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Multivariate Analysis of Tea (*Camellia sinensis* (*L.*) *O. Kuntze*) Clones on Biochemical Characters in Southwestern Ethiopia

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Abstract: Information on genetic diversity with respect to biochemical characteristics is very important for improving tea quality. Thirteen introduced tea clones were characterized at Jimma agricultural research center (Melko and Gera research stations) with the aim of identifying the diversity of tea clones based on biochemical traits. The study was conducted in triplicate of RCBD during the 2017/18 crop season. Data on biochemical traits such as total polyphenols, total antioxidants, β-carotene, ascorbic acid, leaf water content, chlorophyll content, and photosynthetic efficiency were recorded. Cluster analysis showed that the tea clones were classified into four groups, indicating the presence of variability among the examined clones. The maximum distance was observed between clusters III and IV (1216) whereas, the minimum distance (373.39) was between clusters II and IV. The results of principal components analysis exhibited that the first three principal components contributed for 77.22% diversity among the tea clones. In general, the study showed existing of difference among tea clones on considered biochemical parameters. Nevertheless, high biochemical variation among clones does not only a assurance for a high genetic difference; hence, molecular research need to be complemented as corresponding to biochemical studies.

Keywords: Biochemical, Inter-Cluster, Diversity, Multivariate, Quality

1. Introduction

Tea is one of the most important and low-cost beverages in the world with more than 3 billion cups consumed daily worldwide [1] second only to water by people of all ages at all levels of society [2]. Tea originates from Southeast Asia [3] and ranges in latitude from 45°N to 34°S, crossing about 52 countries [4]. The Chinese utilized tea first as a medicinal beverage and later as refreshment, and have done so for the past 3,000 years [5]. The Theaceae family consists of 23 genera distributed mainly in tropical and subtropical regions of Southeast Asia and America. The genus Camellia is the only economically important member of the family, which includes about 82 species [6]. The existing tea cultivar taxonomy, represented by three natural hybrids, encompasses more than 325 species of this genus, and more than 600 popular tea genotypes are currently grown worldwide [7]. Many of them have unique traits such as improved tea-making quality, higher yields, and resistance to biotic or abiotic stresses [4].

The presence and amount of polyphenols, amino acids, chlorophyll contents, carotenoids like b-carotene, lutein, violaxanthin, neoxanthin, catechins, caffeine, and volatile compounds [8] are some of the beneficial biochemical markers used for the organoleptic quality of tea [9]. Although tea produced from the Sinensis plant contains numerous phytochemicals, the polyphenol and flavonoid subcategories are considered the most important in terms of health benefits due to their ability to act as antioxidants by donating electrons or hydrogen protons to reactive oxygen or nitrogen species [10]. Tea is rich in polyphenols, which are associated with a number of pharmacological properties,

including anti-diabetic, anti-bacterial [11], anti-cancer, anti-inflammatory, anti-aging [12], anti-oxidant [13, 14] and anti-malarial [15]. Catechins are one of the major polyphenolic compounds found in the tea plant, accounting for up to 30% of the dry weight of freshly picked tea leaves [16]. Catechins have been reported to be potent biomolecules that confer health benefits associated with regular tea consumption. Moisture content, amino acid content, polyphenol content, ascorbic acid content, b-carotene content, catechins content, chlorophyll-a and b, fermentation rate, theaflavin content, thearubigins content, theophyllin content, theogallin content are some of the biochemicals in fresh leaves or fermented black tea [17].

Based on biochemical compounds present in fresh tea leaves such as fermentation rate, crude fiber content, total polyphenols, total catechins, chlorophyll-a, chlorophyll-b, and total carotenoids, 35 tea germplasm from Sri Lanka were biochemically characterized and significant variation in selected biochemical compounds indicated that tea has high genetic diversity [18]. However, many chemical constituents which are responsible for the quality have not been studied in detail for tea clones in Ethiopia. Therefore, it is time to characterize and evaluate the available tea clones in Ethiopia using biochemical methods to further assist in characterizing the germplasm to identify parents and elite germplasm for tea breeding programs.

