

Effects of Different Fertilizer Rates on Total Polyphenols and Catechins of Selected Clones of Green Tea (*Camellia sinensis* L. [O] Kuntze)

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Abstract: Application of inorganic NPK (nitrogen: phosphorus: potassium) fertilizers on tea (*Camellia sinensis*) has been common among small-scale tea farmers to increase production. However, the cost of fertilizers has been increasing and reducing their net returns. Fertilizers applications also affect the quality of the crop which is mainly dictated by the total polyphenols and catechins; these metabolites are responsible for its anti-oxidant activity, taste and aroma. The focus of this work was on the effect of varying NPK fertilizer application rate on the levels of total polyphenols and catechins of green tea made from three tea cultivars and development of an optimum NPK fertilizer application rate. Samples of three varieties, AHP S15/10, TRFK 6/8 (Tea Research Foundation of Kenya), and BBK 35, were collected from Mettarora, Arroket and Monire tea estates within Sotik highlands. Randomized complete block design sampling was used and sampling was done 6-8 weeks after application of the treatment. The fertilizer used was inorganic plain NPK fertilizer and NPK fertilizer supplemented with Molybdenum. The experimental treatments consisted of six treatments made up NPK 25:5:5; at 0, 75, 150, 225, 300, and 375 kg N/ha/year. The collected samples were processed as non-aerated green CTC (Cut, Tear and Curl) teas at the miniature processing factory unit at Kenya Agricultural and Livestock Research Organisation - Tea Research Institute (KALRO-TRI) at Kericho County. Processed tea samples were milled and chemical analysis done to quantify the levels of biochemicals in the samples. Data obtained were subjected to Analysis of Variance (ANOVA) and mean separation was done using the least significant difference test at $p \leq 0.05$. TRFK 6/8 was found to have the highest mean percentage total polyphenols of 25.90 followed by BBK 35 with a mean percentage total polyphenols of 25.75. AHP S15/10 recorded the least mean percentage total polyphenols of 23.70. A similar trend was observed in the mean percentages of catechins with TRFK 6/8, BBK 35 and AHP S 15/10 recording 20.7%, 18.87% 18.46% respectively. Hence, use of optimum NPK fertilizer application rate specific to each clone supplemented with Molybdenum boosts the levels of polyphenols and catechins in green tea.

Keywords: Cultivars, Polyphenols, Catechins, Fertilizers, Anti-oxidant Activity

1. Introduction

Application of fertilizers is an important part of the normal intensive production of tea [1]. Optimum fertilizer application in association with other appropriate cultural practises produces high yields [2]. As increasing levels of fertilizer application leads to increased yield, concerns arise as to whether the quality of tea is affected. The effect produced can be measured either by

chemical analysis for the flavouring compounds or subjectively by Taster's report [3]. Quantification of bio-chemicals in this report is done through laboratory analyses [3]. The aim of this study, therefore, was to investigate fertilizer uptake in different varieties of tea in Kenya and determine its effects on tea quality parameters variations.

Studies have shown that, after a period of time, there is a reduction of nutrients in the soil under tea plantation either

through utilization by tea plants or through leaching during a rainy season [2]. Loss of nutrients in the soil leads to the reduction in production and also affects quality tea parameters such as total polyphenols levels [4, 5, 6]. Application of inorganic fertilizers has remained to be the only way to enhance the production of tea leaves and also the quality parameters, e.g. Catechins [7]. From previous studies, it has been established that application of fertilizers increases the nutrients in the soil which are in turn used by tea plants [8]. However, with the rising cost of inorganic fertilizer, there is need to determine an optimum fertilizer application rate to reduce the cost of production and at the same time sustain the quality. This project involved analysis of total polyphenols and Catechins from green tea applied with different rates of NPK fertilizers.

The quality of tea, as a drink, is largely determined by the presence or absence of chemical compounds, which are responsible for colour, briskness, brightness, strength and flavour in the infusion [9]. Majority of the chemicals imparting quality are produced during processing of the tea leaves. Biogenesis of such precursors is influenced, on one hand by the genetic and environmental factors and on the other by the cultural practices adopted in the field as well as by the conditions of processing, which can be controlled though biochemical changes that start immediately after plucking. Polyphenols work out to about 15-20% in the black tea, and it comprises the catechins fractions [10]. Compared to black tea, green tea has a higher content of polyphenols, almost around 25-30% on the dry weight basis. All the above constituents may vary in their content according to the type of tea plants, environment, agronomic practices, pest & disease incidence and manufacturing practices [11]. Depending on the type of tea, which is basically due to different ways of processing, tea product may contain different chemicals with varied concentrations [12].

