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Genotypic and seasonal variations of catechin and caffeine content in exotic collection of tea [*Camellia sinensis* (L.) O. Kuntze] germplasm in Sri Lanka

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Abstract

The quality of made tea is largely dependent on the key metabolites *viz.* caffeine and flavan-3-ols. The aim of the present study was to quantify flavan-3-ols and caffeine of Sri Lankan exotic tea germplam. Fresh leaf flavan-3-ols; catechin (+C), epicatechin (EC), epicatechingallate (ECg), epigallocatechin (EGC) and epigallocatechingallate (EGCg), caffeine and gallic acid of 58 accessions (54 exotic and 4 improved cultivars) were performed by High Performance Liquid Chromatography (HPLC). Variation in metabolites among cultivars and seasons were determined. Among the flavan-3-ols, EGCg was the most abundant followed by EGC, ECg, EC and +C. Hierarchical clustering of 58 accessions based on metabolite diversity resulted in two major clusters. High caffeine containing accessions (>30 mg g^{-1}) of Indian origin and improved TRI cultivars clustered together. Low caffeine accessions such as PBGT10, PBGT27, PBGT35, PBGT71, PBGT48, PBGT53 and PBGT54 (< 20 mg g^{-1}) grouped separately. Higher variations in catechins and caffeine content in exotic germplasm is useful in developing tea cultivars of high quality tea.

Key words: Tea quality, *Camellia sinensis*, flavan-3-ols, caffeine, seasonal variations

Tea (*Camellia sinensis* L.), the second most consumed beverage in the world, is a rich source of polyphenolic compounds. Young tea leaves are extremely rich in polyphenolic compounds and the total polyphenols including flavan-3-ols (catechins) in tea ranges from 20 to 30% of the dry weight (Robertson 1992). Polyphenols and their oxidation products are responsible for the quality of black tea (Engelhardt 2010). Five major types of flavan-3-ols; (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechingallate (ECg), (-)-epigallocatechin (EGC) and (-)-epigallocatechingallate (EGCg) have been reported in tea leaves (Sabhapondit *et al*. 2012). Flavan-3-ols are also precursors for the formation of two important compounds of black tea, theaflavins (TFs) and thearubigins (TRs) (Robertson 1992). Caffeine is the most abundant alkaloid in black tea and gives briskness and creaming properties to black tea (Balantine *et al*. 1998). More than 600 tea germplasm accessions representing major varietal types (Banerjee 1992), China type (*C. sinensis* var. *sinensis*) and Assam type (*C. sinensis* var. *assamica* (Masters) Chang) and hybrid Cambod tea (*Camellia sinensis* ssp. *lasiocalyx* (Planchon ex Watt)] are maintained at the Tea Research Institute of Sri Lanka (TRI). Extensive hybridization among the three types resulted in sharing of characteristics and formation of intermediate types which hinder identification of pure

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types in cultivations (Raina *et al*. 2012). The sustainability and profitability of the tea industry primarily depends on the availability of quality planting materials with desirable traits. However, the narrow genetic base of tea cultivations in Sri Lanka owing to the popularity of few commercial clones, renders it more vulnerable to various biotic and abiotic stresses including pests, diseases and drought (Gunasekare 2012). Therefore, collection and introduction of diverse exotic tea cultivars is a routine practice to sustain the tea cultivation. Evaluation of biochemical attributes is a vital step for the selection and identification of potential parental groups to expedite the breeding of quality tea cultivars.

High throughput techniques such as gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), high performance liquid chromatography (HPLC), and nuclear magnetic resonance spectroscopy (NMR) have been widely used to define biochemical diversity of various leaf, root, tuber, and fruit crops (Cevallos-cevallos *et al*. 2009; Price *et al*. 2020). Comprehensive characterization of the phenolic profile of different crops has been applied in the selection and evaluation of new cultivars. Variations in polyphenolic compounds have been successfully used as chemical markers in African Plum (*Prunus salicina* Lindl.) and European grape (*Vitis vinifera*) population in diversity and selection studies (Liang *et al*. 2011; Lampíø 2013). Systematic biochemical and genetic diversity assessment studies of beverage crops like tea, coffee and cocoa is extremely important to identify accessions for variety development targeting quality attributes.

