exhibit anticancer/ antiapoptitic functions; such functions have been reported to be higher in Darjeeling (high altitude at ~27ºN latitude) grown tea plants.

Assay of aroma releasing enzyme β-D glucosidase (EC number 3.2.1.21) activity

Spectrophotometric assay of β-D-glucosidase shows (Figure 6) higher specific activity (U = activity/µg protein)

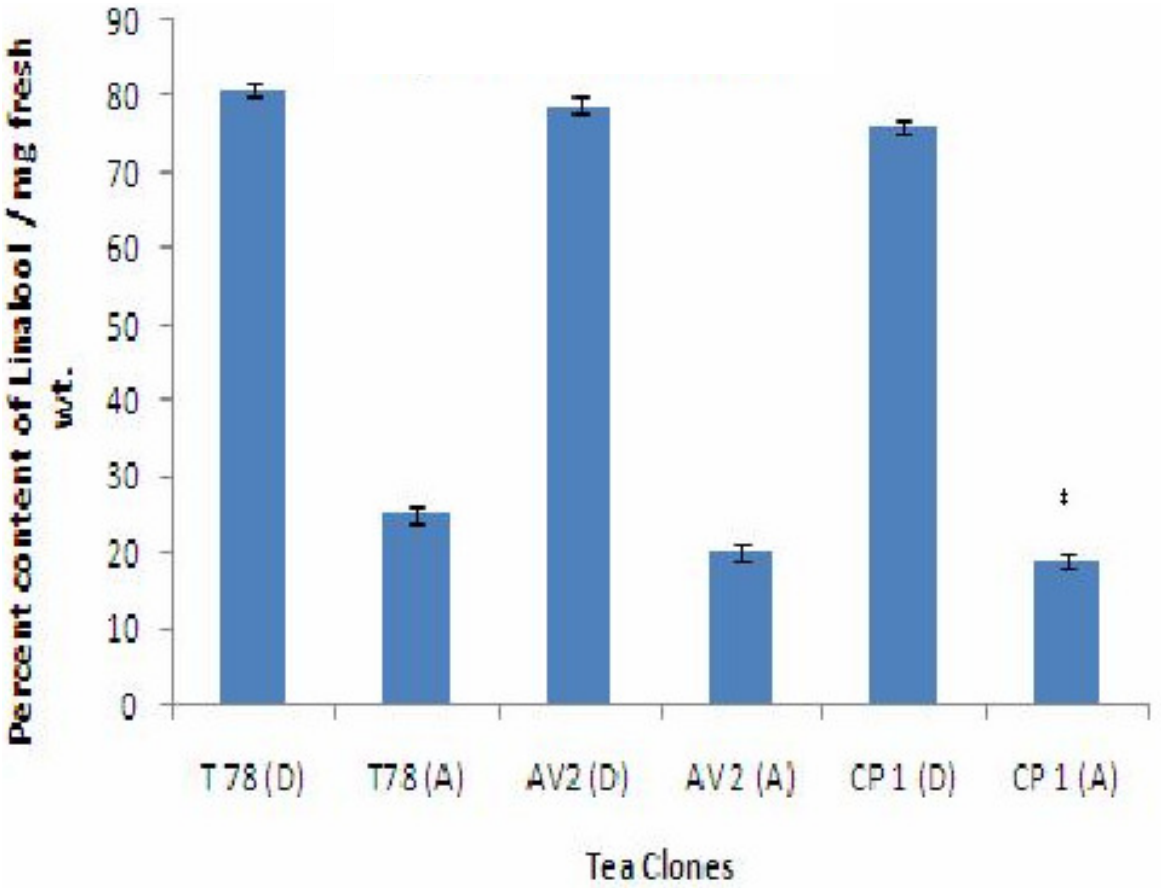


Figure 7. Percent content of linalool in different tea clones viz. T78, AV2, CP1. D = Darjeeling, A = Assam. ($P < 0.001$, for S. D n = 6).