Flavanol, flavonols, glycosides, polyphenolic acids and depsides put together are referred to as total polyphenols and make up about 30% of the dry weight in a tea shoot. Flavanols or Catechins are the major compounds that are oxidizable in the tea leaf. (-) Epigallocatechin (EGC) and (-) epigallocatechin gallate (EGCG) are the predominant catechins present in tea leaf. There are four types of Catechins; Epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate (Figure 1) [13]. The Catechins play a significant role during oxidation ([14]. Tea polyphenols include phenolic acids and flavonoids, which may account for up to 30% of the dry weight in fresh green leaves. Flavonols are phenol derivatives synthesized in substantial amounts and widely distributed among plants. Other bio-chemicals such as the arubigins and the aflavins are present in black tea [15].

Young tea shoots are extremely rich in many bio-chemicals compared to other parts of the tea plant [16]. The levels of those bio-chemicals vary depending on a number of factors such as the type of clone, season of plucking, compositing of soil, fertilizer rates applied and method of processing among other factors.

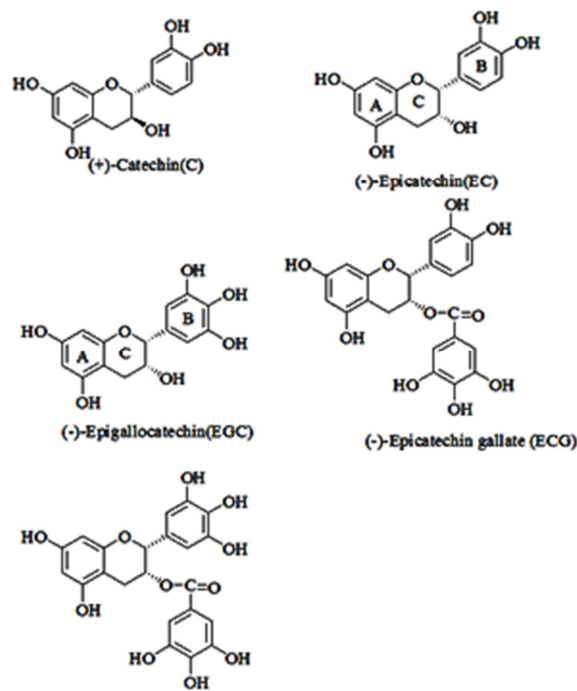


Figure 1. Types of Catechins.

EGCG has been found to be in high concentration in green tea. It is the most active and most researched of tea ingredients. The beneficial health effect is that, it is effective in neutralizing free radicals than vitamin C and twenty five (25) times more powerful than vitamin E [17].

2. Materials and Methods

2.1. Sample Collection

The samples were collected from three different areas within Sotik highlands; Mettarora, Arroket and Monieri and the varieties collected included; AHP S15/10, TRFK 6/8, and BBK 35 respectively. These samples were plucked from different fields of different spacing and sizes. Arroket samples were plucked from field number 13, of size 39 (13' x 3') plants per plot excluding guard rows, with a spacing of 1.22m x 0.76m (4' x 2.5') 10766 plants/ ha. Monieri samples were plucked from field number 38, of size 45 (15' x 3') plants per plot excluding guard rows, with a spacing of 1.22m x 0.76m (4' x 2.5') 10766 plants/ ha. Mettarora samples were plucked from field number 26, of size 45 (15' x 3') plants per plot excluding guard rows, with a spacing of 1.22m x 0.76m (4' x 2.5') 10766 plants/ ha. The experimental treatments consisting of six treatments made up as follows: NPK 25:5:5; at 0, 75, 150, 225, 300, and 375 kg N/ha/year. Sampling was done 6-8 weeks after application of the treatment. The samples were processed as non-aerated green CTC teas at the miniature processing factory unit at KALRO-TRI, Kericho.

2.2. Preparation of Non-aerated Green Tea

After 6 weeks of application of NPK fertilizers, two leaves

and a bud, the crop shoots normally harvested for manufacturing [18], were plucked and put in a polyethylene bag to weigh approximately 50 grams and labelled. The collected tea leaves were steamed at 90°C for 1 minute. This was to kill enzyme polyphenol oxidase which oxidises polyphenols and catechins to thearubigins and theaflavins. The tea sample was then crushed in a CTC machine and dried in a fluid bed drier for 30 minutes to obtain non-aerated green tea.

