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Shoot Epicatechin and Epigallocatechin Contents Respond to Water Stress in Tea [*Camellia sinensis* (L.) O. Kuntze]

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An experiment was conducted to determine the association of tea catechins to water stress in tea, with the objective of determining their suitability as indicators for predicting drought tolerance in tea (*Camellia sinensis*). The study consisted of six tea clones (BBK 35, TRFK 6/8, TRFK 76/1, TRFK 395/2, TRFK 31/30, and TRFK 311/287) and four levels of soil water content (38, 30, 22, and 14% v/v), which were arranged in a complete randomized design and replicated 3 times. The treatments were maintained for a period of 12 weeks. Tea shoots were sampled for catechin analysis during the 6th week of water treatment, in which fresh shoots with two leaves and a bud were plucked and steamed for 2 min, and dried at 70 °C to constant weight. Subsequently, the samples were ground and analyzed for catechins using an HPLC system. The total catechins showed significant correlation with shoot growth (r = 0.65, $P = 0.006$), soil water content (r = 0.54, $P = 0.0066$), and water stress index (r = 0.67, $P = 0.0004$). The epicatechin (EC) correlated with shoot growth (r = 0.58, $P = 0.0032$), soil water content (r = 0.62, $P = 0.0014$), and water stress index (r = 0.63, $P = 0.0010$). Similarly, epigallocatechin (EGC) correlated with shoot growth (r = 0.65, $P = 0.0006$), soil water content (r = 0.50, $P = 0.0133$), and water stress index (r = 0.60, $P = 0.0021$). However, epigallocatechin gallate (EGCg) and epicatechin gallate (ECG) showed no significant response to changes in soil water content. The shoot contents of EC and EGC in the six clones showed varied responses, with a distinct pattern in the water-stress tolerant clones (TRFK 6/8 and TRFK 31/30). The results suggest a potential use for EC and EGC as indicators in predicting drought tolerance in tea.

Key words: catechins; drought stress; flavan-3-ol; free radicals

Plants are known to accumulate organic osmolytes such as proline, glycine betaine, non-reducing sugars, and polyols[1,2] in response to stress factors. Though these organic compounds are species-specific their role is not clearly defined, but it is generally accepted that they contribute to ameliorating stress in plants.[2–4] Most stress-related organic compounds are secondary plant metabolites, and tea (*Camellia sinensis*) contains large amounts of polyphenols, mainly catechins, that belong to the flavan-3-ol class. Flavonoids play a key role in quality determination in black tea,[5] and in fruits,[6] but their role as indicators of desiccation tolerance in tea has not been explored. The precursors of most flavonoids are malonyl-CoA, derived from carbohydrate metabolism and *p*-coumaroyl-CoA, from the phenylpropanoid pathway.[7–9] Phenylpropanoids, which include flavonoids, isoflavonoids, and stilbenes, are derived from deamination of phenylalanine by phenylalanine ammonia-lyase (PAL). Flavonoid biosynthesis is dependent on structural and regulatory genes; structural genes encode enzymes catalyzing the biosynthesis, while regulatory genes control the expression of the genes.[8,10–14] This implies that the availability and quantity of certain flavonoids in plant tissues is an indication of plant response to either internal or external stimuli.

As observed in some plants, the production of certain secondary metabolites serves as a signal generated by an external factor, and manifested at the molecular level, prior to morphological symptoms. For instance, increased biosynthesis of the phytoalexin medicarpin in alfalfa has been observed in response to fungal attack.[15] Therefore, the production of stress-related metabolites is modulated by genes whose functions may include transcription of specific enzymes or encoding of specific receptors that regulate other effecter genes. The dynamics of the biosynthesis of organic compounds in response

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Abbreviations: EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate; RWC, relative water content; TF, theaflavins; TR, thearubigins to a stress factor might provide leads to plant improvement in agriculture. An understanding of flavonoid dynamics in the plant system might yield information for plant genetic manipulation and crop management strategies that might improve crop value. Isolation of the source of a known organic compound or precursor might present an opportunity to increase the by-product through the introduction of some accumulation mechanisms *via* metabolic engineering.[16]