Tea Research Institute of Sri Lanka (TRI) has recently established an exotic collection with tea seed batches received from different sources and developed through vegetative propagation by TRI. A recent taxonomic study revealed that this exotic germplasm mostly represented China types with less numbers of Assam types. However, since most of the cultivated teas in Sri Lanka are predominantly Assam hybrids, accessions of the exotic germplasm with desirable traits could be used to produce new cultivars with a broad genetic variation. Although a few exotic accessions have been previously studied in terms of biochemical profiling (Punyasiri *et al*. 2017), flavan-3-ols and caffeine content of more than 80% of total exotic germplasm collection remains unknown to date.

The objective of the present study was to discover the variation of the five types of flavan-3-ols; (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechingallate (ECg), (-)-epigallocatechin (EGC) and (-)-epigallocatechingallate (EGCg), gallic acid and caffeine in tea accessions of diverse origins selected from tea germplasm maintained at TRI and utilize for breeding purposes targeting speciality teas with health benefits.

Materials and Methods

Plant material

A total of 54 exotic accessions of Sri Lankan tea germplasm were selected based on morphological, floral traits and the countries of origin (Ranatunga *et al*. 2017). Out of 54 accessions, eight accessions have resemblance to Assam/Cambod hybrids and the rest of exotic accessions resemble China types. Additionally, four improved tea cultivars were also included for comparison. All exotic accessions are maintained as a living collection at the field gene bank, TRI, Talawakelle, Sri Lanka (St. Coombs Estate; located Lat 6.94N, Long 80.66E, altitude 1382m amsl).

Preparation of tea samples and biochemical analysis

Tender tea shoots (two apical leaves and the bud) from each progeny was collected (ca. 50 g) and brought to the laboratory at 4 °C. Samples were immediately stored at 80 °C and then freeze-dried for 24 h (Labconco® Corporation, MS, USA). Freeze-dried leaves were ground to a fine powder and sealed in triple-laminated aluminium foil packages and stored until further analysis (Punyasiri *et al*. 2015). The sampling was done in wet season (June and July 2019) and in dry season (February and March 2020). The dry matter content was determined by the loss of moisture in mass at 103 °C (ISO-1573, 1980). Caffeine, C, EC, ECg, EGC, EGCg, and gallic acid were quantified using Agilent 1260 HPLC Infinity System on a phenyl-bonded column with UV detection at 278 nm (ISO 14502-2, 2005). Quantification of individual compounds was determined using Relative Response Factors (RRFs) of caffeine. All chemicals used in the experiments were of HPLC grade (Sigma-Aldrich, USA).

Statistical Analysis

All statistical analyses were carried out with the Statistical Analysis Software package (SAS Version. 9.1, SAS Institute Inc., USA) and biplots were drawn using PAST3 software. Heatmap was generated using ClustVis, online software at http://biit.cs.ut.ee/clustvis. The clustering was based on the Euclidean distance and Ward's minimum variance method.

Results and Discussion

Genotypic and seasonal variations of flavanols and caffeine

The concentration of gallic acid, caffeine, major flavan-3-ols, *viz*. EGC, EC, EGCg, ECg, C, and total catechins (TC) in the extract of 54 exotic accessions and 4 TRI developed cultivars were quantified using HPLC. Among the major flavan-3-ols detected in the extracts, EGCg was the most abundant followed by EGC, ECg, EC and +C. Except EGCg, the means of rest of other biochemical constituents were significantly different between Assam/Cambod type and China type accessions in wet season. In the dry season, means of EGCg and total catechins were not significantly different between two major varietal types. The minimum and maximum concentrations, means, and other statistical parameters of the catechins and caffeine present in wet and dry seasons are given in Table 1. The ranges of individual flavan-3-ols, gallic acid and caffeine during wet and dry seasons are present in a box plot diagram (Figure 1).

In the wet season, PBGT107 possessed the highest EGCg (119.52 mg g^{-1}) content. In dry season, accession AI-4 showed the highest EGCg content followed by AI-9 and AI-2. Among the accessions tested, TRI 2043 possessed the lowest EGCg content in both seasons. Recent study conducted in India which were representing majority of Assam and Cambod types, detected EGCg ranged between 52.2 and 111 mg g^{-1}(Deka *et al*. 2020). In both wet and dry seasons, EGC was the second most abundant flavan-3-ols and the mean values of China type accessions were significantly higher than Assam/Cambod types. Irrespective of the seasons, the lowest EGC content was observed in TRI 2043 whereas PBGT61 (57.32 mg g^{-1}) and PBGT117 (59.74 mg g^{-1}) recorded higher content in wet and dry seasons, respectively.

