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Tarun Kumar Misra
Department of Botany,
University of North Bengal,
Raja Rammohunpur, Siliguri,
Darjeeling, W.B., India,
Pin – 734013.

Dr. Ashis Kumar Nanda
Department of Chemistry,
University of North Bengal,
Raja Rammohunpur, Siliguri,
Darjeeling, W.B., India,
Pin – 734013

Dr. Palash Mandal
Department of Botany,
University of North Bengal,
Raja Rammohunpur, Siliguri,
Darjeeling, W.B., India,
Pin – 734013.

Dr. Anirudha Saha
Department of Botany,
University of North Bengal,
Raja Rammohunpur, Siliguri,
Darjeeling, W.B., India,
Pin – 734013.

Correspondence
Dr. Anirudha Saha
Department of Botany,
University of North Bengal,
Raja Rammohunpur, Siliguri,
Darjeeling, W.B., India,
Pin – 734013.

Climatic variation of polyphenols, flavour index, PPO activities and antioxidative properties in hand made orthodox tea (*Camellia sinensis* (L.) Kuntze) obtained from the different variety of tea cultivars grown in North Bengal

Tarun Kumar Misra, Dr. Ashis Kumar Nanda,Dr. Palash Mandal, Dr. Anirudha Saha

Abstract
Climatic variation of Phenolics (mainly EGCG and ECG) Flavouring index (TF: TR and HPS, TC), PPO activities and anti-oxidative properties in fresh tea shoots to handmade orthodox tea, consisting of one apical bud and two adjoining leaves sampled from TV-1, TV20, TV26, TV29, TV30 (Tocklai Vegetative) clone and Tingamara, Dangri Manipuri and Sundaram B/5/63(Seed jat), Tinali 17, Takda7,8 grown in Terai, Hill and Dooarsregion in North Bengal, were investigated during three harvest season (March, June and December) in 2007, 2008,2009 and 2010. The EGCG, ECG of all clones was lower in cool months of December in four years (average 4.90 - 2.15, 2.93-0.63). Thereafter, the levels of total Phenolics in terms of EGCG, ECG increased throughout the warmer months from March to September. Antioxidant activity showed similar trends which increased from 1st harvest (March) to 3rd harvest (December). All clones showed nearly 100% antioxidant activity at 2nd and 3rd harvest season which higher than standard synthetic antioxidant BHA (Butylated hydroxyl anisole). However, seasonal diurnal variation of phenolics showed different results according to clones used. TF: TR (0.138 to 0.036%), HPS (22.235-10.14%) and TLC (5.73-3.49) also seasonally vary. PPO activity plays a crucial role and depends on the clone. These results seem to suggest that the harvest time is crucial to determining the antioxidant potential of fresh tea shoots and end product.

Keywords: *Camellia sinensis,* climatic variation, PPO activity, antioxidants, Flavour index.

Introduction

Traditional Chinese medicine has recommended green tea for headaches; body aches and pains, digestion, enhancement of immune system, detoxification, as an energizer and to prolong life (Misra *et al*. 2016[11]). Many Researchers revealed that there has been increasing interest in finding plants with high antioxidant capacities since they can progression of many chronic diseases (Mandal *et al*., 2010[9]).Young tea shoots contain more than 35% of their dry weight in polyphenols (Serafini *et al*., 1996[14]). It has been reported that Phenolic compounds that are present in young tea shoots (also referred to as fresh green leaves, fresh tea shoots, or flushes) are known to be one of the main factors in determining the quality of the resulting tea drink (Misra *et al*., 2008[10]). There were studies related to chemical composition of tea shoots and its constituent catechins are best known for their antioxidant properties, which has led to their evaluation in a number of diseases associated with reactive oxygen species (ROS), such as cancer, cardiovascular and neurodegenerative diseases. The natural polyphenols in orthodox tea include (-)-epigallocatechin-3 gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3- gallate (ECG), and epicatechin (EC). Other minor catechins, (+)-gallocatechin (GC), (-)-gallocatechin gallate (GCG), (-)-catechin gallate (CG) and (+)-catechin (C) are also present in tea. The highest concentration is of EGCG followed by, ECG, EGC and EC in decreasing order (Nakabayashi *et al*. 1991[12]). General structure of a catechin(A),Structures of the 12 tea catechins(B) and Retro Diels-Alder fragmentation of a catechin (Daniel *et al*. 2000[3]) given below:

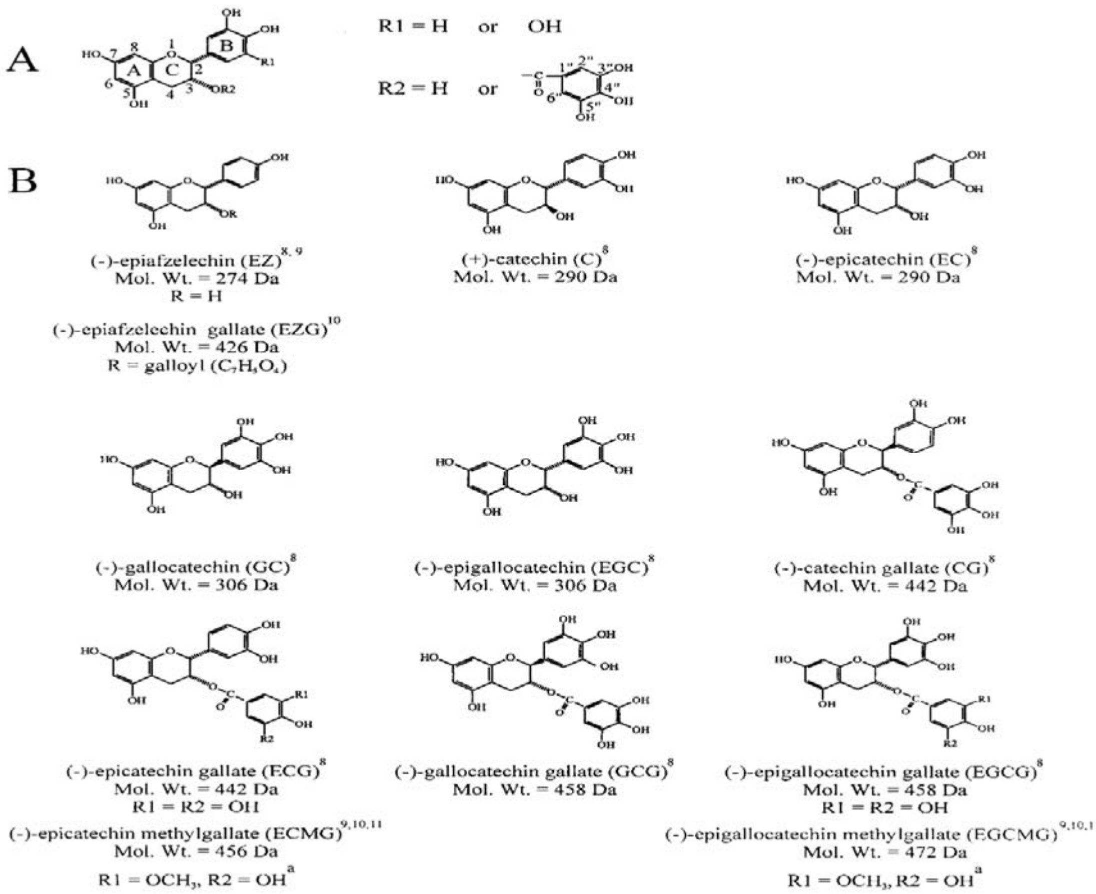

Fig 1: Catechin structures. (A) General structure of a Catechin. (B) Structures of the 12 Tea catechins.

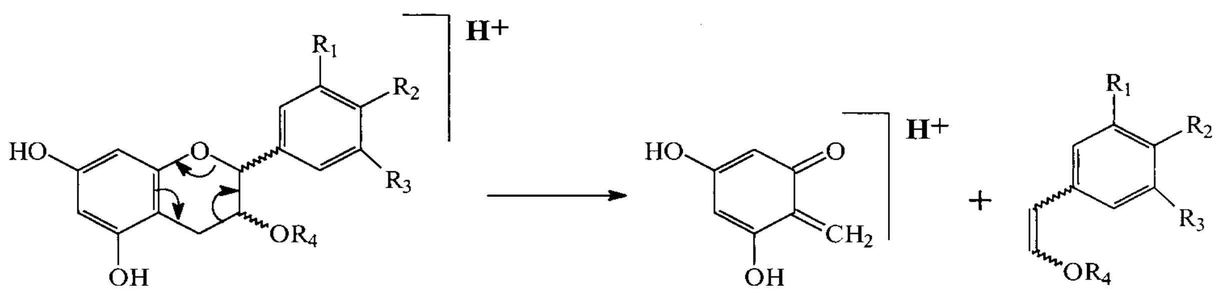

Fig 2: Retro Diels-Alder fragmentation of a Catechin

However the variation of chemical content present in tea shoots in different harvest time and change during processing in particular in North Bengal has not been studied and also challenges before tea industries to get a clear comprehension of biochemical reactions which take place during cultivation to processing which are necessary for safe keeping the constituents in end product. The primary polyphenols are oxidized during the fermentation process and are transformed to compounds with tanning properties. The oxidized polyphenols in tea are responsible for briskness, strength, colour, taste and pungency of the tea infusion. These polyphenols remain intact in tea as dried of leaves following plucking and finally drying in orthodox tea manufacture inactivates the enzyme polyphenol oxidase. Therefore, suitable season of manufacturing can be devised to upgrade the end product. Another major area, seems to be nearly absent in all Industries i.e. controlling reactions in processing. The requirements of tea processing have undergone a major shift over the years. Then the question is what is responsible for this shift? For finding the solution this studies has been under taken for studying different chemical composition in leaf and end product season wise manufacturing process in young tea shoots of eight tea clones in North Bengal i.e. Terai, Dooars and Hill region.

Materials and Methods

Collection and preparation of tea samples

Fresh tea shoots (*C. sinensis*), comprises of one apical bud and two adjoining leaves, around 250 g, were hand plucked from commercial clones, TV-1, TV-20, TV26, TV-29, TV-30, Tingamara, Dangarimonipuri and Sundaram(B/5/63), Tinali17, Takda7,8. The clones are initially identified by Bagan babu(Head of the workers) and Assistant Manager of the TE and finally by the Tea experts. The clones are found together in the same plantation in Paharghumia TE located at Naxalbari area in Terai, Bagrakote Tea garden in Dooars and Phuguri in Darjeeling Hill of North Bengal. The clones were or less same age. The samples were collected from these commercially produced clones at three harvest season (30th March, 30th June and 5th December) in 2007, 2008, 2009 and 2010 respectively. There were no statistically differences between years in terms of chemical profiles among shoots and handmade orthodox tea therefore the results of four years were pooled. Thehandpick freshly collected tea shoots of 16 plants per clone/jat were processed for sample preparation following the usual manufacturing procedure of orthodox tea. For this leave were withered for 10-14 hrs depending on the moisture contents in the leave, fermented in regulated 30^{o}C temperature and moisture for 40 to90 minutes then oven dried in an oven not exceeding the temperature above 65^{o}C. Immediately after plucking samples were oven dried in the in the same temperature for comparison. For enzyme extraction leaf and intermediate product were taken followed by standard procedure.

Estimation of polyphenols
The samples for analysis were prepared following the conditions developed for optimized extraction of tea polyphenols with slight modification (Vasisht, *et al*. 2003[16]). Accurately weighed, about 2 g of moderately fine powder of tea sample was taken in a vacuum flask and 100 mL of boiling water was added to it. The flask was stopper and kept on a rotary shaker for 5 min. The contents were filtered quickly while still hot using vacuum and the marc was washed with 10 mL of boiling water. The volume of the extract was adjusted to 100 mL with cold water and 1mL of this extract was diluted to 25 mL of mobile phase (water: methanol: acetic acid :: 70: 30: 0.5). The diluted extract was filtered through a 0.45 µm membrane filter and a constant volume of 5 µ l was injected for each analysis. The amount of EGCG and ECG in the extract was calculated from the area under the curve corresponding to the respective peaks of two polyphenols and their standard plots.