2.3. Colorimetric Analysis of Total Polyphenols

2.3.1. Extraction of Polyphenols

Extraction and analysis of Total Polyphenols (TP) was done according to ISO procedures [3]. Polyphenols were extracted with 70% methanol from a tea portion of finely ground leaf tea at 70°C. The polyphenols in the extract were determined calorimetrically using Folin-Ciocalteu phenol reagent. This reagent contained phosphor-tungstic acids as oxidants, which on reduction by readily oxidized phenolic hydroxyl groups, yielded a blue colour with a broad maximum absorption at 765nm. This was due to the formation of the so-called tungsten and molybdenum blues. The Folin-ciocalteu phenol reagent reacted with a wide range of polyphenol compounds and although the response could vary with individual components, a selection of Gallic acid as a calibration standard enabled useful total polyphenol data to be obtained.

2.3.2. Analysis of Total Polyphenols

Five standards labelled A, B, C, D and E, corresponding to approximately 10µg, 20µg, 30µg, 40µg, 50µg of anhydrous Gallic acid respectively were used. 0.2 ± 0.01g of the test samples were weighed into extraction tubes. 70% methanol/water extraction mixture contained in a dispenser was placed in the water bath set at 70°C and allowed at least 30 minutes for extraction mixture to equilibrate. The extraction tubes containing the samples were then placed in the water-bath set at 70°C. 5.0 mL of the hot methanol/water extraction mixture was dispensed into the extraction tubes, corked and mixed using a vortex mixer. The extraction tubes were heated in the water bath for 10 minutes and mixed on the vortex mixer after the 5th and the 10th minute. The extraction tubes were removed from the water bath and allowed to cool to room temperature. Centrifugation of the tubes followed at 3500 rpm for 10 minutes. The supernatant was carefully decanted into a graduated tube. Extraction was repeated, and the two extracts combined to make up to 10ml with methanol/water extraction mixture. 1ml of the sample extract was transferred into a one-mark 100mL volumetric flask, and filled to the mark using distilled water. 1mL of diluted sample extract was pipetted into a test tube, and then 5.0mL of dilute Folin-Ciocalteu phenol reagents added and mixed. Within 3-8 minutes after the addition of Folin-Ciocalteu phenol reagent, 4.0 mL of sodium carbonate solution was pipetted into each test tube and mixed. The mixture was allowed to stand for 60 min at room temperature and absorbance measured in 10mm path length cell against water on the spectrophotometer set at a wavelength of

765nm. The total polyphenol content expressed as percentage by mass of the sample dry matter basis was calculated using the formula:-

$$WT = \frac{(D \text{ sample} - D \text{ intercept}) \times V \text{ sample} \times d \times 100}{Sstd \times Msample \times 1000 \times WDM, sample}$$

Where;

WT -Percentage Total Polyphenols

D sample -optical density of the sample test solution

D intercept-optical density at the point of the best fit linear calibration

M sample -mass (g) of the sample test portion

V sample -sample extraction volume in millilitres (10ml of tea leaf extract)

D -dilution factor used prior to the calorimetric determination

WDM sample -is the dry matter

2.4. HPLC Analysis of Catechins

2.4.1. Extraction of Catechins

Extraction of Catechins was done according to the ISO procedures [3]. 2005.0.2 ± 0.01g of the test samples were weighed into extraction tubes. 70% methanol/water (v/v) extraction mixture contained in a dispenser was placed in the water bath set at 70°C and allowed at least 30 minutes for extraction mixture to equilibrate. The extraction tubes containing the samples were then placed in the water-bath set at 70°C. 5.0 mL of the hot methanol/water extraction mixture was dispensed into the extraction tubes, corked and mixed using a vortex mixer. The extraction tubes were heated in the water bath for 10 minutes and mixed on the vortex mixer after the 5th and the 10th minute. The extraction tubes were removed from the water bath and allowed to cool to room temperature (24°C). Centrifugation of the tubes followed at 3500 rpm for 10 minutes. The supernatant was carefully decanted into a graduated tube. Extraction was repeated and the two extracts combined to make up to 10ml with methanol/water extraction mixture.

