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Metabolite profiling and characterization of somaclonal variants in tea (*Camellia* spp.) for identifying productive and quality accession

Jibu Thomas, R. Raj Kumar, A.K.A. Mandal *

Plant Physiology Division, UPASI Tea Research Foundation Tea Research Institute, Valparai 642 127, Coimbatore District, Tamil Nadu, India

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Abstract

A study has been undertaken to characterize 15 field grown somaclonal variants derived from cotyledonary tissues of UPASI-10 using morphological, physiological and biochemical characters. Although variants were derived from UPASI-10, a very few variants possessed unique "Chinery" characters while others exhibited "Assam" characters. However, no variant showed identical morphological characters aligning with the parent. Somaclonal variants showed distinct variation in terms of photosynthetic carbon assimilation, stomatal conductance and diffusion resistance. Proline accumulation and water use efficiency showed marginal variations among the variants. SE 8 and SE 10 recorded higher values of membrane stability index denoting their tolerant nature against stress. Class interval analysis based on physiological parameters grouped these plants into three clusters. Three variants grouped under good category representing higher values of productivity index followed by five variants under moderate category. Green leaf constituents and quality profile of made tea produced with crop shoots of variants exhibited wide variation. Center point radar graph analysis of quality constituents grouped these plants into three clusters. Variants SE 2 and SE 13 were segregated distinctly representing their black tea characters. When considering both the quality and productivity indices, SE 3 and SE 7 fall under moderate category and in future these two variants may be subjected to further quality tests for commercial exploitation.

Keywords: *Camellia*; Somaclonal variants; Physiology; Volatile flavour constituents; Tea; Productivity and quality

1. Introduction

Tea (*Camellia sinensis* L.) belongs to the family Theaceae. It is the oldest non-alcoholic beverage in the world. It is cultivated in about 32 countries in the world and plays an important role in the economy of those tea growing countries. The economic importance of the genus *Camellia* is mainly due to tea which is consumed in the form of "fermented tea" (black tea), "non-fermented tea" (green tea) and 'semi-fermented tea" (oolong tea). More than 700 chemical constituents have been reported in tea leaves, among which flavonoids, amino acids, vitamins, alkaloids and polysaccharides gain much importance for human health. Recent studies have demonstrated that tea is more than a mere stimulant and it may owe its special medicinal properties due to the high level of polyphenols present in the consumed beverage (Astill et al., 2001). Polyphenols account for about 25–35% of total dry weight of freshly plucked tea leaves of which two-thirds is contributed by catechins alone (Saravanan et al., 2005). A number of studies have been performed to assess the effects of tea polyphenols on the mutagenicity of carcinogens (Weisburger, 1996). Antioxidant and free radical scavenging properties of polyphenols have also been examined (Roberts and Fernando, 1981).

Since the beginning, commercial tea gardens were established from seeds (Venkataramani and Sharma, 1975). Tea being out crossing species, a lot of genetic variations existed among the populations. At present there are about 600 cultivated varieties available world wide. A large number of present day elite clones are the result of selections from these bushes followed by vegetative multiplication. However, the pedigree of these clones remained unknown.

* Corresponding author. Tel.: +91 4253 235301; fax: +91 4253 235302.
E-mail address: jibuupaasi@gmail.com (J. Thomas).