2. Materials and Methods

2.1. Description of the Study Site

The experiment was conducted in 2017/2018 at Jimma Agricultural Research Centers (JARC) Melko and Gera. Melko is located at 7°46′N and 36°E latitude and longitude at an elevation of 1750 m, with an average temperature of 11.7°C minimum and 25.9°C, maximum over the last 5 years, rainfall of 1511.7 mm, relative humidity of 68.4%, wind speed at 1 m at 2.448 km/h, average monthly soil temperature at 5 cm at 24.9°C, average annual sunshine hours of 73.95 hours. The soil at Melko is a reddish-brown soil (Eutric Nitosol) with a pH of 5.2.

Gera is located at 7°7′N and 36°E latitude and longitude, at an elevation of 1940 m. The average temperature for the past five years was a minimum of 11.1°C and a maximum 23.9°C, precipitation was 1558.9 mm, relative humidity was 71.7%, wind speed at 1m was 1.92 km/hrs, average monthly mean soil temperature at 5 cm was 22.46°C and average annual sunshine hours were 61. 76 hours. The Gera, station was also characterized by red soil and was very fertile with a loam type [19].

2.2. Experimental Materials

Thirteen Assam tea clones collected from various tea gardens (Wushwush, Gumero, Chewaka) and JARC at Melko and Gera tea gardens were used in the experiment (Table 1).

Table 1. *Description of tea clones used for the study.*

Serial no.	country of introduction	tea clones	sources of tea clones
1	Kenya	11/4	Wushwush
2	Kenya	6/8	Wushwush
3	Kenya	FNF	Wushwush
4	Kenya	11/56	Wushwush
5	Kenya	31/11	Chewaka
6	Kenya	S-15/10	Chewaka
7	Kenya	Melko-1	JARC
8	Kenya	Melko-2	JARC
9	India	Chai	Gumero
10	India	BB-35	Gumero
11	India	SR-18	Gumero
12	India	B9	Gumero
13	India	L6	Gumero

2.3. Experimental Design and Management

The experiment was superimposed on a tea plantation that was effectively established with three replications of RCBD at the Gera and Melko research station in 2005. Twelve-year-old tea trees were mid-pruned with shears at 50 cm above ground level in December 2017 as reported by [20] earlier. After these treatments, tea trees were returned to normal plucking or shoot replacement cycles; all biochemical data were recorded in spring (pre-monsoon) season in May.

2.4. Data Collected

2.4.1. Chlorophyll Content

Chlorophyll content was measured from mature third leaves using a chlorophyll content meter (serial number = 001952 USA, Opti-science CCM-200). Four leaves were collected from five plants per plot.

2.4.2. Photosynthetic Efficiency

Photosynthetic efficiency was measured from mature third leaves using a chlorophyll fluorometer (OPTI-SCIENCES; OS-30). Four leaves per plant were collected from five plants in each plot.

2.4.3. Leaf Moisture Content

The moisture content of tea leaves was determined according to Chinese National Standard GB8304-87. Specifically, each sample was heated in a thermostatic oven at 103°C for 4 hours and weighed before and after heating using an electronic balance. The moisture content of the wet base was then estimated using the following equation.

Moisture Content = M1-M2/M1*100 (1)

Where: M1 = mass of sample before drying
M2 = mass of sample after drying

2.4.4. Plucking of Tea

During harvesting for yield and biochemical analysis, the shoots were plucked at the 2/3 internode position between the second and third leaves, counting from the second leaf [17]. Two leaves and a bud were harvested and placed in the water proof bags, immediately transferred to a shaded area and then transported to a ventilated drying room where they were placed in drying containers made of wire mesh and wood. To ensure uniform drying of the tea leaves, close follow-up and turnover was done until the tea leaves were ready for milling. The dried tea leaves were ground and analyzed for each of the following.