In the case of drought tolerance, elucidation of secondary metabolites that are associated with drought can be a key to developing crop cultivars for dry environments. It has been observed that though drought-tolerance genes are present in plants, they are often poorly expressed during stress.[17] This suggests, a need to identify and possibly to manipulate the biosynthesis signaling pathway so as to trigger the potentially protective genes into action. However, against this background, there are no known attempts to use rich tea polyphenols to address the challenges facing the crop, one of which is recurring drought. Similarly, no attempt has been reported to use flavonoids as a tool in plant selection or crop management. Hence there is a need to determine the quantitative variations of polyphenols in tea clones, and to evaluate their correlation with quantifiable traits such as leaf yield and response to fertilizers, and more importantly, tolerance to moisture stress and other stress-inducing factors.

The objective of this study was, among other things, to determine the influence of soil water deficit on catechins and their correlation with drought stress in tea. It was hypothesized that tea plants exposed to increasing soil water deficits show a decline in shoot growth, and that the severity of this response can be predicted by tea polyphenols.

Materials and Methods

Set-up of rain-out shelter. The study was conducted in a rain-out shelter measuring 17 m by 6.5 m on the ground and with a height of 2.5 m. The roof was raised and curved to give a dome shape with a radius of 0.5 m above the 2.5 m height, and an extended eve of 0.3 m all round. The roof design was to facilitate the flow of rain water out of the structure and to enhance uniform distribution of solar radiation inside. The roof was covered with an ultra violet-treated 200-micron film of clear polythene sheet (Sunselector AD-IR 504) designed to transmit 82% of photosynthetically active radiation and 65% of diffused light with 88% thermicity. The sides were covered with the same polythene, but to 1 m in height from ground level on the longer sides, and to 2.5 m on the shorter sides. The 1.5 m of uncovered space along the longer sides was to allow free air flow in and out of the structure. A door measuring 1 m wide and 2 m high was made in the middle of one of the shorter sides, covered with chicken wire, but without polythene.

Plant materials and soil media. Six tea clones in the study were BBK 35, TRFK 6/8, TRFK 76/1, TRFK 395/2, TRFK 31/30, and TRFK 311/287. The clones were developed from stem cuttings, and were raised in the nursery in the usual way.[18] They were transplanted after 6 months into 1000-gauge black polythene pots measuring 0.3 m in diameter and 0.3 m in depth. Each clone had 24 potted seedlings, giving a total of 144, and they were later arranged into 72 experimental units.

Soil medium for raising seedlings in the nursery and in the pots after transplanting was sub-soil and top soil in a ratio of 3:1. The analysis of the soil was as follows: 3.5% N, 0.16% P, 169 ppm K, 255 ppm Ca, pH 4.3, and 9.3% organic matter. Its textural class was clay with 25% sand, 67% clay, and 8% silt. The field capacity of the soil was 42% soil water content, determined by time-domain reflectometry soil moisture meter (Trime-FM2, Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands).

Prior to the study, the potted tea plants were allowed to establish themselves for 2 months in the tea nursery, where they were watered adequately twice a day. Thereafter, the plants were transferred to the rain-out shelter and arranged according to the various treatments.

Application of treatments. The study consisted of two factors: four varying soil water contents subjected to six different tea clones. The soil water content levels were 38, 30, 22, and 14% v/v, and, they were subjected to the six clones listed above, which gave a total of 24 treatments. The treatments were arranged in completely randomized design and were replicated 3 times, giving a total of 72 experimental units, each unit having two potted plants. The predetermined soil water content in each experimental unit was maintained within ±2%, and measurement was done daily at 9:00 h and 14:00 h during the 12-week period of the study.