Irrespective of the season, the mean values of ECg content in China types were significantly lower than Assam/Cambod types. The highest concentration of ECg was observed in PBGT118 and AI-6 in wet and

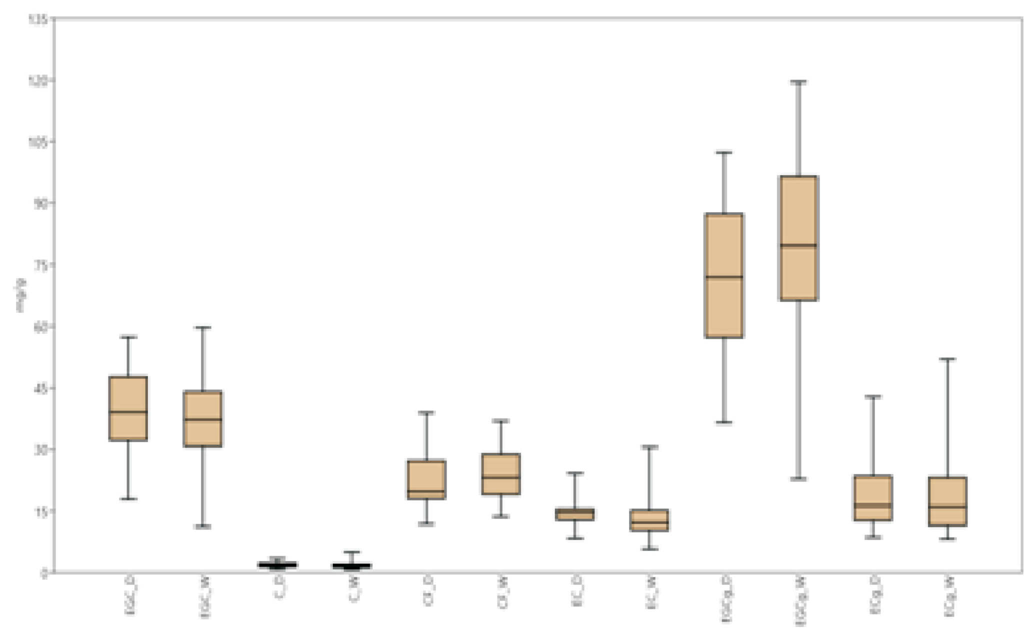

Figure 1. Box plots explaining wet and dry seasons variation of flavan-3-ols and caffeine in tea germplasm accessions. C:catechin; EC: epicatechin, ECg: epicatechingallate, EGC: epigallocatechin, EGCg: epigallocatechingallate, CF: caffeine, W: wet season, D: dry season

Table 1. Summary statistics of selected biochemical constituents in Assam/Cambod types and China type accessions tested in exotic germplasm