High performance liquid chromatography
The analytical determinations of EGCG and ECG were carried out using reverse phase- high performance liquid chromatography in isocratic mode. The Waters HPLC system equipped with automated gradient controller, 510 pumps, U6K injector, 481 detector, 746 data module and Waters µ-bondapak C18 column (3.9 x 300 mm), was used for the analysis. Elution was carried out at ambient temperature between 24 to 28°C using water: methanol: acetic acid (70: 30: 0.5) as a mobile phase at a flow rate 1.0 mL/min All extracts were prepared in triplicate and each extract was analyzed in triplicate. The UV detection was carried out at 280 nm.

Standard solution
A standard solution of EGCG was prepared by dissolving 4.72 mg of EGCG in 50 ml methanol. Standard plot for HPLC analysis was prepared by injecting in triplicate a constant volume of 5 µl of serially diluted concentrations containing 9.85, 19.70, 39.40, 78.80, 157.60 and 315.20 ng/5 µ l of EGCG and noting AUC corresponding to each concentration. The standard solution of ECG was prepared by dissolving 3.02 mg of ECG in 10 mL methanol. The stock solution was diluted to make 9.40, 18.80, 37.60, 75.20, 150.40 and 300.8 ng/5 µl dilutions of ECG. A constant volume of 5 µl of each concentration was injected in triplicate. The standard plot was prepared as described for EGCG and same conditions of analysis were used for the two catechins.

Linearity of HPLC systems and its sensitivity
Linear regression was obtained by plotting the peak area versus concentration of a series of dilutions for each phenolic compound. The regression lines, expressed as correlation coefficients, were linear (r^2 =1 and 0.9998 for EGCG and ECG respectively) in the experimental range. Sensitivity (defined as the lowest measurable concentration of a compound in the sample) was determined as that concentration which generated a peak at least three times higher than the baseline noise range depicted below.

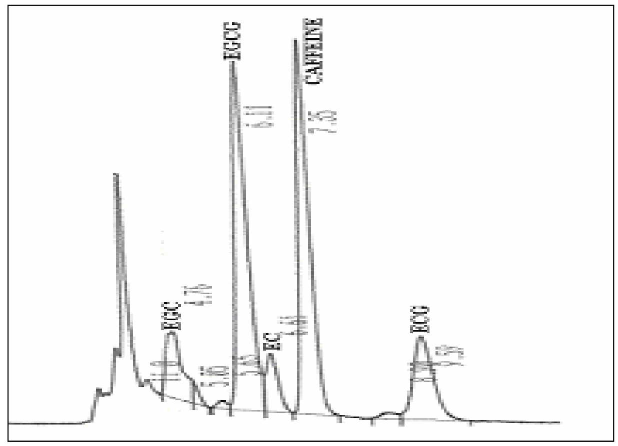

Fig 3: A typical HPLC chromatogram of tea extract

Linearity and sensitivity of the detection method for EGCG and ECG

Compound range studied Correlation coefficient Sensitivity			
	(ng/5µL)	(r^2)	(ng/5µL)
EGCG	9.85-315.2	1	9.85
ECG	9.40-300.8	0.9998	9.40

Recovery and Reproducibility
The percent recovery of analytic in the method was established by spike the sample 100 % level. The per cent recovery of EGCG from triplicate set of analysis was 95.98 ± 0.49. Reproducibility was found to be within limits by repeating the analysis at different times.

Extraction and estimation of enzyme activities
Leaf tissues/intermediate product was homogenized with potassium phosphate buffer pH 6.8 (0.1 M)containing 0.1 mM EDTA, 1% PVP and 0.1 mM PMSF in pre-chilled mortar pestle. The extract was centrifuged at 4^{o}C for 15 min at 17,000g in a refrigerated cooling centrifuge. The supernatant was used for the assay of the following: polyphenol oxidase (PPO) as per standard method of Upadhyaya *et al*. 2008[15].

Polynophenol oxidase activities
PPO was assayed using pyro-gallol as substrate with minor modifications, 5.0 ml of assay mixture and 1.0 ml of enzyme extract. After incubations at 25^{o}C for 5 min, the reaction was stopped with additions of 1.0 ml of 10% H_2SO_4.The purpurogallin formed was read at 430 nm. One unit of enzyme activity is defined as that amount of enzyme, which forms 1 lmol of purpurogallin formed per minute under the assay conditions.

Extraction and estimation of TF, TR, TC and HPS

The theaflavin (TF), thearubigin (TR), high polymerized substances (HPS) and total liquor color (TLC) of the tea samples were determined by the process shown in Figure 4. The tea samples obtained from different clone/jat were analyzed for various chemical constituents by standard procedure Masoud*et al*., 2006[9].