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Although selections are very popular among the tea breeders, hybridization between promising parents have also resulted in many clones through F1 hybrids or biclonal seed stocks. Even though conventional tea breeding has been practiced for several decades and is successful in developing many clones, the process has its own limitations. The time consumed in releasing a planting material for commercial exploitation is exorbitant. Therefore, an alternative approach has been made to develop new accessions through somaclonal variation. Plants regenerated from somatic tissue through tissue culture showed morphological/biochemical/genetic variations in the field. It is reported that many cultivars have been developed through somaclonal variation in commercial crops. Despite somaclonal variation being a powerful tool, no serious attempt has been made to develop new variants in tea using the potential of this technique. Das (1992) observed wide range of chromosomal variation in leaf and cotyledon derived callus while Chen and Liao (1983) observed the change in ploidy level among anther derived plantlets of tea. Reports are scanty on morphological or biochemical variations exist among the regenerants. In our laboratory, we have regenerated plants through somatic embryogenesis from cotyledon (Raj Kumar and Ayyappan, 1992). Molecular analysis through inter simple sequence repeats (ISSR) showed genetic variation in 15 plants among these somatic embryo derived populations (Jibu Thomas et al., 2005). In the present report, an attempt has been made to characterize these somaclonal variants (SE) for identifying the potential productive and quality variant for further multiplication and commercialization.

2. Results and discussion

2.1. Morphological characters

Leaves of the parental line (UPASI-10) are dark green in colour, elliptic, smooth blunt tip, erect posture, distantly serrated and large in size. When compared to parental line, somaclonal variants possessed yellow, pale or dark colour leaves, ovate or elliptic in shape, smooth or rugose venation, erect, semierect or horizontal in posture and small to large in size. Most of the variants had acute leaf tip and close serration except SE 5. Although variants were derived from "China" hybrid, UPASI-10, only a few variants possessed unique "Chinery" character (elliptic, dark green, smooth, erect to semierect, small/medium size leaves) while others exhibited "Assam" characters (broad/ovate, pale to yellowish green, rugose, horizontal, medium to large leaves). No variant showed identical morphological characters aligning with the parent. Since the variants possessed both "Assam" and "China" characteristics, these could not be categorized into their respective groups. Somaclonal variation appeared to result from both preexisting genetic variation within the explants and variation induced during culture phase (Evans et al., 1994). Heritable variations are stable through sexual cycle while epigenetic variation is unstable even under asexual propagation. In the present study, the variants were subjected to morphological, physiological and biochemical tests, but it was not possible to determine the type of variation that exists at present.

2.2. Physiological behavior

Somaclonal plants showed a considerable variation in almost all the physiological parameters studied (Table 1). A significant variation was noticed in carbon assimilation rate (Pn) that ranged from 5.7 μmol CO_2 assimilated $m^{-2}\,s^{-1}$ (SE 6) to 8.59 μmol CO_2 assimilated $m^{-2}\,s^{-1}$ (SE 11). Similarly, significant variation in terms of stomatal conductance (Gs) and diffusion resistance (R) was noticed among the somaclonal variants. The highest stomatal conductance of 0.9 $cm\,s^{-1}$ was recorded in SE 11 and the lowest was noticed in SE 6 (0.2 $cm\,s^{-1}$) while diffusion resistance of 4.17 $s\,cm^{-1}$ was noticed in SE 6 and the lowest of 2.5 $s\,cm^{-1}$ was observed in SE 11. There was no significant difference in water use efficiency (WUE) among the variants. Strong positive correlation existed between photosynthetic carbon assimilation and stomatal conductance among the variants ($r = 0.991^{**}$).

Membrane stability determined by measuring conductivity of electrolytes that leached out from leaves subjected to high temperature is widely used as a measure of high temperature tolerance of crops because of the adaptation to stress associated with viability of cells (Saadalla et al., 1990). The membrane stability index (MSI) ranged from 55.50 (SE 11) to 72.30 (SE 10) denoting the diversity of stress tolerance nature among the variants while strong

Table 1
Photosynthetic rate (μmol CO_2 assimilated $m^{-2}\,s^{-1}$), stomatal conductance ($cm\,s^{-1}$), diffusion resistance ($s\,cm^{-1}$), water use efficiency (ratio), membrane stability index (%) and relative injury (%) of somaclonal variants of tea