	Assam/Cambod types (n=12)					China types (n=46)				
	Min ($mg\ g^{-1}$)	Max ($mg\ g^{-1}$)	Mean* ($mg\ g^{-1}$)	SD	CV%	Min ($mg\ g^{-1}$)	Max ($mg\ g^{-1}$)	Mean* ($mg\ g^{-1}$)	SD	CV%
Wet season										
GA	0.23	0.62	0.38[a]	0.11	29.58	0.16	0.56	0.29[b]	0.11	37.16
EGC	11.14	48.39	31.61[b]	9.81	31.05	14.05	59.74	38.32[a]	9.08	23.71
C	1.31	4.99	2.43[a]	1.08	44.41	0.76	4.04	1.55[b]	0.61	39.62
Caff	14.59	36.86	30.40[a]	6.32	20.78	13.6	35.26	22.41[b]	5.24	23.37
EC	6.62	30.52	17.15[a]	6.03	35.14	5.76	23.6	12.05[b]	3.31	27.47
EGCg	22.76	110.02	80.70[ns]	26.14	32.39	44.03	119.52	80.06[ns]	18.08	22.58
ECg	20.46	41.81	28.95[a]	7.36	25.41	8.11	51.94	15.92[b]	8.06	50.64
TC	86.98	202.76	160.83[ns]	32.70	20.33	89.97	201.82	147.90[ns]	25.97	17.56
Dry season										
GA	0.18	0.85	0.44[a]	0.19	42.03	0.12	0.33	0.19[b]	0.05	25.43
EGC	17.84	42.47	28.46[b]	6.71	23.58	19.24	57.32	42.06[a]	8.98	21.36
C	1.33	3.25	2.32[a]	0.68	29.16	0.87	3.38	1.70[b]	0.52	30.73
Caff	19.35	38.93	32.82[a]	5.88	17.92	11.8	34.81	19.57[b]	4.71	24.05
EC	10.04	24.16	16.45[a]	3.95	24.01	8.27	20.3	13.95[b]	2.39	17.10
EGCg	36.49	102.35	79.48[ns]	21.57	27.14	42.52	92.52	69.55[ns]	14.70	21.13
ECg	21.93	42.8	29.14[a]	6.93	23.80	8.52	30.03	15.54[b]	4.87	31.35
TC	100.96	182.68	155.84[a]	24.27	15.57	98.07	178.47	142.79[b]	17.02	11.92

* Mean values between Assam/Cambod and China types followed by different letters are significantly different at p =0.05 according to t test. SD: standard deviation of population mean; CV%: coefficient of variance of population

dry seasons, respectively. The lowest ECg content was recorded in PBGT63 in wet season whereas PBGT124 in dry season. The range of ECg content recorded in the exotic accessions of the present study was consistent with previous studies conducted with recommended tea varieties of Sri Lanka (Punyasiri *et al*. 2017) and India (Deka *et al*. 2020).

The means of EC content were significantly different between those two major varietal types. The Indian introduction AI-6 recorded the highest values of EC in both wet and dry seasons. Recent studies on Sri Lankan (Punyasiri *et al*. 2017) and Indian (Deka *et al*. 2020) tea cultivars reported similar ranges of EC whereas higher range was (22.51 to 34.95 mg g^{-1}) reported in Kenyan tea accessions (Lubanga *et al*. 2021). Although, mean values of minor components such as gallic acid and (+)catechin were lower, their coefficient of variation (CV %) was higher indicating the high genetic variations among the selected accessions (Table 1). Mean values of sum of all individual flavan-3-ols (total catechins) content in wet season were 160.83 mg g^{-1} and 147.90 mg g^{-1} between Assam/Cambod and China types but the values were not significantly different. However, mean values of total catechins of Assam/Cambod type accessions were significantly higher than China types in dry seasons. Similarly, Deka *et al*. (2020) observed higher mean values of TC in monsoon period irrespective of varieties. Another study reported that the total flavan-3-ol content in 403 Chinese tea cultivars varied from 56.6 to 231.9 mg g^{-1} which is similar to our observation (Jin *et al*. 2014). In India, the higher TC content was recorded in Assam types (mean 231 mg g^{-1}) than China types (mean 157 mg g^{-1}) (Sabhapondit *et al*. 2012). In the present study, Assam introductions and TRI developed varieties which are morphologically grouped in Assam types showed higher TC content.

Caffeine content of Assam types ranged from 14.59 to 36.86 mg g^{-1} (mean 30.40 mg g^{-1}) and from 19.35 to 38.93 mg g^{-1} (mean 32.82 mg g^{-1}) in wet and dry seasons, respectively. Caffeine content of China types ranged from 13.60 to 35.26 mg g^{-1} (mean 22.41 mg g^{-1}) and from 11.80 to 34.81 mg g^{-1} (mean 19.57 mg g^{-1}) in wet and dry seasons, respectively. Irrespective of the seasons, it is observed that the means of caffeine content of Assam/Cambod types was significantly higher than China types. In other tea growing countries, the caffeine content varied between 12 – 59 mg g^{-1} in China (Chen and Zhou 2005), and 19.6 - 43.7 mg g^{-1} in Kenyan (Muthiani et al. 2016) tea germplasm. In addition to low EGCg content, PBGT 35, PBGT48, PBGT53 and PBGT71 recorded lowest content of caffeine in both seasons.