2.4.2. Analysis of Catechins

High performance liquid chromatography (HPLC) analysis was done according to the procedure by ISO14502-2:2005[3]. 1mL of sample was pipetted into a test tube and diluted to 5mL with a stabilizing solution (10% (v/v) acetonitrile with 500µg/mL of EDTA and 500µg/mL ascorbic acid), filtered and loaded into 2mL vials. HPLC fitted with an SPD-20 UV-Visible detector and C₆, 25cm x 4.6 i.e. column was used for determination at 278nm. The solvent systems for gradient elution included: Mobile phase A (9:2:89 (v/v/v) Acetonitrile: Acetic acid: EDTA) and mobile phase B (80:2:18 (v/v/v) Acetonitrile: acetic acid: EDTA) at a flow rate of 1mL/min. The column temperature was set at 35 ± 0.5°C and the injection volume of 20µL used. The conditions for the binary gradient was set at 100% solvent A for 10 min then over 15 min a linear gradient to 68% mobile phase A, 32% mobile phase B and held at this composition for 10 min. The conditions were again reset to 100% mobile phase A and

allowed to equilibrate for 10 min before the next injection. Catechin identification was done by comparing the retention times of samples and standards under similar conditions. The standards used were Catechin (+) C, Epigallocatechin (-) EGC, Epicatechin (-) EC, Epigallocatechin gallate (-) EGCG and Epicatechin gallate (-) ECG. Total Catechins (TC) were expressed as a percentage by mass on a sample dry matter basis and given as a summation of individual Catechins as;

$$\% \text{ Total Catechin} = [(\% \text{EGC}) + (\% \text{C}) + (\% \text{EC}) + (\% \text{EGCG}) + (\% \text{ECG})]$$

Where;

ECG -Epicatechin gallate

EC -Epicatechin

EGCG -Epigallocatechin gallate

C- Catechin

2.5. Statistical Analysis

Data obtained were subjected to one-way Analysis of Variance (ANOVA) using the SAS 9.1 statistical software. The Significant Difference (LSD) test was used for mean separation where statistically significant differences were observed. Data were presented as a mean of the triplicate determinations \pm standard deviation (SD).

3. Results and Discussion

3.1. Total Polyphenols

As outlined in the procedure, polyphenols content of fresh tea leaves were determined during different supply of plain and molybdenum supplemented NPK fertilizers. From the results, each clone responded differently to fertilizer rates. On plain NPK fertilizers application, variety BBK 35 had higher percentage total polyphenol levels at an application rate of 300 Kg NPK/HA (figure 2). The reason could be that the optimum NPK fertilizer application rate specific to clone BBK 35 was 300Kg NPK/HA. At this rate BBK 35 was able to effectively utilize nutrients in the fertilizers applied which translated to the high levels of total polyphenols.

Table 1. Total polyphenols processed from clone BBK 35 grown in Monieri, Sotik Estate.

Fertilizer Rates (NPK/HA) (kg)	Total Polyphenols (%)
0	25.69 ^{bdec} \pm 0.32
75	24.01 ^f \pm 0.82
150	24.45 ^{fc} \pm 0.38
225	24.77 ^{fd} \pm 0.15
300	26.14 ^{bdac} \pm 1.43
375	25.52 ^{dec} \pm 0.25
7M+0	27.00 ^{ba} \pm 1.17
8M+75	27.15 ^a \pm 0.99
9M+150	26.20 ^{bdac} \pm 1.16
10M+225	26.38 ^{bac} \pm 0.13
11M+300	27.10 ^{ba} \pm 0.97
12M+375	26.36 ^{bac} \pm 0.99
Mean	25.75
LSD	1.46
CV	3.33

AHP S15/10 on the other hand was able to utilize the nutrients in fertilizers applied which lead to a higher percentage total polyphenols at an 75Kg NPK/HA and TRFK 6/8 had a higher percentage total polyphenols levels at an application rate of 375kg NPK/HA (table 2). Again, each clone had a different application rate which yields high levels of polyphenolic compounds.