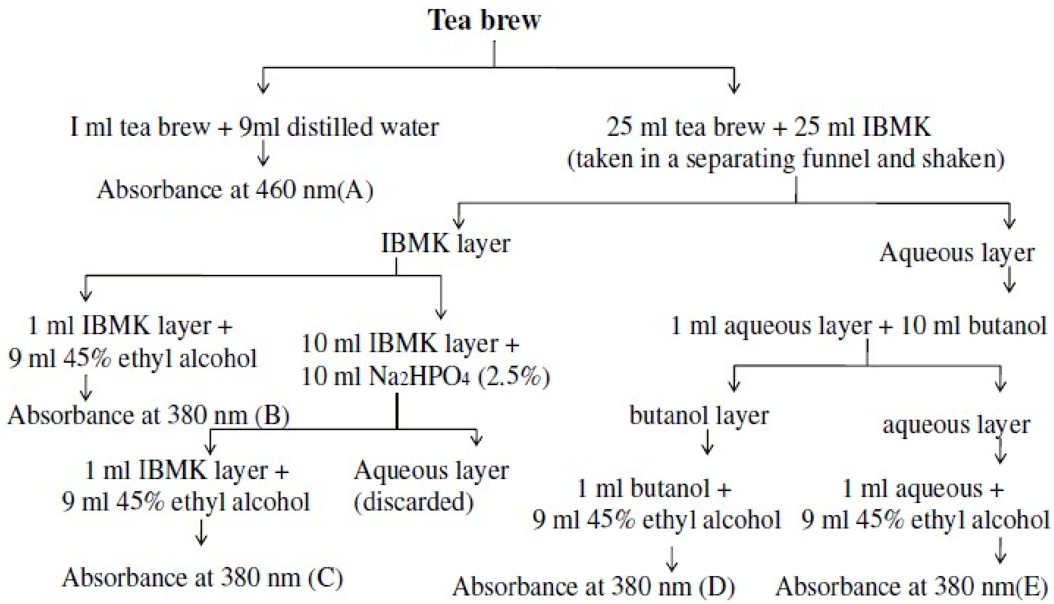

Fig4: Method for determination of TF, TR, HPS and TLC.

TF (%)	=4.313 x C
TR (%)	=13.643 x (B+D-C)
HPS (%)	=13.643 x E
TLC	=10 x A
TF	=theaflavin
TR	=thearubigin
HPS	=high polymerized substances
TLC	=total liquor color
IBMK	= iso-butyl methyl ketone
Na2HPO4	= disodium hydrogen phosphate

DPPH based free radical scavenging activity

The free radical scavenging activities of each fraction were assayed using a stable DPPH, following standard method of Blois MS. 1958[2]. The reaction mixture contained 2 ml of 0.1mM DPPH and 0.1 ml of each methanol fraction. Simultaneously, a control was prepared without sample fraction. The reaction mixture was allowed to incubate for 5 min at room temperature in the dark and scavenging activity of each fraction were quantified by decolourization at 515 nm. Percentage of free radical scavenging activity was expressed as % inhibition from the given formula:

$$\text{Percent inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

Antioxidant capacities of the samples were compared with those of BHA and the blank.

Chemicals

All chemicals and reagents were of AR or HPLC grade (E. Merck). (-) EGCG and (-) ECG were obtained from Sigma-Aldrich Chemicals Ltd. Distilled water was used wherever water is mentioned.

Statistical Analysis

The experiment was a completely randomized design with three replications. Data were subjected to analysis (version 11.0 SPSS. Inc.) of variance (ANOVA) and P< 0.01 was regarded as significant.

Results and Discussion

The EGCG, ECG content of young fresh tea shoots belongs to clones is given in table 1. The statistically important differences on phenolics in terms of EGCG &ECG were obtained in different harvest times in tea clones (p<0.01). Total phenolic content were continuously increased from 1st harvest to 3rd harvest in all clones, which found to be highest 4.9, 2.93 & lowest 2.15, 0.63% in TV26, TV20 respectively (table 1). Misra *et al*. 2016[11] previously reported that fresh tea shoots are extremely rich in total phenolic compounds which can constitute up to 300 mg/g of dry material. It is also reported that green tea shoots is very rich for total phenolics and total phenolic content of green tea shoots 4-5 times higher than phenolic rich Ashwagandha (*Withania somnifera*) plant Misra *et al*., 2008[10].

The great difference of tea shoots in terms of total phenolics at different harvest time is supposed to the effect of change of ecological parameters. North Bengal takes less sunlight in December month (3rd harvest) than the other harvest times. This may affect the biosynthesis of total phenolics. On the other hand, in this month in North Bengal region, the differences between day and night temperatures are also higher and the rainfall was irregular. In this stress conditions, tea clones produce more phenolics. It has been shown (figure 5) that the biosynthesis of Phenolic compounds in tea shoots can be effectively induced by stronger sunlight and length of daytime i.e. Seasonal or diurnal variation. That is why in shaded tea flushes the concentrations of polyphenols are much lower.

Fig 5: Seasonal variation of EGCG (R=0.998, $p<0.05$).

Same findings also observed by Mahanta *et al*. 1992[7]. On the basis of this information, the differences in total phenolic levels between fresh tea shoots harvested in different months in North Bengal may not be just a temperature effect but also a day length and sunlight effect. However, further studies are required to elucidate the induction of the biosynthesis of total phenolics by day length and sunlight exposure correlating to the UV index. Previously, under field conditions, the phenolic composition of tea shoots varies considerably with seasonal, genetic, and agronomic factors and mechanisms that induce seasonal variations on total phenolic content in tea shoots may include one or all three of the following environmental conditions i.e day length, sunlight, and/or temperature, which vary markedly across seasons. Achuthan *et al*. 2003[1] said that the highest total phenolic levels in tea shoots are important for public by reducing the risk of atherosclerosis and coronary heart disease, which can be caused by oxidation of low-density lipo proteins. The present total phenolic content results would indicate that there is potential to produce higher quality black tea during the December months in North Begal. These results are in agreement with the findings of Yao *et al*. 2005[17] which found more phenolics occurred in relatively warmer months in the shoots. On the other hand, the differences were observed on total phenolic content in shoots among tea clones (table 1). Obanda *et al*. 1997[13] showed the level of phenolics in green tea shoots varied among clones. Antioxidant activity of fresh tea shoots in ten clones is given in Table 1. There were statistically differences among harvest times in all tea clones except TV1 ($p<0.01$). Antioxidant activity was increased from 1st harvest to 3rd harvest times in all tea clones (table 1). Free radical scavenging activity was found to be between 99.97-74.10% Antioxidant activity (% Free radical scavenging) of BHA (200 mg/l) was 91.18%. All extracts sampled from 3rd harvest in all clones had higher antioxidant activity than BHA (Table 1). Similar to 3rd harvest, all clones also showed higher antioxidant activity at 2nd harvest time than BHA, except TV30. However, in 1st harvest time, only Dangri Manipuri clone had higher antioxidant activity than BHA (table 1). The other clones had lower antioxidant activity than BHA at 1st harvest time. In previous studies conducted on tea by Karori *et al*. 2007[6], the antioxidant activity of different tea products in different solvent was found between 56-83%. Halliwell *et al*. 1999[4] revealed that commonly consumed products such as tea, coffee and cocoa have possessing significant amount antioxidant activity. The results for antioxidant activity clearly outline that tea shoots could be one of the richest sources among plants in terms of antioxidant activity. The great difference of tea shoots for antioxidant activity at different harvest time is supposed to the effect of change of ecological parameters. The composition of tea shoots varies with climate, season, variety, and age of the shoot. There were strong relationships between free radical scavenging activity and total phenolics in all harvest dates in all tea clones. Julianiet *al*. 2002[5] are also reported strong relationships between antioxidant activity and phenolics in tea shoots which support our findings.