Cluster analysis

A heatmap was generated to identify grouping pattern of exotic germplasm accessions based on the flavan-3-ols and caffeine content in both dry and wet seasons using hierarchical cluster analysis (Figure 2). The heatmap was generated using Euclidean distance and Ward's linkage clustering method. In the hierarchical cluster analysis, accessions studied were grouped into two main clusters (A and B). Further, the cluster A divided into A-I, A-II, and similarly B also divided into B-I and B-II (Figure 2). Out of the 58 accessions, 11 belonged to Assam and Cambod types (clustered as A). Cluster A included six Indian originated accessions (AI-1, AI-2, AI-4, AI-6, AI-9 and SI-1), three TRI developed cultivars (TRI 2043, TRI 3073 and TRI 4079) and two China type exotic accessions PBGT100 (Japan origin) and PBGT118 (Azerbaijan origin). All catechins except EGC, positively contributed for grouping of accessions in cluster A. The accession TRI 2043 was unique due to its origin, high pubescence density, pigmented leaves

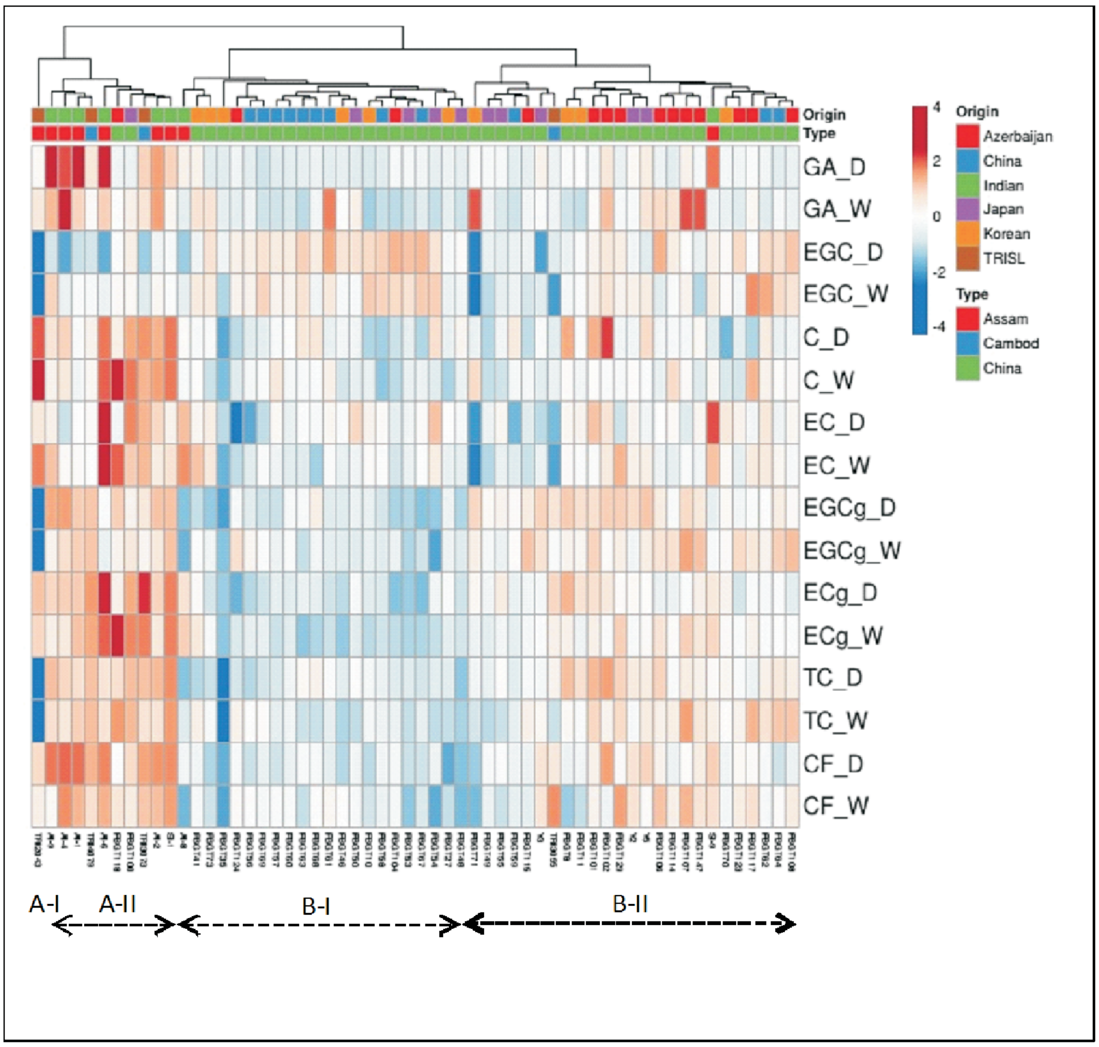

Figure 2. The heatmap generated using Euclidean distance and Ward's linkage clustering method. Red and blue colour patterns indicate positive and negative contributions of individual catechins and caffeine for clustering of 58 tea accessions, respectively. (W: wet season, D: dry season)

and tolerant to blister blight disease. This accession has been separated from other ten accessions in cluster A-II and formed a single sub cluster A-I. EGCg and total catechins of TRI 2043 in both seasons were negatively contributed to separate from main cluster A. In addition to high yielding, TRI 3073 and TRI 4079 were considered as moderate and high quality black tea producing cultivars, respectively. It is worthwhile to determine the black tea quality potential of Indian introductions and other two exotic accessions which are grouped in same cluster. Except two accessions (PBGT100 and PBGT118), all China type accessions were grouped in cluster B. Therefore, Assam/Cambod accessions clustered in cluster A could be distinct from the cluster B. Cluster B consisted of two sub clusters as B-I and