On fertilizer supplemented with Molybdenum, all the three clones BBK 35, AHP S15/10 and TRFK 6/8 had one fertilizer application rate of 8M+ 75Kg NPK/HA (figure 3). Comparing those applied with plain NPK fertilizer (figure 2) and those supplemented with Molybdenum, those applied with NPK fertilizer supplemented with Molybdenum had a higher percentage polyphenols levels. Plants also use molybdenum to convert inorganic phosphorus into organic forms [19]. Molybdenum could have assisted in the conversion of Nitrogen, Phosphorus and Potassium nutrients to a form which could be easily utilized by the tea plants. TRFK 6/8 and AHP S15/10 were the most sensitive to the treatments with the highest CV of 4.70% and 4.43% respectively but BBK 35 was least sensitive response to treatment rates.

Table 2. Total polyphenols processed from clone AHP S15/10 grown in Mettarora, Sotik Estate.

Fertilizer Rates (Npk/Ha) (kg)	Total Polyphenols (%)
0	23.73 ^{bac} \pm 1.50
75	24.15 ^{ba c} \pm 1.62
150	23.65 ^{bc} \pm 0.78
225	22.57 ^{dc} \pm 1.15
300	24.01 ^{bac} \pm 0.86
375	23.99 ^{bac} \pm 0.84
7M+0	23.69 ^{bc} \pm 1.01
8M+75	25.48 ^a \pm 0.36
9M+150	21.63 ^d \pm 1.37
10M+225	24.81 ^{ba} \pm 0.64
11M+300	22.64 ^{dc} \pm 0.95
12M+375	24.11 ^{bac} \pm 0.74
Mean	23.70
LSD	1.78
CV	4.43

Table 3. Total polyphenols processed from clone TRFK 6/8 grown in Arroket, Sotik Estate.

Fertilizer Rates (NPK/HA)	Total Polyphenols (%)
0 kg	25.57 ^a \pm 0.54
75 kg	25.56 ^a \pm 0.71
150 kg	26.23 ^a \pm 1.09
225 kg	24.76 ^a \pm 0.57
300 kg	26.31 ^a \pm 1.18
375 kg	26.67 ^a \pm 0.46
7M+0 kg	25.98 ^a \pm 1.07
8M+75 kg	26.22 ^a \pm 0.86
9M+150 kg	26.07 ^a \pm 2.05
10M+225 kg	25.26 ^a \pm 1.94
11M+300 kg	25.99 ^a \pm 0.13
12M+375 kg	24.87 ^a \pm 1.63
Mean	25.90
LSD	2.05
CV	4.70

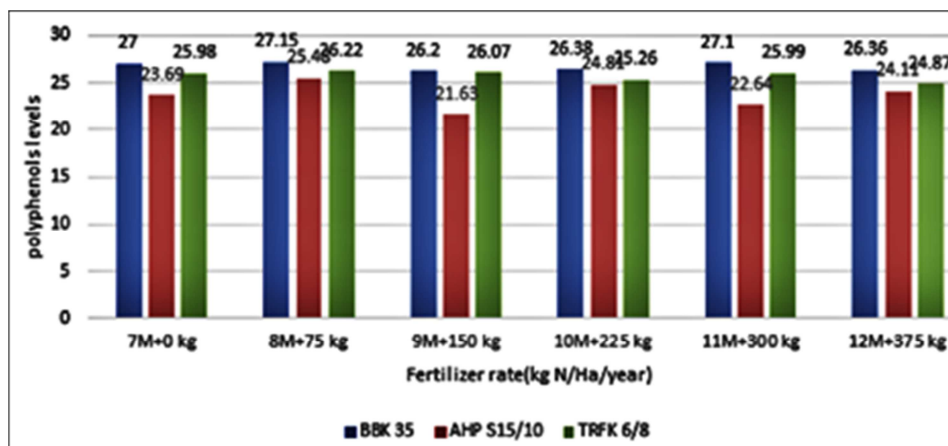


Figure 2. Effect of molybdenum supplemented NBK fertilizer on total polyphenols.