The cause of this shift is due to the overall change in planting materials from jats to clones, important aspect is the changes in chemical profile of tea leaf over the years due to: closer plantation, Cultural practices eg. Prune/unprune, high fertiliser and other inputs (foliar application), Shade. Mineral Nutrition are very important in relation to Quality, it seems from this research is phosphorus increases the level of quality, Zn &Mn attributes namely EGCG, ECG, EGC, Caffeine, TF & TR, Nitrogenreduces the quality at high dose by increasing chlorophyll and undesirable amino acids increases VFC content but reduces flavour index, potash reduces catechin content but increases fibre content, light intensity directly regulate the catechins biosynthesis, it is found that optimum shade and diurnal variations the activity of PAL, key enzyme for catechins biosynthesis resulting higher content of catechins. Tea Leaf Polyphenol Oxidase (PPO) which is responsible for Oxidizes dihydroxy polyphenol, varied clone to clone(figure 6),increases by abiotic stress; influence on pH, moisture contents of soil, P deficiency decreases PPO activity. The amount of H_2O_2 produced is greater at pH 5.5, the optimum pH for PPO activity, than at pH 4.5. Hence, an observed increase of theaflavins in black teas fermented at pH 4.5 appears to be due to lower turnover of formed theaflavins into thearubigins.

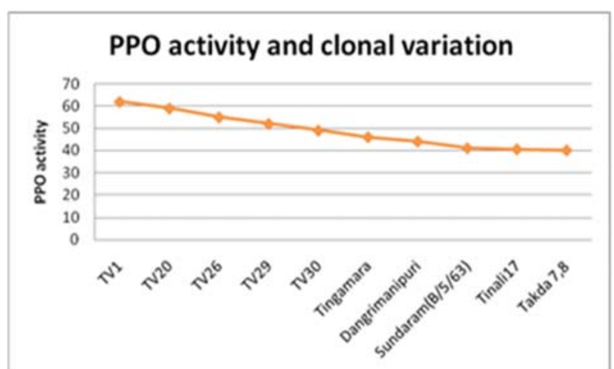

Fig 6: shows the polyphenol oxidase activity and Seasonal variations

It is also found that less moisture content in the soilincreases the catechin content in tea. During processing TF is reduced by rise of temperature which is important for brightness and briskness but effect on strength due to TR is not that significant. It will possible if rise in temperature be regulated due to chemical reactions during fermentation.

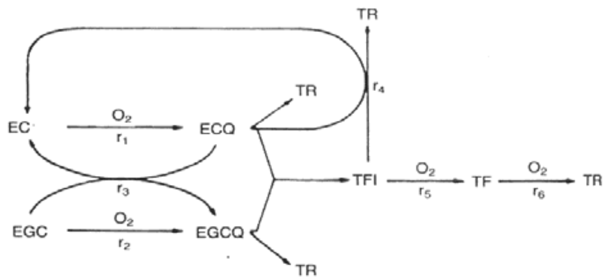

Fig7: Equation shows the formation of TF & TR from EC.

This equation in figure 7, clearly shows that which are the factors affecting the formation and degradation of TF & TR are

- Coupled Oxidation
- Oxygen
- pH
- Catechin composition of green leaf
- Temperature

Air plays important role during withering in accumulation of desirable Amino acids and is independent of the loss of moisture. High temperature of withering results in plain and soft liquor. Depending upon the environment optimum withering is achieved within 12 to 14 hrs increasing the overall tea quality. Humidified hot air can reduce the fermentation time with no adverse effect on quality in cooler ambient temperature. Hot air without humidification is detrimental. Also seems that the quality of cultivars varies with flushes. Earlier plants were grown under low fertiliser regime. Later certain sections of tea industry have gone for higher input with resultant impact on chemical constituents of tea shoots responsible for quality. All these factors influence the level of chemical constituents significantly. Hence many of the basic findings in the past may have to be revised. Despite excellent efforts, changes in different micro environments were not possible earlier due to lack of precise control of physical parameters. Different Factors also affecting fermentation time like Fineness of plucking, Nature of cultivars, Degree of wither or percentage of moisture in withered leaf, Temperature, aeration, thickness of bed etc and Catechin composition of the green leaf.