B-II. It is interesting to note that majority of Chinese originated accessions suitable for green tea and Oolong tea production were grouped in cluster B-I along with several exotic accessions *viz*. Korea, Japan. One Assam originated accession (AI-8) and two Azerbaijan originated accessions (PBGT104 and PBGT124) also grouped in cluster B-I. EGC contents in both seasons positively contributed for grouping accessions in cluster B-I whereas caffeine, ECg, EGCg and TC contents negatively contributed.

The exotic accessions grouped in this cluster *viz*. PBGT 10, PBGT 27, PBGT 35, PBGT 48, PBGT 53 and PBGT 54 which showed low caffeine content (< 20 mg g^{-1}) in both seasons can be considered as potential genetic resources for development of tea with low caffeine content. On the other hand, cluster B-II represents mixture of accessions having different origins. Majority of Azerbaijan originated accessions (11 accessions) along with each Assam type (SI-9) and Cambod type (TRI 3055) accessions were grouped in the same cluster.

The newly characterized exotic accessions have high potential for producing specialty teas opening new vistas for the Sri Lankan tea industry. Biochemical characterization of tea germplasm using high throughput techniques is very useful in utilization of accessions to improve the existing cultivars. In the present study, a significant influence of genetic variation on individual catechins, caffeine and genetic diversity was observed for all accessions, exotic as well as TRI improved cultivars.

Various studies reported considerable variations in biochemical constituents in tea leaves due to genotypic, seasonal and geographical variations (Wu *et al*. 2012; Kottawa-Arachchi et al. 2013; Punyasiri *et al*. 2017; Deka *et al*. 2020; Lubanga *et al*. 2021). However, this is the first comprehensive study targeting exotic tea germplasm collection in Sri Lanka.

Among individual catechins, ECg showed constant pattern in both seasons indicating less environment influence. Several studies indicated ECg contents in fresh leaves could be a reliable marker for identifying high-quality black tea producing accessions (Yao and Xu, 2005).