2.5. Determination of Total Polyphenol Content

Ten grams of crushed tea leaves were mixed with 100 ml of methanol, homogenized in a homogenizer (PLTYRON®2500E, Switzerland) for 1 minute, and held in a 20°C water bath for 60 minutes. The samples were then centrifuged at 2500 rpm for 15 min and the supernatant was stored for analysis (first extraction). Residuals were re-extracted under the same conditions and the supernatant was collected for analysis (second extraction). Both supernatants (first and second extractions) were mixed, the combined methanol extracts were evaporated and dried in an oven at 40°C, and the extracted samples were stored at 4°C. After the samples were extracted, the total polyphenol content was determined through the reduction of the folin-ciocalteau reagent by phenolic compounds and the subsequent formation of a blue complex. To 10 ml of the extract, 2 ml of 2 N folin-ciocalteau reagent was added. Instantly, 2 ml of 7.5% sodium carbonate solution was added. The mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 765 nm using a UV-spectrophotometer T80 China. Gallic acid was used as a standard substance and the measured values were compared to a standard curve prepared with gallic acid solution. Total polyphenol contents articulated as mg of gallic acid equivalents per gram of sample (mg GAE/g sample). All samples were prepared in triplicate for each analysis and the average absorbance at 765 nm was determined. To prepare the calibration curve, 0.5 g of gallic acid was precisely weighed and placed in a 10 ml measuring flask, dissolved in 10 ml of absolute methanol, and the gallic acid stock solution prepared to 100 ml with 80% of the same solvent. From this stock solution 0, 2, 3, 4, 5, 6, 7 ml were added to a 100 ml flask and diluted to obtain 0, 15, 30, 60, 90, 120, 150 mg/l of gallic acid in methanol. Then 0.5 ml of each sample was placed in a test tube and mixed with 2 N Folin-ciocalteau reagent and 2 ml of 7.5% sodium carbonate. The test tubes were covered with aluminum foil and allowed to stand at room temperature for 30 minutes, after which the absorbance at 765 nm was measured using a UV spectrophotometer (T80 China).

2.6. Determination of Ascorbic Acid (Vitamin C)

Vitamin C was measured as described [21]. A 1% starch indicator solution was prepared in a beaker by adding 0.5 g soluble starch to 50 ml distilled water. The mixture was mixed well and cooled before use. Iodine solution was prepared by dissolving 5 g potassium iodide and 0.268 g potassium iodate in 200 ml distilled water in a beaker. To this solution, 30 ml of 3M sulfuric acid was added and diluted to a final volume of 500 ml with distilled water. Finally, the solution was labeled as iodine solution. The vitamin C standard solution was prepared by dissolving 0.250 g ascorbic acid in 100 ml distilled water in a beaker and diluted to 250 ml with distilled water in a female flask. Finally, the flask was labeled with the vitamin C standard solution. The vitamin C of the sample was determined by titrating 10 ml of the extract with iodine solution until the endpoint was reached. Ten milliliters of sample was taken in a measuring flask, 10 drops of 1% starch solution was added and shaken well until the endpoint was reached. The endpoint of the titration is the first permanent trace of the dark blue-black color due to the starch/iodine complex after the solution was shaken for 20 seconds. The amount of iodine solution used in the titration was recorded by subtracting the starting volume from the final volume. The titration was repeated with further extractions of the sample solution until the results agreed at 0.1 ml, and the average value was used for the final calculation. The vitamin C standard solution was titrated against the iodine solution using the same procedure as for the sample titration, and the volume of iodine solution used in the titration was recorded by subtracting the starting volume from the final volume. The milliliters of titrant used for each flask were calculated by averaging the obtained measurements (average volume = total volume/number of trials). The volume of titrant required for the standard solution was calculated and recorded. Finally, the amount of ascorbic acid in the tea sample (mg /100 g) was estimated from the volume of titrant required for the standard solution.