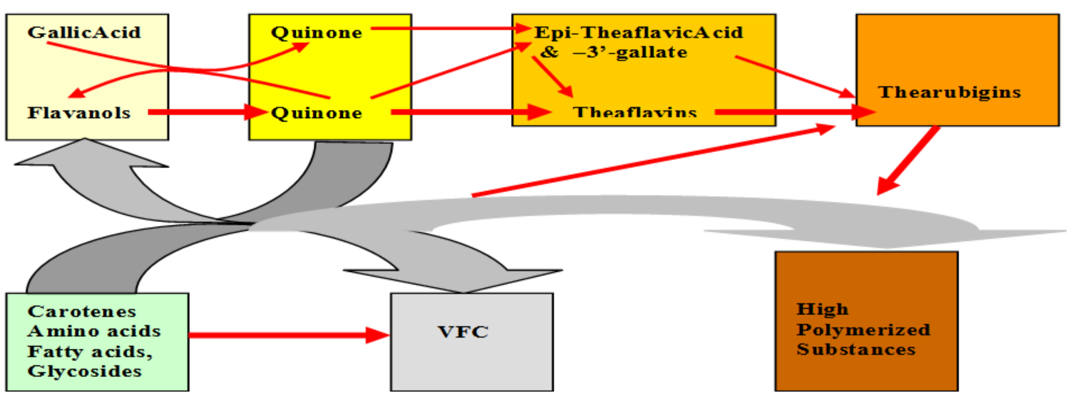

Fig8: Equation of formation of volatile flavoring compounds

Table 2: TF, TR, HPS and TC of handmade Orthodox Tea

Tea Sample	TF%	TR%	HPS	TLC	FI::TF:TR
TV1	0.337±0.01	9.175±0.01	14.562±0.01	4.77±0.01	0.037
TV20	0.268±0.03	7.352±0.02	11.25±0.2	3.89±0.02	0.036
TV26	0.395±0.02	2.855±0.01	10.14±0.32	4.65±0.03	0.138
TV29	0.285±0.01	6.520±0.01	22.235±0.41	5.73±0.01	0.044
TV30	0.358±0.02	7.321±0.02	12.156±0.23	3.49±0.01	0.049
Tingamara	0.289±0.03	7.754±0.05	13.895±0.15	4.21±0.02	0.037
Dangrimanipuri	0.356±0.04	9.654±0.04	19.658±0.24	5.01±0.01	0.037
Sundaram(B/5/63)	0.263±0.01	6.589±0.01	11.965±0.28	3.75±0.02	0.040
Tinali1,7	0.402±0.02	9.602±0.2	14.456±0.39	4.62±0.01	0.042
Takda 7,8	0.451±0.01	9.653±0.2	17.365±0.02	4.38±0.02	0.047

All data are presented as the mean of three experiments; $P < 0.01$ regarded as significant.

The major quality attributes of tea are flavor, aroma, color, and strength. The TF: TR called flavor index should be within 0.08 to 0.1 for a good quality tea. The TF: TR ratio of are shown in table 2. Low TF content in black tea may be due to over fermentation and/or long periods of storage (Yao *et al*. 2006[18]). Similar observations were made by Masoud *et al*. 2006 [9] on different manufactured orthodox and CTC teas. The primary polyphenols are oxidized during the fermentation process and the oxidized polyphenols in black tea are responsible for briskness, strength, colour, taste and pungency of the black tea infusion (figure 8). Among the different teas, obtained from TV26 was found to have a greater amount of VFC followed by TV30, Takda7,8, TV29, Tinali17,Sundaram(B/5/63), TV1, Dangrimanipuri, Tingamara and TV20with 0.138 to 0.036%VFC respectively. The Total Liquor colourvalues of different tea infusions was found to have the highest in TV29value of 5.73 and lowest inTV30 value of3.49 compared to other teas. Highly polymerized substances were found in TV29 and lowest in TV26, value 22.235 and 10.14 respectively. As each tea was processed from different tea cultivars that were grown in various agro-climatic conditions, the difference in polyphenol content of these various teas is reasonable.

Conclusion

It can be concluded that tea shoots are a valuable product, based on its rich and beneficial nutrient composition. It seems that the most important chemical components such as EGCG & ECG were highest at the last harvest time in Terai of North Bengal. However, certain growing conditions and cultural management techniques affecting the chemical components of tea shoots will be the subject of further research projects.

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Table 1: EGCG, ECG and free radical scavenging activity of handmade Orthodox Tea