Caffeine is well known for its stimulant properties. Caffeine contributes to the creaming properties of black tea. Therefore tea with a low caffeine level is considered to be of inferior quality (Robertson 1992). All Indian introductions and TRI developed varieties showed relatively higher amount of caffeine (~30 mg g^{-1}) than exotic accessions indicating the suitability for black tea production. The threshold level of caffeine toxicity found to be 400mg/day, meanwhile the recent investigations indicate that above 300mg/day caffeine consumption is associated with lower birth weight (Chin *et al*. 2008), cancer risk, male infertility, insomnia, calcium imbalance, and etc. and also adult who are highly susceptible to caffeine intake due to genetic variation in enzyme which associated with caffeine metabolism (Kuribara 2016). Therefore, the demand for decaffeinated or caffeine-less tea is being raised due to its various health benefits. Further, caffeine less tea would be a suitable alternative for caffeine sensitive people to acquire medicinal properties of tea. There are several industrial methods available for caffeine-less tea and several attempts have been made to lower the caffeine content in manufactured tea using genetic engineering (Mohanpuria *et al*. 2011) methods. However, natural breeding of caffeine-less tea cultivar would be a promising and cost effective way to obtain less caffeine tea with existing quality (Ogino *et al*. 2019). Further, selection of less caffeine accessions with other desirable quality attributes from existing genetic stock have been reported in Japan and China (Ogino *et al*. 2009; Wu *et al*. 2012). The study facilitated identification of low caffeine exotic accessions *viz.,* PBGT10, PBGT27, PBGT35, PBGT71, PBGT48, PBGT53 and PBGT54 which could be useful in developing tea cultivars with low caffeine content through breeding.

The study further facilitated identification of exotic accessions with high EGCg content. EGCg exhibits anti-cancer effects through a variety of mechanisms, multi anti-diabetic activities and anti- obesity actions through suppressing fat accumulation in humans (Sayama 2015). Hence, accessions PBGT 118 and AI-8 that are characterized with high EGCg content could be immensely useful in future tea breeding programmes to use as parents to develop tea cultivars with enhanced medicinal properties.

Implications on breeding diverse tea cultivars

Clustering pattern of heatmap revealed that, it is worthwhile to determine the genetic variations and phylogenetic relationship of exotic collections of Azerbaijan origin with high-throughput molecular marker techniques. The exotic accessions which grouped in cluster B-I and B-II can be incorporated into the cultivar development programme so as to cater to the requirements of green tea and oolong tea which are in high demand due to increasing consumption. It also indicated that the accessions in cluster A are more suitable for black tea production whereas accessions in cluster B are appropriated for the development of green teas and speciality teas with health promoting aspect.

A study on genetic structure of Sri Lankan tea germplasm revealed that it is predominantly represented by Cambod type accessions followed by Assam types (Ranatunga *et al*. 2017). Further, availability of less number China type accessions has been identified as a major gap and it was emphasized to acquire more China type accessions and develop China hybrids in order to broaden genetic base of the Sri Lankan tea germplasm. Exotic germplasm collection comprises introduction of seed stocks from many countries which have the potential diversity for widening the genetic base. We report the first comprehensive study on characterization of exotic tea germplasm accessions for their variability in catechin and caffeine content. Interestingly, 46 exotic accessions which are having taxonomic affinity towards China type show discrete levels of caffeine and EGCG two important metabolites in tea. Therefore, these accessions could be immensely useful in future hybridization programmes as potential parents for generating diverse progenies. The progenies so obtained will lay the foundation for developing future tea cultivars with broadened genetic base.

Today, tea has received considerable attention due to the health benefits of the compounds it contains. Currently, tea breeding programme in Sri Lanka is focused on developing cultivars suitable for producing specialty teas ie. low caffeine, high EGCG etc with enhanced medicinal properties. Hence, exotic accessions identified in the study could be utilized in breeding programmes to develop such cultivars. As identified in previous studies, recurrent use of the same parents in tea breeding in Sri Lanka contributed to narrow genetic diversity in the cultivated gene pool (Gunasekare 2012). Hence, exotic collection is a valuable resource for tea breeding programmes as a new source of variation.

Conclusion

Genotypic variations of green leaf flavan-3-ol and caffeine contents were detected in the selected exotic tea accessions of different origins in the field gene bank of Sri Lanka. This study confirmed the seasonal fluctuation of biochemical compounds in tea accessions. Most of the accessions which resemble the Assam types, cluster together with TRI developed cultivars indicating the close ancestral relationships between these two groups. The results indicate that further investigations on black tea quality potential of accessions of Indian origin would be useful for identification of high quality tea cultivars. Several exotic accessions which resemble China types could be useful in identifying tea clones with low caffeine content. Information generated in this study would help to identify diverse exotic accessions as parents for breeding new cultivars with enhanced medicinal properties.

Conflict of interest: The authors declare no conflicts of interest.

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