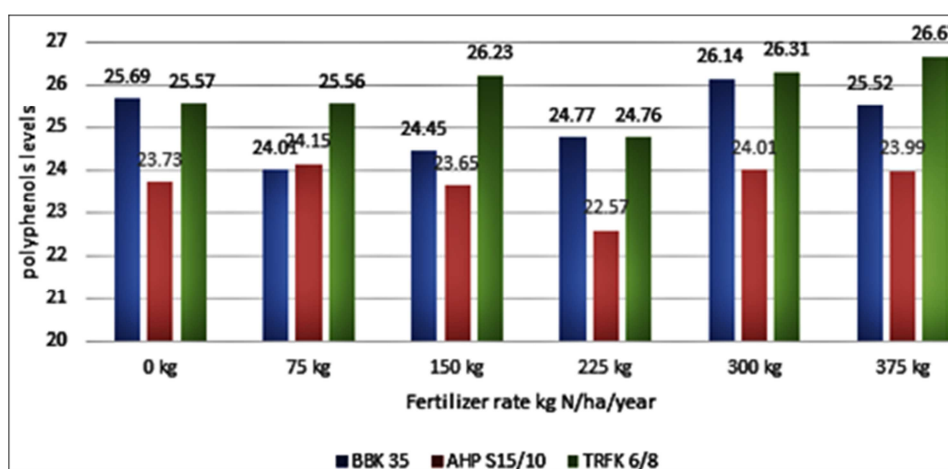


Figure 3. Effect of plain NPK fertilizer rates on polyphenols levels.

3.2. Total Catechins

The Catechins contents of tea leaves plucked from different treatments with different levels of nitrogen are presented in tables 4 and 5.

The values in different treatments show the variations but were statistically non-significant. Similarly, treatments with molybdenum were at peak in pushes grown in presence of 10M+225 kg NPK/HA and the lowest amount was observed in treatment 7M+0kg NPK/HA (figure 4). It has also found out that the quantity of total Catechins in samples treated with molybdenum were higher than those without molybdenum [4]. On NPK fertilizer not supplemented with Molybdenum, BBK 35 had a higher percentage total Catechins. From the results obtained, BBK 35 was the most sensitive to fertilizer rates applied. It was able to effectively utilize the nutrients in the fertilizers applied which was used in the synthesize of bio-chemicals hence higher levels of polyphenolic compounds. On the samples applied with NPK fertilizers supplemented with Molybdenum, BBK 35 had higher levels of percentage total polyphenols. Molybdenum could have assisted in the conversion of nutrients in the NPK fertilizers applied hence the tea plants used the nutrients effectively. It has also been observed that there were higher

polyphenolic levels in samples from tea plants applied with NPK fertilizers supplemented with Molybdenum [12]. BBK 35 and AHP S15/10 were the most sensitive to the treatments with the highest CV of 8.87% and 8.63% respectively but TRFK 6/8 was least sensitive response to treatment rate with CV of 5.84%.

Table 4. Total Catechins processed from clone BBK 35 grown in Monieri, Sotik Estate.

Fertilizer Rates (Npk/Ha) (kg)	Total Catechins (%)
0	14.42 ^d ± 0.45
75	14.08 ^d ± 0.28
150	15.95 ^{bd} ± 0.84
225	14.96 ^{dc} ± 0.85
300	15.95 ^{bdc} ± 1.03
375	15.11 ^{bdc} ± 1.47
7M+0	15.95 ^{bdc} ± 1.28
8M+75	16.05 ^{bdc} ± 0.37
9M+150	17.46 ^{ba} ± 2.83
10M+225	16.62 ^{bac} ± 2.58
11M+300	18.52 ^a ± 1.26
12M+375	18.59 ^a ± 0.52
Mean	16.14
LSD	2.42
CV	8.87

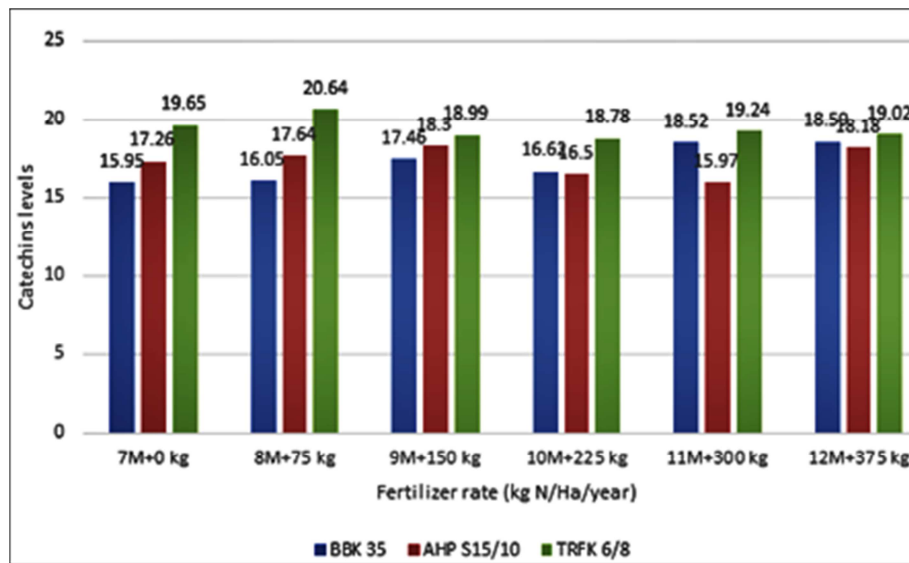


Figure 4. Effects of Molybdenum supplemented fertilizer rate on catechins levels.