Plant	Physiological parameters					
	Pn	Gs	R	WUE	MSI	RI
UP10	8.20	0.36	2.78	1.21	69.9	29.2
SE 1	7.20	0.33	3.03	1.12	59.8	38.2
SE 2	7.01	0.31	3.23	1.18	68.6	31.3
SE 3	7.08	0.32	3.17	1.38	68.7	30.3
SE 4	6.71	0.29	3.50	1.20	58.9	40.1
SE 5	6.54	0.28	3.54	1.16	61.9	37.6
SE 6	5.70	0.24	4.17	1.35	71.1	27.9
SE 7	6.37	0.28	3.57	1.40	69.7	29.1
SE 8	6.03	0.26	3.86	1.39	72.3	27.7
SE 9	6.71	0.29	3.45	1.37	65.7	33.4
SE 10	6.92	0.31	3.26	1.34	72.3	26.5
SE 11	8.59	0.40	2.50	1.14	55.5	43.8
SE 12	8.52	0.39	2.57	1.13	62.3	37.4
SE 13	7.26	0.34	2.98	1.21	64.3	35.2
SE 14	7.57	0.35	2.83	1.16	62.1	37.9
SE 15	7.82	0.37	2.70	1.20	57.9	39.8
SE	0.68	0.01	0.04	0.10	1.64	1.66
CD ($P = 0.05$)	1.39	0.03	0.08	0.20	3.35	3.40

negative relationship existed between membrane stability index and relative injury (RI) of membrane ($r = 0.993^{**}$). Similar trend was noticed in relative injury also (Table 1). It was found that the relative injury and the membrane stability index are inversely related. The high values of membrane stability index in some variants indicated their tolerant nature against different kinds of biotic and abiotic stress (Mohamed Sathik et al., 1998). Somaclonal variants exhibited relatively higher membrane stability against stress confirming their genomic origin (Shivshankar et al., 1991). The variants which exhibited higher degree of MSI and lower degree of RI can be exploited for future breeding programme (Shanahan et al., 1990). Besides the adaptive mechanism against stress, phenological features are also responsible for MSI/RI. The higher injury indicated extend of lipid peroxidation which in turn is a resultant of higher oxidative stress caused by high stress (Blum and Ebercon, 1981).

2.3. Biochemical characteristics

Green leaf constituents of somaclones exhibited very wide variation (Table 2). Total polyphenol ranged from 22.73% (SE 15) to 30.38% (SE 13). Catechins varied between 12.43% (SE 12) and 17.30% (SE 4). Similar trends were noticed in terms of amino acids and total chlorophyll contents (Table 2). Diversity of the catechin fractions was prominent in all the plants. Among the major catechin fractions, EGCG recorded as high as 12.69% in SE 13 (Table 3). Tea, like fruits and vegetables contains natural flavonoids with potent antioxidant properties (McKay and Blumberg, 2002). Polyphenols account for about 25–35% of total dry weight of freshly plucked tea leaves of which two-thirds is contributed by catechins alone. It is true that the black tea quality depends on the level of catechins. According to Wanyoko (1977) and Owuor and Othieno (1988) the polyphenolic theaflavins and thearubigins present in the black tea are formed by the enzymic oxidation of the catechins in the green leaf and therefore their concentration in the fresh green tea may influence black tea "quality". Due importance is warranted to characterize the variants based on the black tea parameters and volatile constituents. Except SE 4 (4.76%), SE 8 (4.98%) and SE 10 (4.79%), the caffeine content ranged from 2.3% to 3.7%. Baumann et al. (1996) reported that the variation in caffeine content could be attributed due to the impairment in the synthesis of adenine nucleotides, which are precursors of caffeine or by the conversion of 7-methylxanthine to caffeine via theobromine (Kato, 2001). A trigger in the genes coding catechin formation may be assumed a likely reason for the variations in the catechin content. Environmental factors also might have resulted in the altered synthesis of some of the individual catechins (Jibu Thomas et al., 2005). Present study corroborates the findings of Cloughley (1982) who has reported that biochemical content of the crop shoots has been influenced by genetic factors. Total chlorophyll content of the tea shoots confirmed the visual observations (Table 2).