Relative water content. The relative water content (RWC) was determined in the 3rd leaf in the tea shoots. One leaf in each experimental unit was randomly picked during the 6th week of water treatment, and their fresh weights (fr.wt) were taken immediately. The leaves were re-hydrated with distilled water at 4 °C for 24 h, and the turgid weight was taken ($t_{.wt}$). They were subsequently oven-dried at 70 °C, and dry weights (d.wt) were taken. The leaf RWC was then calculated using the following formula: RWC = $(fr_{.wt} - d_{wt}/(t_{wt} - d_{wt})$.[19,20]

Growth measurements. Shoot and leaf growth in the tea were determined in relation to soil water content. The procedure for shoot growth measurement was described previously.[21]

Water stress index. The water stress index of the clones was calculated based on shoot growth, as detailed previously.[21]

Sampling for determination of total polyphenols and catechins. Tea shoots for determination of total polyphenols and shoot catechins were sampled during the 6th week of water treatment. About 500 g of fresh shoots with two leaves and a bud were plucked in each of the experimental units, and were immediately steamed for 2 min. The samples were then placed in labelled paper bags and dried in an oven at 70 °C for 24 h. The dry samples were ground using a blender, sealed in paper bags, and safely stored in a dark, dry environment until laboratory analysis.[7)] Analysis of total polyphenols and catechins followed the ISO procedure.[22,23)] The procedure for the analysis of total polyphenolcontent and the results have been presented elsewhere.[21)]

HPLC analysis for catechins. The tea catechins were quantitatively analyzed using an HPLC system (Shimadzu LC 20AT, Kyoto, Japan) with a Gemini 5μ c6-phenyl Phenomenex column. Two hundred mg of ground samples was extracted with 5 ml of warm (70 °C) 70% methanol. The mixture was warmed in a water bath (70 °C) for 10 min and then cooled. The cool mixture was centrifuged at 3,500 rpm for 10 min, and the extract was decanted into a 10-ml cylinder and topped-up to 10 ml. One ml of the extract was diluted 5-fold and passed through a 0.5-μm pore filter before injection into the HPLC column. Mobile phase A consisted of 9% (volume fraction) acetic acid, and mobile phase B consisted of 80% (volume fraction) acetonitrile. The flow rate was 1.0 ml/min, and the injection volume was 20 μl. The column was operated at 40 °C, and UV spectra peaks were detected at 278 nm. The chromatographic peaks in the samples were identified by comparing their retention times with chemical standards.[23,24)]

Data analysis. Regression analysis was done using a Gompertz exponential function (GenStat 5 release 4.2). The functional Gompertz model provided the best fit curve, with relatively low residual and better adjusted R^2 values than other growth models, and it was therefore used in this study. Pearson correlation analysis was performed using SAS (Ver.8.1 e).

Results

Relative water content (RWC)

The RWC and shoot growth in the tea declined with decreases in soil water content. On average, the RWC of the tea leaves was 93% between soil field capacity (which was 42% soil water content), and 28% soil water content, but large reductions occurred when the soil water content fell below 28% (Fig. 1). Shoot growth followed the same pattern (Fig. 2), but with slight differences among the clones. The plants sustained their physiological functions at soil water deficits of less than 30% (42-28/42*100), but as the soil water deficit exceeded 32%, a sharp reduction in RWC and shoot growth was observed (Figs. 1 and 2).

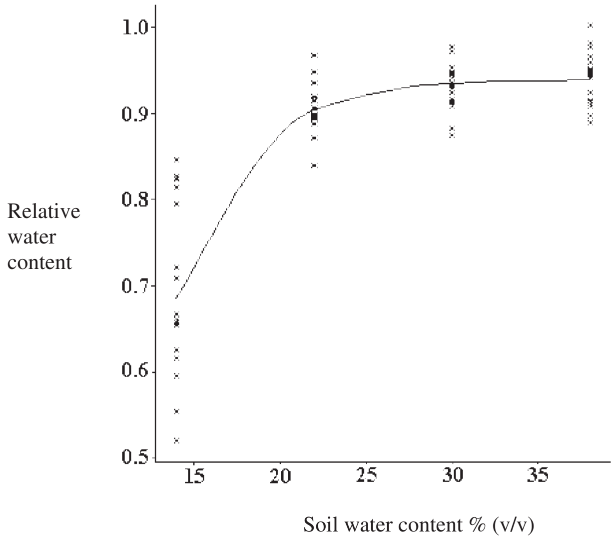


Fig. 1. Influence of Soil Water Content on Relative Water Content in Tea Leaf.