Ascorbic acid =Ascorbic acid in sample (mg) x volume made up (ml) x 100 (2)

2.7. Determination of Beta-Carotene

The extraction and determination of total β-carotene content followed the method described in [22]. Briefly, 1 g of sample powder was mixed with 1 g of $CaCl_2.2H_2O$ and 50 ml of extraction solvent (50% hexane, 25% acetone, 25% ethanol) and shaken gently for 30 minutes. After adding 15 ml of distilled water, the solution was shaken frequently for another 15 minutes. The organic phase containing β-carotene was separated from the aqueous phase using a separating funnel and filtered through whatman filter paper No. 1. To avoid degradation of the carotenoids, extraction was performed under reduced light and the extracted samples were stored for analysis. After the samples were extracted, absorbance was measured at 450 nm using a UV spectrophotometer T80 China and compared to a β-carotene standard. A β-carotene standard

(Sigma Aldrich) was used as a standard and compared to the standard solution. To prepare the calibration curve, 0.01 g of β-carotene standard was accurately weighed and dissolved in 20 ml of a solvent similar to the extraction solvent used for sample extraction (50% hexane, 25% acetone, 25% ethanol) and the β-carotene standard stock solution was prepared to 100 ml with the same solvent. From this stock solution, 0, 2, 3, 4, and 5 ml were added to a 100 ml flask and diluted with the same solvent to yield 0, 0.1, 0.2, 0.4, and 0.8 mg/l of β-carotene standard solution. Then, 0.5 ml of each sample was placed in a test tube, covered with aluminum foil, and the absorbance at 450 nm was measured using a UV spectrophotometer (T80 China). All samples were prepared in triplicate for each analysis and the average absorbance at 450 nm was determined.

2.8. Determination of Total Anti-Oxidant

Samples were extracted according to the method described [23]. Ten grams of crushed tea leaves were mixed with 100 ml of methanol, homogenized in a homogenizer (PLTYRON®2500E, Switzerland) for 1 minute and kept in a water bath at 20°C for 60 minutes. The samples were then centrifuged at 2500 rpm for 15 min and the supernatant was saved for analysis (first extraction). The residue was re-extracted under the same conditions and the supernatant was collected for analysis (second extraction). Both supernatants (first and second extractions) were mixed and the methanol extract was evaporated to dryness in an oven at 40°C. After the samples were extracted, the total antioxidant content was determined according to the methods [23], including DPPH assay (2, 2-diphenyl-1-picrylhydrazyl) or free radical scavenging assay. The percentage of free radical scavenging activity was calculated from the absorbance of the solvent extract, methanol, and standard solution (Equation 3). For IC50 values, a solvent extract of the sample was taken in each test tube at a concentration range of 200, 400, 600, 800, and 1000 µl, prepared to 1 ml with solvent, and 2 ml of 0.1 mM DPPH was added. The mixture was shaken well and incubated at room temperature and in the dark for 30 minutes. The decrease in absorbance of the resulting solution was measured at 517 nm with a UV spectrophotometer T80 China. All experiments were performed in triplicate and averaged.

$$\text{Radical scavenging activity (\%)} = \frac{Ac - At/As}{Ac} \times 100 \qquad (3)$$

Where: Ac-Absorbance of control

At- Absorbance of test solution

As- Absorbance of standard Solution

The IC50 value was described as the amount of sample that scavenges 50% of DPPH radicals, calculated from the percentage of radical scavenging activity by plotting DPPH free radical scavenging activity versus sample concentration.

2.9. Statistical Analysis

2.9.1. Cluster Analysis

In this study, biochemical characteristics were used to classify clones into homogeneous groups. The data were subjected to cluster analysis to determine the variation among the clones; hierarchical clustering using similarity coefficients among the 13 tea clones was employed. Clustering was performed by employing the method of mean chain clustering strategy of observation using the proc cluster procedure [24] in SAS version 9.3. The number of clusters was determined by examining three statistics: pseudo F, pseudo t2, and cubic clustering criteria.

2.9.2. Divergence Analysis Between Clusters

Genetic divergence between clusters was determined using the generalized Mahalanobis's D^2 statistic [25] with the formula:

$$D^2p = (Xi — Xj)\ S^{-1}(Xi — Xj)$$

Where:

D^2p= the distance between any two groups i and j; Xi and Xj = the p mean vectors of clones i and j, respectively, S^{-1} = the inverse of the pooled covariance matrix.

The D^2 values obtained for the pairs of clusters were tested at 5% and 1% levels of significance against the values in the table for p degrees of freedom, while p is the number of variables considered [26].