Most of the tea samples registered values greater than 0.8% and 8.0% in terms of theaflavins and thearubigins, respectively (Table 4). TR to TF ratio of made tea samples of SE 6, SE 8 and SE 9 registered higher values (>10). Briskness index of made tea sample prepared with SE 2

Table 2
Polyphenols (%), catechins (%), amino acids (%), chlorophylls (%) and proline (μmol g^{-1}) on fresh weight basis among the somaclonal variants

Plant	Biochemical parameters				
	Polyphenols	Catechins	Amino acids	Chlorophylls	Proline
UP10	27.23	17.68	2.52	3.42	0.41
SE 1	23.85	13.63	2.56	3.31	0.49
SE 2	28.26	16.15	2.79	3.17	0.45
SE 3	26.92	15.38	2.39	3.22	0.37
SE 4	30.28	17.3	2.75	3.06	0.44
SE 5	27.11	15.49	2.55	3.68	0.40
SE 6	26.93	15.39	3.62	3.03	0.36
SE 7	24.48	13.99	2.54	3.40	0.32
SE 8	28.60	16.34	2.51	2.76	0.33
SE 9	22.86	13.06	2.87	2.95	0.36
SE 10	26.78	15.3	3.65	2.71	0.38
SE 11	24.06	13.75	3.85	2.32	0.55
SE 12	21.75	12.43	2.99	3.31	0.44
SE 13	30.38	17.36	3.54	3.56	0.45
SE 14	28.21	16.12	2.72	3.48	0.49
SE 15	22.73	12.99	2.71	2.90	0.53
SE	1.17	0.89	0.24	0.22	0.03
CD ($P = 0.05$)	2.40	1.82	0.49	0.45	0.06

Table 3
Catechin fractions and caffeine in dry weight basis among the somaclonal variants

Plant	Relative distribution of catechin fractions (%)					Caffeine (%)
	EGC	+Cat	EC	EGCG	ECG	
UP10	1.15	0.87	1.40	12.59	0.62	2.53
SE 1	0.47	0.80	1.54	10.24	0.58	2.30
SE 2	0.40	0.68	1.30	13.28	0.49	2.37
SE 3	0.41	0.70	1.07	12.69	0.51	3.37
SE 4	0.52	1.52	2.26	12.56	0.44	4.76
SE 5	0.22	0.81	1.56	12.29	0.61	3.19
SE 6	0.43	0.98	1.40	11.89	0.69	2.90
SE 7	0.38	0.78	1.07	11.11	0.65	2.34
SE 8	0.59	1.17	1.43	12.18	0.97	4.98
SE 9	0.42	0.56	1.23	10.28	0.57	3.37
SE 10	0.38	0.80	1.42	11.97	0.73	4.79
SE 11	0.77	0.85	1.35	10.32	0.46	2.79
SE 12	0.49	0.64	1.36	9.64	0.30	2.45
SE 13	1.33	1.31	1.38	12.69	0.65	3.52
SE 14	1.30	0.80	1.52	11.96	0.54	3.76
SE 15	0.72	0.93	1.41	9.56	0.37	2.85
SE	0.18	0.15	0.43	0.77	0.18	0.65
CD ($P = 0.05$)	0.36	0.31	0.89	1.57	0.36	1.33

EGC, epigallo catechin; +Cat, simple catechins; EC, epicatechin; EGCG, epigallo catechin gallate; ECG, epicatechin gallate.