$P < 0.001$, SE 0.05, n = 72, adjusted $r^2 = 79\%$

Effect of soil water content on catechins in tea shoots

The total catechin in tea shoots was influenced by shoot growth, and it correlated with soil water content (Table 1 and Fig. 3). The catechins determined by HPLC were epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCg), of which EGCg and ECG were higher in amount (Table 1). However, not all catechins in the tea shoots responded similarly to varying soil water contents. The EGC and EC contents were found to correlate with the soil water content (Table 2). The EGC content decreased in TRFK 6/8, TRFK 76/1, TRFK 395/2, and TRFK 31/30, but increased in TRFK 311/287 and BBK 35 ($P < 0.001$), with declines in soil water content (Table 1). The reduction of EGC content was gradual in TRFK 31/30, TRFK 395/2, and TRFK 6/8, as indicated by the gradients of their curves (generated from regression estimates), which were 1.17×10^{-3}, 1.18×10^{-3}, and 1.18×10^{-3} respectively. Similarly, EC content in the shoots of the six clones reduced with decline in soil water content. The rate of decline, as indicated by the gradient of the curves were 4.9×10^{-2}, 1.0×10^{-3}, 1.16×10^{-1}, 1.0×10^{-3}, 1.0×10^{-3}, and 2.95×10^{-1} for clones BBK 35, TRFK 6/8, TRFK 76/1, TRFK 395/2, TRFK 31/30, and TRFK 311/287 respectively (Table 1).

Discussion

The results clearly show that total shoot catechins responded to changes in soil water content. The total shoot catechin content in the six clones ranged from 9 to 13%, and that of the individual variants were EGCg 4–6%, EGC 3–5%, ECG 1–2%, and EC 0.8–1.5%. However, among the catechins, only EGC and EC significantly correlated with changes in soil water

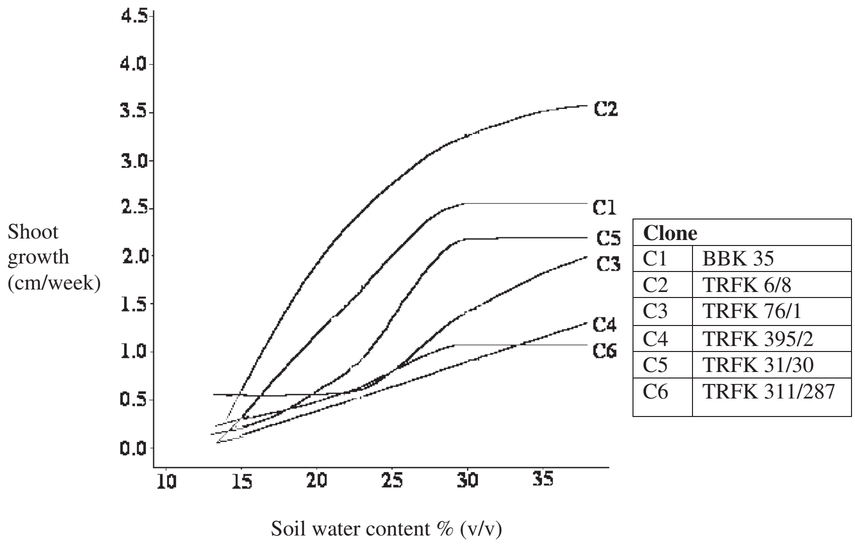


Fig. 2. Influence of Soil Water Content on Shoot Growth of Six Clones of Tea.
$P < 0.001$, SE 0.76, n = 72, adjusted $r^2 = 64\%$