Cultivars	Season	EGCG(mg/G)			EGCG (%)			ECG(mg/G)			ECG (%)			% of free radical scavenging activity		
		Terai	Dooars	Darjeeling	Terai	Dooars	Darjeeling	Terai	Dooars	Darjeeling	Terai	Dooars	Darjeeling	Terai	Dooars	Darjeeling
TV1*	March	23.12±0.29	17.52±0.22	19.81±0.25	2.31	1.75	1.98	8.75±0.18	8.82±0.15	11.02±0.11	0.87	0.88	1.1	89.32±.32	89.52±.12	86.32±.36
		44.05±0.52	46.20±0.59	46.21±0.62	4.4	4.6	5.2	19.35±0.13	22.15±0.14	23.16±0.19	1.93	2.2	2.3	99.49±.46	99.59±.56	99.89±.56
TV1*	June	5.82±0.28	6.21±0.25	8.92±0.24	0.58	0.62	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.21	0.26	0.29	82.12±.25	82.22±.35	82.12±.25
		25.56±0.36	29.51±0.39	36.56±0.33	2.55	2.95	3.65	9.36±0.19	9.82±0.17	15.23±0.17	0.936	0.98	1.5	92.86±.36	92.96±.46	93.86±.56
TV1*	December	18.02±0.27	17.01±0.21	18.91±0.21	1.8	1.7	1.89	6.24±0.18	6.41±0.14	6.82±0.11	0.62	0.64	0.68	82.86±.26	81.86±.56	82.96±.16
		39.02±0.21	41.25±0.24	49.02±0.29	3.9	4.1	4.9	17.55±0.11	26.01±0.15	27.82±0.15	1.75	2.6	2.78	96.72±.19	95.72±.29	96.82±.39
TV20*	March	21.12±0.39	17.52±0.22	19.81±0.25	2.1	1.7	1.98	7.75±0.18	9.82±0.15	10.02±0.11	0.77	0.98	1	86.72±.17	85.72±.27	86.32±.27
		46.05±0.51	47.20±0.59	52.12±0.62	4.6	4.72	5.2	29.35±0.13	22.15±0.14	23.16±0.19	2.93	2.21	2.31	98.22±.23	99.22±.33	98.23±.37
TV20*	June	7.82±0.28	6.21±0.25	8.92±0.24	0.78	0.62	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.219	0.26	0.29	79.14±.28	78.14±.28	79.54±.68
		27.56±0.36	29.51±0.39	36.65±0.33	2.75	2.95	3.65	10.36±0.19	9.82±0.17	15.23±0.17	1.03	0.98	1.52	92.6±.29	93.6±.19	92.61±.59
TV20*	December	18.02±0.25	17.01±0.21	18.91±0.21	1.8	1.7	1.89	7.24±0.18	7.41±0.14	6.82±0.11	0.724	0.74	0.68	85.36±.41	84.36±.51	87.36±.11
		39.02±0.29	41.25±0.24	49.02±0.29	3.9	4.12	4.9	16.55±0.11	26.01±0.15	27.82±0.15	1.65	2.61	2.78	77.47±.21	78.47±.31	75.47±.41
TV26*	March	23.12±0.29	17.52±0.22	19.81±0.25	2.31	1.75	1.98	9.75±0.18	8.82±0.15	11.02±0.11	0.975	0.88	1.1	86.27±.29	85.27±.49	86.87±.09
		48.05±0.52	46.20±0.59	52.21±0.62	4.8	4.62	5.2	18.35±0.13	24.15±0.14	23.86±0.19	1.83	2.41	2.38	98.94±.11	97.94±.12	98.94±.51
TV26*	June	4.82±0.28	6.21±0.25	8.92±0.24	0.48	0.621	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.219	0.26	0.29	87.32±.45	88.32±.55	87.52±.55
		21.56±0.46	29.51±0.39	38.56±0.33	2.15	3.85	3.65	6.36±0.19	10.82±0.17	15.53±0.17	0.63	1.08	1.55	96.66±.35	95.66±.45	96.69±.15
TV26*	December	17.02±0.22	17.01±0.21	18.91±0.21	1.7	1.89	1.89	6.24±0.18	6.41±0.14	6.92±0.11	0.62	0.64	0.69	78.52±.52	78.92±.62	78.72±.62
		37.02±0.23	41.25±0.24	49.02±0.29	3.7	4.12	4.9	15.55±0.11	29.01±0.15	27.72±0.15	1.55	2.9	2.77	89.25±.40	87.25±.50	89.45±.42
TV29*	March	19.12±0.32	17.52±0.22	19.81±0.25	1.9	1.75	1.98	8.75±0.18	8.82±0.15	11.52±0.11	0.87	0.88	1.15	88.25±.39	89.25±.29	88.25±.29
		48.05±0.55	46.20±0.59	52.03±0.62	4.8	4.62	5.2	14.35±0.13	23.15±0.14	23.66±0.19	1.43	2.31	2.36	99.0±.24	99.12±.25	99.12±.25
TV29*	June	3.82±0.26	6.21±0.25	8.92±0.24	0.38	0.62	0.89	2.19±0.21	2.60±0.25	2.62±0.24	0.21	0.26	0.26	78.36±.39	78.96±.19	78.66±.32
		22.56±0.56	29.51±0.39	36.56±0.33	2.25	2.95	3.65	11.36±0.19	11.82±0.17	15.93±0.17	1.13	1.18	1.59	96.77±.33	97.77±.33	96.79±.13
TV29*	December	15.02±0.21	17.01±0.21	18.91±0.21	1.5	1.7	1.89	5.24±0.18	6.41±0.14	6.52±0.11	0.52	0.64	0.65	69.25±.30	69.95±.32	69.75±.38
		39.02±0.21	41.25±0.24	49.02±0.29	3.9	4.12	4.9	19.55±0.11	27.01±0.15	27.92±0.15	1.95	2.71	2.79	86.87±.16	86.81±.46	86.88±.26
TV30*	March	29.12±0.29	17.52±0.22	19.81±0.25	2.9	1.7	1.98	8.75±0.18	8.82±0.15	11.42±0.11	0.87	0.88	1.14	87.29±.19	87.99±.59	87.39±.59
		49.05±0.52	41.20±0.59	52.32±23	4.9	4.12	5.2	29.35±0.13	24.15±0.14	23.56±0.19	2.93	2.41	2.35	96.55±.33	97.55±.37	96.58±.38
TV30*	June	6.82±0.28	6.21±0.25	8.92±0.24	0.68	0.62	0.89	2.19±0.21	2.60±0.25	2.95±0.24	0.21	0.26	0.29	74.10±.11	78.10±.21	74.29±.19
		35.56±0.36	29.51±0.39	38.56±0.33	3.56	2.95	3.85	8.36±0.19	13.82±0.17	15.63±0.17	0.83	1.38	1.56	97.46±.42	97.86±.62	97.66±.22
TV30*	December	18.02±0.27	17.01±0.21	19.91±0.21	1.8	1.7	1.99	6.24±0.18	6.41±0.14	6.88±0.11	0.62	0.64	0.688	76.32±.28	76.82±.18	76.12±.18
		39.02±0.21	41.25±0.24	47.02±0.29	3.9	4.12	4.7	27.55±0.11	25.01±0.15	27.85±0.15	2.75	2.5	2.78	86.47±.50	88.47±.52	86.47±.29
Tingamara*	March	19.12±0.27	17.52±0.22	19.81±0.25	1.9	1.75	1.98	8.75±0.18	8.82±0.15	11.42±0.11	0.87	0.88	1.14	89.98±.39	89.78±.19	89.78±.59
		45.05±0.50	46.20±0.59	49.21±0.62	4.5	4.62	4.92	17.35±0.13	26.15±0.14	23.96±0.19	1.73	2.61	2.39	99.28±.22	99.28±.21	99.27±.22
Tingamara*	June	7.82±0.20	6.21±0.25	8.92±0.24	0.78	0.62	0.89	2.19±0.21	2.60±0.25	2.32±0.24	0.21	0.26	0.23	77.24±.22	75.24±.32	77.29±.21
		35.23±12	29.51±0.39	46.56±0.33	3.5	2.95	4.65	10.36±0.19	11.82±0.17	15.73±0.17	1.03	1.18	1.57	94.19±.48	94.99±.28	95.19±.45
Tingamara*	December	8.02±0.24	17.01±0.21	18.91±0.21	0.8	1.7	1.89	6.24±0.18	6.41±0.14	6.52±0.11	0.62	0.641	0.65	75.24±.20	76.26±.22	75.22±.22