Table 4
Variations in black tea quality parameters among somaclonal variants

Plant	Quality parameters						
	TF	TR	TR:TF	HPS	TLC	CI	BI
UP10	0.96	8.61	8.97	9.23	3.15	5.38	27.51
SE 1	0.77	7.44	9.66	10.97	3.90	4.18	25.08
SE 2	1.07	9.40	8.79	10.74	4.85	5.31	31.10
SE 3	1.18	9.93	8.42	9.22	3.64	6.16	25.93
SE 4	1.14	9.51	8.34	9.77	3.89	5.91	19.32
SE 5	0.89	8.87	9.97	9.77	4.76	4.77	21.81
SE 6	0.72	7.85	10.90	9.28	4.89	4.20	19.89
SE 7	0.95	9.22	9.71	7.84	2.58	5.57	28.88
SE 8	0.83	8.34	10.05	8.27	3.75	5.00	14.29
SE 9	0.86	8.72	10.14	7.87	2.72	5.18	20.33
SE 10	0.88	7.74	8.80	9.37	3.58	5.14	15.52
SE 11	0.91	8.25	9.07	9.66	3.55	5.08	24.59
SE 12	0.86	8.27	9.62	7.64	2.40	5.41	25.9
SE 13	0.93	8.59	9.24	9.74	4.85	5.07	20.90
SE 14	1.04	8.74	8.40	9.22	4.40	5.79	21.67
SE 15	0.95	9.11	9.59	9.77	4.40	5.03	25.00

Values represent mean of two observations. TF, theaflavins (%); TR, thearubigins (%); HPS, high polymerized substances (%); TLC, total liquour colour; CI, colour index; BI, briskness index.

was as high as 31.10. Though the TR:TF was high in SE 8, higher caffeine content of the green leaf brought down the briskness index to 14.29. In the case of volatile flavour components, about 50% of somaclones registered higher values of flavour index (Table 5). The aroma compounds are classified into two groups. Group I consisted of hexanal, 1-penten-3-ol, *trans*-2 hexenal, 2-hexanol, 1-pentanol, 6-methyl-5-hepten-2-one, *cis*-3 hexenol and 1-octen-3-ol. Compounds fall in group II are ∞ pinene, linalool oxide I, linalool oxide II, citronellal, benzaldehyde, linalool, phenyl acetaldehyde, geranyl acetate, methyl salicylate, nerol, geraniol, alpha ionine, benzyl alcohol, phenyl ethanol, beta ionine, *cis*-neroldol and methyl jasmonate. Quality of the black tea is correlated with polyphenol and catechin content (Mayer and Harel, 1991). Though the parental material, UPASI-10, fall under the moderate category in terms of quality parameters (Venkataramani and Sharma, 1975) certain variants gained higher values in terms of a number of quality parameters. This confirms the potential role of somaclonal variation in evolving high quality tea accessions as it has been proved in other crops.

Table 5
Major volatile constituents and flavour index of somaclonal variants

Plant	Group I	Group II	FI
UP10	19.33	13.36	0.95
SE 1	17.72	15.62	0.88
SE 2	18.56	17.96	0.97
SE 3	21.00	18.19	0.87
SE 4	21.18	17.98	0.85
SE 5	20.91	18.02	0.86
SE 6	17.75	15.37	0.87
SE 7	18.79	17.18	0.91
SE 8	17.54	16.73	0.95
SE 9	16.52	16.48	1.00
SE 10	17.77	16.72	0.94
SE 11	16.75	17.19	1.03
SE 12	17.39	17.15	0.99
SE 13	16.75	17.24	1.03
SE 14	18.26	16.54	0.91
SE 15	17.85	16.65	0.93

Values represent sum of group I and group II components. FI, flavour index.

2.4. Productivity and quality indices

Class interval analysis based on the analyzed parameters grouped these plants into three clusters (Fig. 1). Results strengthened the findings of Jibu Thomas et al. (2004) on the positive relationship existed between stress related parameters and productivity. Based on the interaction, variants SE 1, SE 11 and SE 15 were categorized under good values of productivity index followed by SE 3, SE 7, SE 8, SE 12 and SE 14 under moderate category. Remaining seven somaclones recorded very low values of productivity index. Synchronization among the physiological parameters such as stomatal conductance, photosynthetic carbon assimilation and water use efficiency will have an added advantage in increasing the yield potential. In view of the values for quality parameters, variants SE 2 and SE 13 were segregated distinctly representing their black tea characters.