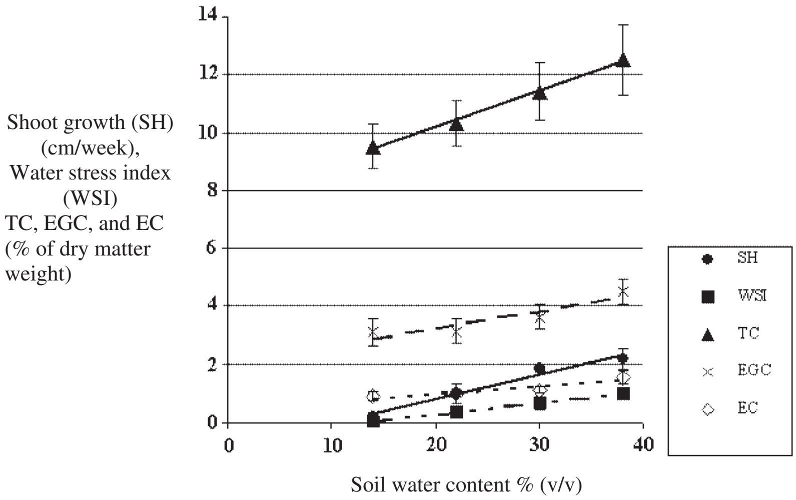


Fig. 3. Linear Correlation of Total Catechins (TC), Epigallocatechin (EGC), Epicatechin (EC), Shoot Growth (SH), and Water Stress Index (WSI) with Soil Water Content.

The relationship was derived using means from analyzed data for six clones (n = 24) at each level of soil water content. The *P* values of analyzed data are TC ($r^2 = 0.99$, $P = 0.07$), EC ($r^2 = 0.86$, $P = 0.0075$), EGC ($r^2 = 0.86$, $P = 0.04$), SH ($r^2 = 0.97$, $P = 0.0003$), WSI ($r^2 = 0.99$, $P = 0.0001$).

content (Table 2). It can be argued that the observed response is due to the principal amount of the specific catechin in the leaf. Organic compounds that are synthesized in small quantities, such as EGC and EC, can be expected to exhibit significant changes when the plant is under stress, but this was not the case. The quantity of EGCg compared closely to that of EGC, and the content of ECG compared closely to that of EC, yet both EGCg and ECG showed low correlations with soil water content (Table 2). The above observation therefore rules out the influence of principal amount as the basis of the differential drought stress response.

The observed differential response was due to the chemical structures of the catechins. Both EC and EGC have 3′,4′-dihydroxyl groups in the B-ring, and H^+ for EC and OH^- for EGC at position 5′ in the B-ring (Fig. 4). Though ECG (epicatechin gallate) and EGCg (epigallocatechin gallate) also have the 3′,4′-dihydroxyl group in the B-ring, the notable difference is the carboxyl-group attached to position 3 in the C-ring. It has been found that the molecular structure, and particularly the hydroxyl groups, influence the radical-scavenging activity of flavonoids.[25] The configuration of the hydroxyl group in the B-ring has been associated with potency of free radical scavenging.[25,26] Plants under water stress produce oxygen-derived free radicals, reactive oxygen species, which are associated with tissue damage.[27,28] These include superoxide anions

Table 1. Total Catechins (TC), Epicatechin (EC), Epicatechin Gallate (ECG), Epigallocatechin (EGC), and Epigallocatechin Gallate (EGCg) Contents (% of dry matter) in Tea Grown at 14, 22, 30, and 38% Soil Water Content Levels