2.9.3. Principal Component Analysis

Principal component analysis was performed using SAS version 9.3 [24] with a correlation matrix. The goal of this analysis was to reduce the observed variables to a small number of principal components that accounted for the majority of the variance of the observed variables. A high alpha value for a particular component is interpreted as an indication that the component has a strong one-dimensional structure or that the dimension can reliably explain the total variance. In general, alpha values above 0.70 are considered reliable.

3. Results and Discussion

3.1. Cluster Analysis for Biochemical Traits

The 13 clones were classified into four clusters according to the D^2 value based on the mean of the tea clones (Table 2). Cluster I was the most abundant with six clones (46.16%), followed by cluster II with five clones (38.46%), cluster III with one clones (7.79%) and cluster IV one clone (7.79%) accounting for the total diversity in the population.

Cluster I consisted of tea clones collected from four locations: three tea gardens (Chewaka, Gummaro and Wushwush) and one from research station (JARC). This includes 31/11 from Chewaka, SR-18 and Chai from Gumero, FNF and 11/56 from Wushwush and Melko-1 from JARC. Cluster II holds five tea clones from three tea farms and one from research station, thus S-15/10 from Chewaka, BB-35 and L6 from Gumero, 6/8 from Wushwush and Melko-2 from JARC. Cluster III is 11/4 clone from Wushwush plantation and cluster IV is only B9 clone from Gumero plantation; different clones from three different plantations

and one research center were classified into different clusters, indicating the existence of genetic diversity within the plantations. This study was partially in agreement with [18], who reported high genetic diversity in Sri Lankan tea germplasm based on chlorophyll content, carotene, and total polyphenols.

Tea clone 11/4, classified alone in the third cluster, had the highest chlorophyll content and fresh leaf moisture content, the longest height to the first branch, the most mature petiole, the lowest hundred bud weight, and the second highest number of buds per branch. Tea clone B9 was distinguished by the lowest chlorophyll content and photosynthetic efficiency.

Table 2. *Distribution of tea clones into four clusters based on D^2 analysis.*

Cluster number	No. of clones	Percent (%)	Clones
1	6	46.15	Chai, FNF, 31/11, 11/56, Mlk1 and SR-18
2	5	38.46	BB-35, 6/8, L6, Mlk2 and S-15/10
3	1	7.69	11/4
4	1	7.69	B9

Cluster Mean Analysis of Biochemical Traits

Cluster I had the highest photosynthetic efficiency and moderate scores for traits such as chlorophyll content, total polyphenols, total antioxidants, and β-carotene, with ascorbic acid being the lowest. Cluster II was distinguished by the highest total polyphenols and ascorbic acid. However, tea clones belonging to this cluster showed moderate values in chlorophyll content and photosynthetic efficiency (Table 3). Cluster III was characterized by the highest fresh leaf moisture and chlorophyll content, but showed moderate values for total polyphenols, total antioxidants, β-carotene, ascorbic acid and photosynthetic efficiency. Finally, cluster IV showed the highest values for total antioxidants and β-carotene. This cluster also had moderate values for fresh leaf water content and ascorbic acid.

In general, the lowest values for each cluster were as follows. Cluster I was ascorbic acid, and Cluster II was raw leaf moisture content, antioxidants, and β-carotene. Cluster III, however, showed mostly high and medium values for the traits considered. Finally, cluster IV was distinguished by the lowest values for chlorophyll content, total polyphenols, and photosynthetic efficiency.

Table 3. *Cluster mean and mean difference for seven biochemical traits.*

Variables	Cluster mean				Cluster mean difference			
	I	II	III	IV	I	II	III	IV
CC	26.35	19.736	32.63**	11.09*	3.23	-3.38	9.51	-12.03
FLMC	56.05	55.31*	57.78**	56.9	0.09	-0.65	1.82	0.94
TPP	27.29	27.58**	27.33	25.93*	-0.01	0.28	0.03	-1.37
TAO	23.66	23.61*	25.85	26.51**	-0.37	-0.42	1.82	0.25
BC	8.73	8.44*	8.95	9.47**	0.04	-0.25	0.26	0.78
AA	0.64*	0.68**	0.65	0.65	-0.01	0.002	0.04	2.48
PE	0.43**	0.41	0.42	0.39*	0.005	-0.01	0	-0.03