When considering quality and productivity indices, none of the accessions fall under good values for both productivity and quality where as SE 3 and SE 7 fall under moderate category in both the cases, which may be subjected to further tests for commercial exploitation. It is anticipated that the finding from the current study would be beneficial to the breeders in identifying the productive and superior quality clones. Although somaclones are derived from known parental tissues, these were not commercially exploited; however, these developed accessions provide an important reservoir of recombined or reactivated genes that can be utilized in in vitro breeding programmes. The findings will serve as a valuable database on the performance of somaclones specific to desired trait in order to characterize its potential.

3. Experimental

3.1. Induction of somaclonal plants

Somaclonal plants were raised with single line cotyledon culture of a "China" hybrid clone "UPASI-10" (UP10) adopting the protocol of Raj Kumar (2001). Germination of somatic embryos and successive field transfer of plantlets were carried out during 1998 at Experimental Farm of United Planters' Association of Southern India (UPASI) Tea Research Institute located in the Anamallais, Tamil Nadu, India. All cultivation practices were carried out uniformly according to standard recommendations of UPASI (Hudson et al., 2002). Field planted five year old

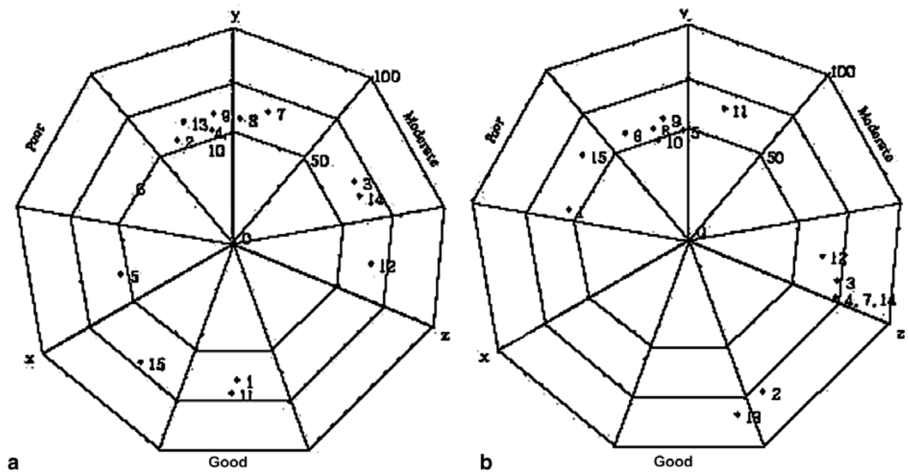

Fig. 1. Center point radar graph segregation of variants into productive (a) and quality (b) accessions.

somaclonal variants (SE 1–SE 15) were subjected to trait specific analysis using morphological, physiological and biochemical tools.

3.2. Morphological characterization

Field established plants were subjected to the observation of leaf characters such as colour, shape, venation, apex, posture, serration, and size in accordance with National Bureau of Plant Genetic Resources (NBPGR), New Delhi to compare the morphological similarity that existed among the variants.

3.3. Physiological parameters

Stomatal conductance (Gs) of the fully expanded leaves of each plant was measured individually with a portable Porometer (Delta T, Steady State porometer, LI-COR Inc., USA) at a light intensity of 900–950 $\mu mol\ m^{-2}\ s^{-1}$ while diffusion resistance (r) was computed using the values of Gs.

Photosynthetic rate (Pn) was monitored with an infra red gas analyser (ADC, UK. models: LCA-3) fitted with a Parkinson's leaf chamber (Model PLC-3) for CO_2 analysis using mother leaf adopting the protocol of Jeyaramraja et al., 2003. Here again physiologically matured mother leaves were used as the test material. Water use efficiency (WUE) was computed as the ratio of carbon dioxide assimilated to water transpired per unit area of leaf (Harborne, 1973).