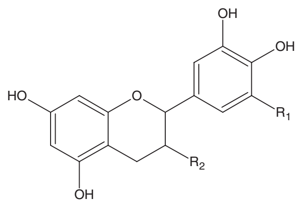
Catechins	Tea clone	Soil water content % (v/v) 14	22	30	38
		← (Catechin content % of dry matter) →			
Total catechin (TC)	BBK 35	8.32 (0.50)*	8.56 (0.24)	11.08 (1.63)	9.74 (0.82)
	TRFK 6/8	10.48 (0.96)	12.55 (0.65)	11.59 (0.24)	11.98 (0.38)
	TRFK 76/1	9.91 (1.21)	10.21 (1.58)	14.43 (0.35)	17.00 (1.38)
	TRFK 395/2	7.02 (0.91)	7.43 (1.75)	10.76 (0.62)	11.51 (0.38)
	TRFK 31/30	9.14 (1.56)	10.50 (1.29)	9.80 (0.63)	12.44 (1.16)
	TRFK 311/287	11.49 (0.48)	9.44 (2.04)	12.63 (1.54)	12.27 (0.46)
Epicatechin (EC)	BBK 35	0.95 (0.07)	1.00 (0.06)	1.15 (0.11)	1.46 (0.11)
	TRFK 6/8	1.35 (0.25)	1.01 (0.07)	1.31 (0.32)	1.87 (0.05)
	TRFK 76/1	0.87 (0.30)	0.93 (0.08)	1.47 (0.18)	2.33 (0.16)
	TRFK 395/2	0.58 (0.06)	0.74 (0.14)	1.00 (0.07)	1.46 (0.10)
	TRFK 31/30	0.65 (0.07)	1.10 (0.42)	0.73 (0.07)	1.05 (0.16)
	TRFK 311/287	0.93 (0.14)	0.90 (0.09)	0.97 (0.03)	1.19 (0.09)
Epicatechin gallate (ECG)	BBK 35	0.86 (0.04)	1.09 (0.05)	1.58 (0.35)	1.22 (0.11)
	TRFK 6/8	0.81 (0.09)	1.39 (0.16)	0.85 (0.23)	0.99 (0.08)
	TRFK 76/1	1.05 (0.06)	1.07 (0.29)	2.12 (0.25)	2.32 (0.26)
	TRFK 395/2	0.58 (0.05)	0.84 (0.30)	1.38 (0.23)	1.15 (0.37)
	TRFK 31/30	1.02 (0.17)	1.61 (0.72)	0.98 (0.72)	1.62 (0.32)
	TRFK 311/287	0.92 (0.04)	0.95 (0.02)	1.38 (0.17)	1.18 (0.09)
Epigallocatechin (EGC)	BBK 35	2.56 (0.007)	1.86 (0.91)	3.24 (0.44)	3.47 (0.43)
	TRFK 6/8	4.60 (0.51)	4.80 (0.63)	4.85 (0.77)	5.48 (0.50)
	TRFK 76/1	2.77 (0.61)	3.34 (0.21)	4.17 (0.70)	5.30 (0.48)
	TRFK 395/2	2.52 (0.41)	2.74 (0.46)	3.19 (0.11)	4.42 (0.43)
	TRFK 31/30	2.34 (0.30)	2.83 (0.14)	2.45 (0.15)	3.56 (0.28)
	TRFK 311/287	3.81 (0.13)	3.69 (0.52)	3.88 (0.23)	4.79 (0.44)
Epigallocatechin gallate (EGCg)	BBK 35	3.75 (0.41)	3.90 (0.54)	4.69 (0.75)	3.29 (0.42)
	TRFK 6/8	3.49 (0.95)	4.91 (0.43)	4.16 (1.23)	3.08 (0.09)
	TRFK 76/1	4.36 (1.14)	4.62 (0.93)	6.19 (0.50)	6.33 (1.26)
	TRFK 395/2	3.19 (0.44)	3.01 (0.98)	4.82 (0.36)	4.02 (0.30)
	TRFK 31/30	5.00 (1.05)	5.54 (3.83)	5.48 (0.55)	5.97 (1.30)
	TRFK 311/287	5.58 (0.19)	4.74 (0.72)	6.00 (1.26)	4.65 (0.06)

*Values in brackets are standard deviations.

Table 2. Correlation Analysis between Shoot Growth (SH), Leaf Relative Water Content (RWC), Soil Water Content (SWC), Water Stress Index (WSI), Total Catechins (TC), and the Catechin Variants: Epigallocatechin Gallate (EGCg), Epigallocatechin (EGC), Epicatechin Gallate (ECG), and Epicatechin (EC)

	SH	RWC	WSI	SWC	TC	EGCg	EGC	ECG	EC
SH	1.000								
RWC	0.70 (0.0001)*	1.000							
WSI	0.83 (<0.0001)	0.77 (<0.0001)	1.000						
SWC	0.76 (<0.0001)	0.82 (<0.0001)	0.91 (<0.0001)	1.000					
TC	0.65 (0.0006)	0.44 (0.0330)	0.67 (0.0004)	0.54 (0.0066)	1.000				
EGCg	0.28 (0.1907)	0.26 (0.2230)	0.27 (0.1976)	0.14 (0.5018)	0.66 (0.0004)	1.000			
EGC	0.65 (0.0006)	0.31 (0.1375)	0.60 (0.0021)	0.50 (0.0133)	0.73 (<0.0001)	0.04 (0.8440)	1.000		
ECG	0.38 (0.0656)	0.45 (0.0258)	0.55 (0.0049)	0.48 (0.0164)	0.79 (<0.0001)	0.73 (<0.0001)	0.25 (0.2465)	1.000	
EC	0.58 (0.0032)	0.36 (0.0803)	0.63 (0.0010)	0.62 (0.0014)	0.73 (<0.0001)	0.03 (0.8875)	0.81 (<0.0001)	0.48 (0.0181)	1.000

*P values in brackets.