**,*=highest and lowest cluster mean, AA=ascorbic acid, BC=beta-carotene, CC=chlorophyll content, FLMC=fresh leaf moisture content, PE=photosynthesis efficiency, TAO=total anti-oxidant and TPP=total polyphenol

3.2. Genetic Differences Among Tea Clones Based on Biochemical Traits

The maximum inter-cluster distance was between clusters III and IV (1216), followed by the maximum between clusters IV and I (853.05). The smallest inter-cluster distance was between clusters I and III (49.61), followed by between II and I (104.01), between II and III (274.94) and between II and IV (373.39) (Table 4). This result indicates that the tea clones included in this study are moderately divergent. Crosses containing genotypes belonging to the most divergent clusters can be used in hybridization programs to obtain a broad spectrum of variation and good expression of heterosis and broad variability [27]. Therefore, crosses between clones in these clusters may give a high heterotic response and thereby better isolates, considering the genetic diversity.

Table 4. *Average inter-cluster divergence values obtained for biochemical traits.*

From class	Squared Distance to cluster		
	II	III	IV
I	104.01**	49.61**	853.05**
II		274.94**	373.39**
III			1216**

* =significant at 12.59 and above
**=highly significant at 16.81 and above

3.3. Principal Component Analysis for Biochemical Traits

The principal component analysis of biochemical traits showed high contributions from total polyphenols (0.91), chlorophyll content (0.89), fresh leaf moisture content (0.82), ascorbic acid (0.74), total antioxidants (-0.72) and beta-carotene (0.71). In general, the first three principal

components contributed to 77.22% diversity among tea clones (Table 5). The first principal component accounted for 39.29% of the variability among tea clones and was attributed to discriminating traits such as total polyphenols, total antioxidants, ascorbic acid, and photosynthetic efficiency. On the other hand, the second principal component accounted for 20.54% of the variability among the test tea clones, which was attributed to the variability in raw leaf moisture content and β-carotene. The third principal component explained 17.39% of the total variability among tea clones and was due to variation in chlorophyll content. In general, the variation in the first and second principal components that resulted in the greatest variation (59.83%) was mainly due to the combined effects of total polyphenols, total antioxidants, raw leaf moisture content, β-carotene, ascorbic acid, and photosynthetic efficiency. Therefore, these traits play a major role in classifying tea clones into different groups and should be considered when selecting diverse parents for use in breeding and crossbreeding programs. The first four principal traits accounted for 78% of the total variation in the 20 Sri Lankan tea clones, which is in agreement with the study conducted [27].

Table 5. *Eigenvector and eigenvalues of the first three principal components for biochemical traits.*

Principal component			
Variable	**I**	**II**	**III**
CC	0.333	-0.004	0.893
FLMC	0.342	0.824	0.166
TPP	0.910	-0.083	-0.286
TAO	-0.720	0.186	0.103
BC	0.474	0.708	-0.081
AA	0.740	-0.182	-0.339
PE	0.636	-0.427	0.423
Eigen value	2.750	1.438	1.217
Percent	39.29	20.54	17.39
Difference	1.31	0.22	0.56

AA=ascorbic acid, BC=beta-carotene, CC=chlorophyll content, FLMC=fresh leaf moisture content, PE=photosynthesis efficiency, TAO=total anti-oxidant and TPP=total polyphenol.

4. Conclusion

This study showed that there is considerable genetic diversity among tea clones for several biochemical traits. The presence of genetic diversity is a potential resource for improving tea crops through selection and hybridization. Therefore, the diversity observed for biochemical traits must be exploited to improve the quality of this valuable crop. On the other hand, quantification of other biochemical compound groups such as carbohydrates, lipids, amino acids, caffeine, and theabromine is essential to discover genetic diversity and to select parents for future tea breeding programs based on the biochemical profile of tea clones. Furthermore, the diversity observed in this study should be confirmed using molecular markers such as SSRs and SNPs.

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