Intact maintenance leaves were used for the determination of membrane stability index (MSI) and relative injury (RI) at 40 °C after (Tyagi et al., 1999) with slight modification. Leaf blades were cut into leaf discs of 1 cm diameter immediately after sampling. The samples were washed three times with distilled water to remove electrolytes released from the cut ends of the leaf discs. Leaf samples (0.5 g) were placed into glass vials with 10 ml of distilled water and heated at 40 °C for 1 h while control vials were maintained at 25 °C. Electrolytes were measured directly by an electrical conductivity meter (Mettler Toledo, Model: MO128, Switzerland) then samples were autoclaved to kill plant tissues and electrical conductivity was measured again for the determination of relative injury (RI) and expressed in per cent according to Martineau et al. (1979).

3.4. Biochemical constituents of crop shoots

Green leaf constituents such as polyphenols (Dev Choudury and Goswami, 1983), total catechins (Swain and Hillis, 1959), amino acids (Moore and Stein, 1948), chlorophylls (Harborne, 1973) and proline (Bates et al., 1973) were quantified using outlined protocols in the young crop shoots. Crop shoots were dried overnight at 90 °C and extracted with 70% methanol for quantification of catechin fractions using high performance liquid chromatography (Hewlett Packard Model 1100 Series, USA) fitted with Phenomenex (5 μ Luna Phenyl-Hexyl bonded) column adopting the method of Jibu Thomas et al. (2005) with a gradient elution of mobile A (9.0% acetonitrile acidified with acetic acid) and mobile B (80.0% acetonitrile). Detection was carried out at a UV wavelength of 272 nm. Relative distributions of these constituents are expressed in per cent as per ISO method (1999).

3.5. Quality parameters of made tea

As the quantity of the crop shoot harvested from the individual bushes ranged from 100 to 300 g with respect to the cropping seasons, the miniature crush tear and curl (CTC) manufacture was attempted during the high cropping period corresponding to April–May and September–October. The made tea was assessed for its primary quality constituents and volatile flavour components. Quality constituents such as theaflavins (TF), thearubigins (TR), high polymerized substances (HPS) and total liquor colour (TLC) were analyzed using spectrophotometric method

(Thanaraj and Seshadri, 1990). Colour index and briskness index were computed with the values of TF, TR, HPS and caffeine (Ramaswamy, 1986). Volatile flavour constituents (VFC) analyses of black tea samples were carried out with gas chromatography (Perkin–Elmer, Autosystem XLGC) fitted with 60 m HP- Innowax column (0.25 mm i.d., 0.25 μm thickness film and packed with PEG) provided with Turbomatrix head space sampler (16 HS). The temperature of the injector and detector were maintained at 200 and 250 °C, respectively. GC conditions include the FID detector with carrier gas N_2 at 1.0 ml min^{-1}. The split ratio was maintained at 1:20 with 1μ injection.

The presence of individual components was expressed as percentage relative distribution of peak areas to that of the internal standard, ethyl caproate (50 ppm). Based on the values of group I and II constituents, flavour index (FI) was computed as the ratio between the groups according to Ravichandran and Parthiban (1998).

3.6. Statistical analysis of the data

All the physiological and biochemical data presented are the mean of five replications carried out in various seasons. Data obtained in the present study were statistically analyzed (ANOVA and linear regression) for their significance. Class interval analysis was also performed to categorize the somaclones into different groups (good, moderate and poor). Using the analyzed data, center point radar graph was plotted. For categorization under productivity index, parameters such as photosynthesis, stomatal conductance, water use efficiency, total chlorophyll, diffusion resistance, proline content, membrane stability index and relative injury were considered whereas in the case of quality index, polyphenols, catechins, amino acids, caffeine and chlorophyll contents of green leaf and certain quality parameters of made tea such as theaflavins, thearubigins, colour index, briskness index and flavour index were considered.

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