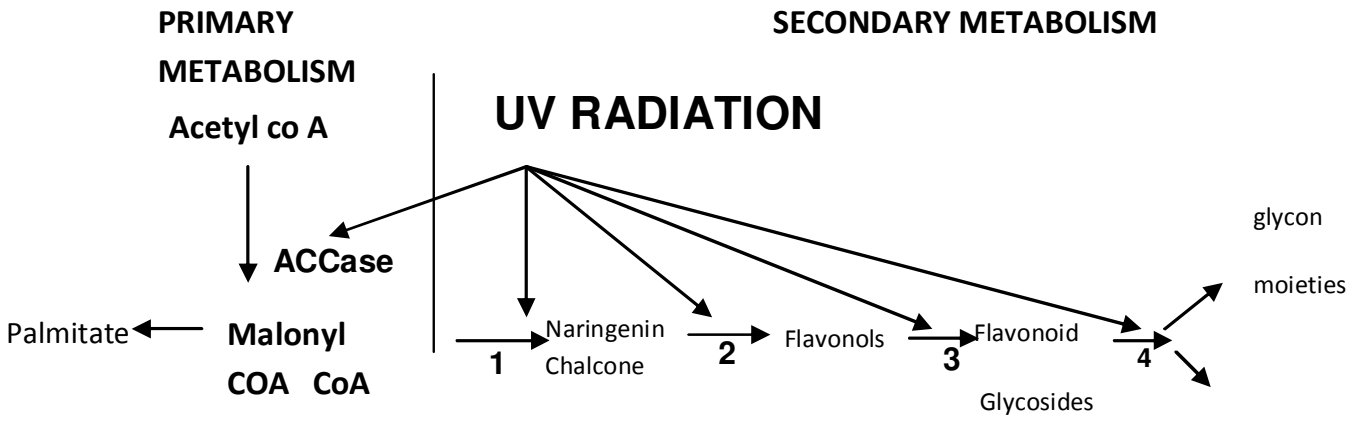
in high altitude (Darjeeling) grown clones than in the same clones grown in low altitude (Assam) - the values appearing as superimposed enhancement over varietal attribute in each variety. The data reveals varietal difference at each altitude with T78 showing higher activity than CP 1 and AV2 at both the altitudes. The enhancement in values of the varieties at high altitude is presumably due to altitude related UV enhancement.

Assay of total linalool content

Spectrophotometric assay of aroma causing aglycon moiety (assayed in comparison to a standard linalool--Sigma Aldrich) known to be produced by activity of β-D glucosidase on a substrate linked β-D glucoside (Moon et al., 1996) provide the data in Figure 7. It is clear from the data that high accumulation of linalool in tea leaf is a function of high altitude environment over and above the varietal difference in values at each altitude. In comparison with data in Figure 6, it is evident that high altitude related UV fluence caused enhancement in activity of the enzyme β-D glucosidase, results in higher production of this aglycon moiety viz. linalool. Linalool has been reported to be high in black tea, oolong tea and green tea (Ogawa et al., 1995). It appears that the high aroma known for Darjeeling orthodox tea (Lachman et al., 2003) is due to the high level of aglycon moiety, viz. linalool, occurring in fresh leaf of Darjeeling grown tea clones.

Conclusion

High accumulation of flavonoids (Figure 1) and flavonoid glycosides (Figure 1, Table1a,Table 1b, Figure 2), and higher activity of PAL *(EC 4.3.1.24)* (Figure 3) for production of flavonoids that function as UV absorbing compounds (Figure 4) and antioxidant (Figure 5) as well as higher activity of β-D glucosidase *(EC 3.2.1.21)* (Figure 6) that splits flavonoid glycosides to cause the production of aroma causing compounds viz. linalool (Figure 7), was found to be higher in high altitudes grown tea plants; the values are found to be comparable to a basic value that reflects a varietal difference, such observation was true for both altitudes. The data presented relate to tea plants growing at high altitudes (Darjeeling) and low altitude (Assam) - both places falling



Scheme 1. Schematic presentation of UV involvement in secondary metabolism. 1: chalcone synthase; 2: 3´–O- Methyl transferees; 3: UDP-glucose flavone : flavonol 7- 0- glucosyltransferase; 4. β-D-glucosidase.

within the subtropical region of the Northern hemisphere viz. on ~27ºN. It is pertinent to mention here that experimental tea leaf were collected during April to June for several consecutive years and hence, the data presented here relate to tea leaf produced during the 1st flush of tea leaf in Darjeeling and Assam. This suggests that high aroma and high antioxidant potential known for Darjeeling tea is a location specific attribute presumably caused by UV mediated epigenetic effect viz. up-regulation of relevant genes under high altitude related high UV fluence.

This study establishes that although UV radiation is generally known to cause cell destruction, (representing a negative effect of UV on plant growth and development), UV stress may in turn up-regulate the production of cell protective and aroma causing compounds that may also serve as photoreceptors for signaling further production of the protective and quality compounds (as represented in Scheme 1). This report presents our comparative study on the flavonoid group of compounds and associated enzymes with respect to antioxidant and aroma (value addition) in leaf of tea plants growing in conventional tea cultivation sites at high altitude (Darjeeling) and low altitude (Assam), between which UV fluence presents the single major variation in environmental factors. The experimental data presented here establishes the biochemical status of Darjeeling tea leaf with reference to the “Darjeeling Tea” of repute. Such a study should also be useful in planning strategies for tea cultivation in non-conventional sites particularly in the face of a global climate change.

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