		49.02±0.23	41.25±0.24	49.98±0.29	4.9	4.12	4.99	16.55±0.11	21.01±0.15	27.22±0.15	1.65	2.1	2.72	89.72±.25	89.82±.35	89.72±..39
Dangrimanipuri*	March	13.12±0.25	17.52±0.22	18.81±0.25	1.3	1.75	1.88	8.75±0.18	7.82±0.15	11.12±0.11	0.87	0.78	1.11	87.20±.22	87.29±.20	87.21±.26
		47.05±0.51	46.20±0.59	49.63±0.62	4.7	4.62	4.96	18.35±0.13	20.15±0.14	23.16±0.19	1.83	2.01	2.31	99.97±.31	99.87±.41	99.97±.10
Dangrimanipuri*	June	9.82±0.28	6.21±0.25	8.92±0.24	0.98	0.62	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.21	0.26	0.29	88.56±22	88.36±42	88.46±27
		35.56±0.36	29.51±0.39	46.56±0.33	3.56	2.95	4.65	11.36±0.19	12.82±0.17	15.23±0.17	1.13	1.28	1.52	97.35±.30	98.35±.32	97.55±.37
Dangrimanipuri*	December	18.02±0.29	17.01±0.21	18.91±0.21	1.8	1.7	1.89	6.24±0.18	6.41±0.14	6.82±0.11	0.62	0.64	0.68	77.12±.20	77.92±.29	77.72±.28
		39.02±0.21	41.25±0.24	49.02±0.29	3.9	4.12	4.9	19.55±0.11	29.01±0.15	27.82±0.15	1.95	2.9	2.78	90.55±.26	90.45±.36	90.55±.42
Sundaram(B/5/63)*	March	23.12±0.29	17.52±0.22	19.81±0.25	2.3	1.75	1.98	8.75±0.18	8.82±0.15	11.02±0.11	0.87	0.88	1.1	89.25±.25	89.45±.85	89.45±.45
		44.05±0.52	46.20±0.59	49.32±0.62	4.4	4.62	4.92	18.35±0.13	28.15±0.14	23.16±0.19	1.83	2.81	2.31	99.97±.19	99.77±.59	99.97±.29
Sundaram(B/5/63)*	June	5.82±0.28	6.21±0.25	8.92±0.24	0.58	0.62	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.21	0.26	0.29	84.26±.31	84.46±.31	84.36±.37
		25.56±0.36	29.51±0.39	39.56±0.33	2.56	2.95	3.95	10.36±0.19	11.82±0.17	15.23±0.17	1.03	1.18	1.52	97.35±.22	97.85±.25	97.45±.28
Sundaram(B/5/63)*	December	18.02±0.27	17.01±0.21	17.91±0.21	1.8	1.7	1.79	7.24±0.18	6.41±0.14	6.82±0.11	0.72	0.64	0.68	85.25±.23	85.95±.21	85.25±.23
		39.02±0.21	41.25±0.24	44.02±0.29	3.9	4.12	4.4	17.55±0.11	23.01±0.15	27.82±0.15	1.75	2.3	2.78	90.55±.30	91.55±.20	90.55±.30
Tinali 17*	March	23.12±0.29	17.52±0.22	16.81±0.25	2.3	1.75	1.68	7.75±0.18	8.82±0.15	11.02±0.11	0.77	0.88	1.1	78.56±.39	78.86±.49	78.56±.39
		49.05±0.19	46.20±0.59	56.21±0.62	4.9	4.62	5.62	18.35±0.13	29.15±0.14	23.16±0.19	1.83	2.91	2.31	99.87±.49	99.17±.19	99.87±.49
Tinali 17*	June	7.82±0.28	6.21±0.25	8.92±0.24	0.78	0.62	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.21	0.26	0.29	84.12±.38	84.82±.88	84.42±.78
		45.56±0.36	29.51±0.39	46.56±0.33	4.56	2.95	4.65	12.36±0.19	17.82±0.17	15.23±0.17	1.23	1.78	1.52	96.35±.22	96.31±.12	96.35±.32
Tinali 17*	December	8.02±0.27	17.01±0.21	18.91±0.21	0.8	1.7	1.89	7.24±0.18	6.41±0.14	6.82±0.11	0.72	0.641	0.68	74.21±.16	73.29±.25	74.81±.26
		42.02±0.21	41.25±0.24	49.02±0.29	4.2	4.12	4.9	19.55±0.11	28.01±0.15	27.82±0.15	1.95	2.8	2.78	91.55±.28	93.57±.18	91.59±.18
Takda 7,8*	March	20.12±0.24	17.52±0.22	19.81±0.25	2.01	1.75	1.98	8.75±0.18	8.82±0.15	11.02±0.11	0.87	0.88	1.1	84.59±.39	82.39±.29	84.99±.19
		46.05±0.50	46.20±0.59	49.21±0.62	4.6	4.62	4.9	14.35±0.13	24.15±0.14	23.36±0.19	1.43	2.41	2.33	98.97±.38	98.99±.35	98.98±.48
Takda 7,8*	June	6.82±0.27	6.21±0.25	8.92±0.24	0.68	0.62	0.89	2.19±0.21	2.60±0.25	2.92±0.24	0.21	0.26	0.29	79.69±.35	77.69±.55	79.29±.15
		45.56±0.30	29.51±0.39	46.56±0.33	4.56	2.95	4.65	13.36±0.19	12.82±0.17	14.23±0.17	1.33	1.28	1.42	97.85±.46	96.85±.76	97.85±.16
Takda 7,8*	December	18.91±0.23	18.01±0.21	18.91±0.21	1.89	1.8	1.89	6.24±0.18	6.41±0.14	7.82±0.11	0.62	0.64	0.78	79.65±.21	78.60±.29	79.25±.20
		49.23±19	43.25±0.24	49.02±0.29	4.92	4.32	4.9	18.55±0.11	27.01±0.15	27.89±0.15	1.85	2.71	2.78	98.55±.41	98.57±.48	98.25±.41

All data are presented as the mean of three experiments; $P < 0.01$ regarded as significant.*unprocessed leave.