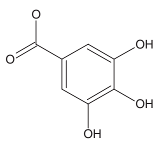
Basic Structure of Catechin

(-)-Epicatechin
R1 = H,
R2 = H

(-)-Epigallocatechin
R1 = OH,
R2 = H

(-)-Epicatechin gallate
R1 = H,

R2 =



(-)-Epigallocatechin gallate:
R1 = OH,

R2 =

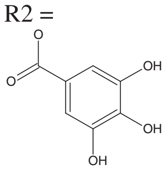


Fig. 4. Chemical Structures of the Catechins.

($O_2^-\bullet$), hydroxyl radicals ($\bullet$OH), and hydrogen peroxide. Reactive oxygen species usually occur within a tolerable balance in the plant tissue, but that balance can be exceeded under stress conditions, with likely damage to the tissue. In order to counter the likely damage caused by free radicals under stress, plants produce antioxidants such as ascorbate, glutathione, carotenoids, and flavanoids, among others.[29,30] The sensitivity of EC and EGC to changes in soil water content suggests that the two catechins are involved in mechanisms that ameliorate the cellular effects caused by plant water stress. Their role in this hypothesized mechanism has yet to be fully elucidated. This is the first report showing an association of individual catechins with water stress in tea. Our results corroborate the earlier observation by Saravanan *et al.*[31] that drought-tolerant tea clones segregate into one group based on the catechin fraction. From this study, it is clear that not all catechin fractions respond to water stress in tea, and similar observations can be expected of other tea polyphenols, as predicted earlier.[21]

In respect to the response of tea clones to water stress, one fundamental question that remains unanswered is whether the catechins can be used as indicators of drought tolerance. Judging from the dynamics of tea EC and EGC in response to soil water content, there is a potential to develop a useful index to predict drought stress tolerance in tea. This would, however, require screening a large pool of tea germplasms that segregate for the drought tolerance trait. For example, in our studies, it was found earlier that clones TRFK 6/8 and TRFK 31/30 had higher water stress indices, indicating that they were more tolerant to water stress than the other clones.[21] Further work revealed some uniqueness of the same clones with regard to both EC and EGC (Table 1). The reduction in EC and EGC in the said clones was marginal and gradual when the soil water content was reduced from 38% to 14%, as indicated by the gradients of the regression curves. In contrast, the other clones in the study exhibited wide fluctuations in shoot contents of EC and EGC within the range of 14 to 38% soil water content, suggesting the possibility of using this criterion to discriminate drought-tolerant and susceptible tea clones. However, given the inherent genetic diversity that exists in tea clones,[7] developing a useful guide as an indicator of drought tolerance would require further germplasm screening. The catechins have also been used in quality determination, particularly in plain black teas.[32] The quality of black tea is influenced by chemicals that arise from oxidized catechin during tea manufacture, and they include theaflavins (TF), thearubigins (TR), and other compounds that contribute to the color and flavor of liquor. TF gives briskness and brightness to black tea, while TR is responsible for its color and taste. Though high levels of TF and TR are desirable for good-quality black tea, high amounts of TR give an unpalatable taste and mask the good effects of TF. Therefore, TR should increase only if TF levels are also rising for the maintenance of high quality black tea.[33)] A regression analysis performed on the quality indices to quantifiable catechins in green tea found EGCg and EC to be suitable for predicting the quality of Kenyan plain black tea.[34)] TF and liquor brightness improved with high amounts of EGCg, but reduced with high amounts of EC. Thearubigins were enhanced by high levels of EC, while tea flavor negatively correlated with EC.[34)] Given that EC reduced with declines in soil water content while EGCg was less affected, the quality of tea under droughty conditions can be predicted. Based on these results, the quality of black tea can be expected to improve during drought periods despite the expected declines in yield. However, there is no certainty as to what extent of water stress is desirable for the quality of black tea, and whether profit gains due to improved quality can compensate for the yield loss associated with drought.

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