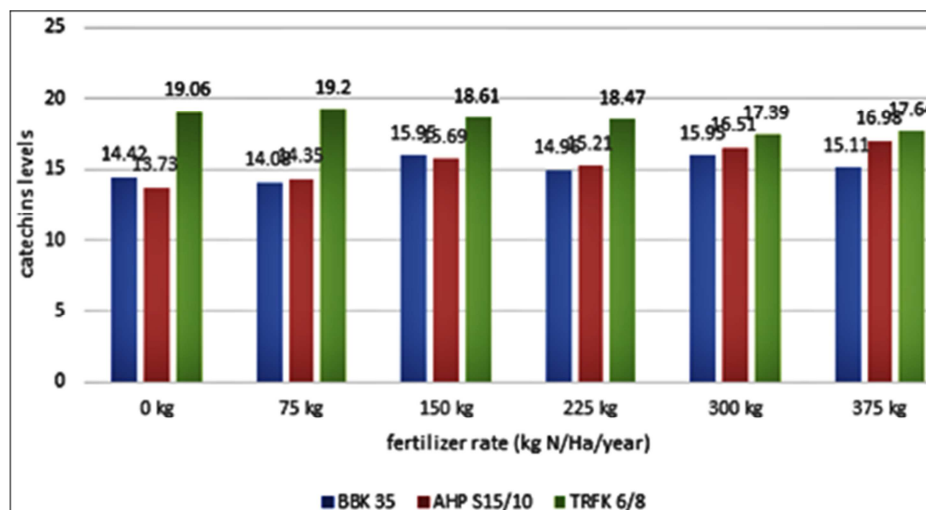


Figure 5. Effects of fertilizer rates on Catechins levels.

Table 5. Total catechins processed from clone AHP S15/10 grown in Mettarora, Sotik Estate.

Fertilizer Rates (Npk/Ha) (kg)	Total Catechins (%)
0	13.73 ^c ± 1.77
75	14.35 ^{ed} ± 2.15
150	15.69 ^{ebdc} ± 0.23
225	15.21 ^{edc} ± 2.13
300	16.51 ^{bdac} ± 0.98
375	16.98 ^{bac} ± 1.66
7M+0	17.26 ^{bac} ± 0.65
8M+75	17.64 ^{ba} ± 0.97
9M+150	18.30 ^a ± 0.97
10M+225	16.50 ^{bdac} ± 1.44
11M+300	15.97 ^{ebdac} ± 1.06
12M+375	18.18 ^a ± 1.45
Mean	16.36
LSD	2.40
CV	8.63

4. Conclusion

This research, therefore, concludes that with an increase in nitrogen fertilizer rates, biochemical are increased up to optimum levels where further increased rates does not lead to a decline in biochemical contents but rather makes tea farming unsustainable venture. it also concludes that tea bushes applied with NPK fertilizers supplemented with Molybdenum are rich in polyphenolic compounds as compared with those applied with plain NPK fertilizers.

From the above results, it can be concluded that application of 8M+ 75Kg NPK/HA fertilizers supplemented with Molybdenum could enhance the production as well as the polyphenolic content of made green tea of all the cultivars. It is recommended that NPK fertilizers supplemented with Molybdenum should be applied at the optimum levels since it boosts the biochemical contents in green tea and at the same time reduces production cost.

Besides, other clones should also be subjected to the same study in order to get a more informed conclusion.

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List of Abbreviations

CTC	Cut, Tear and Curl
EC	Epicatechin
ECG	Epicatechin Gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin Gallate
HPLC	High Performance Liquid Chromatography
KALRO	Kenya Agricultural and Livestock Research Organization
NPK	Nitrogen, Phosphorus and Potassium
PPO	Polyphenol Oxidase Enzyme
TPVA	Tea Processing and Value Addition Programme
TRI	Tea Research Institute
TP	Total Polyphenols

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