Free diterpenes cafestol and kahweol in beans and *in vitro* cultures of *Coffea* species

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Caffeine and chlorogenic acids are the major bioactive metabolites in beans of coffee, which also contains the unique diterpene compounds cafestol and kahweol known to be responsible for hypercholesterolemic effects in humans. The aim of the study was to profile cafestol and kahweol in different plant parts of *Coffea* species and its beans grown in India, and also in *in vitro* raised tissues of commercial Indian species, viz. *Coffea arabica* and *Coffea canephora*. Total quantities of free diterpene levels were found to be comparatively higher in beans of *C. arabica* (1.89 mg g^{-1}) than in *C. canephora* (1.13 mg g^{-1}). Other plant parts, except the roots, showed considerable amount of cafestol and kahweol. These diterpenes were also synthesized in somatic embryos and *in vitro* regenerated plants of both *Coffea* species. Somatic embryos of *C. canephora* and *C. arabica* subjected to different hormonal treatments showed variation in the levels of total diterpenes. In this study, we were able to profile cafestol and kahweol levels in *in vitro* tissues and also in flowers and zygotic embryos of *Coffea* species. The diterpene profiling in *in vitro* tissues would be of relevance in future to obtain somaclonal variants with their reduced levels.

Keywords: Cafestol, coffee beans, kahweol, somatic embryos.

COFFEE breeding and improvement has been a subject of commercial interest due to its international relevance in trade and utility[1]. The genus *Coffea* (Rubiaceae) has about 100 species. Among these, two commercially important species are *Coffea arabica* and *Coffea canephora*. The naturally occurring diterpenes cafestol, kahweol and 16-*O*-methyl cafestol are unique to coffee, and are present in the unsaponifiable fraction of raw coffee mostly esterified to 14 different fatty acids at the C-17 position[2,3] and only a small portion (~ 1–3%) is present in the free-form. These diterpenes find utility in skin care as a sun filter[4] and also in the treatment of dry skin, psoriasis, burns, wounds and blisters[5], and as anti-inflammatory and anti-carcinogenic agents[6]. The cafestol and kahweol-based formulations are known to enhance percutaneous drug delivery[7]. However, the recent toxicological studies of cafestol and kahweol on humans have shown a direct relationship between coffee consumption and increase in blood cholesterol[8], especially low density lipoprotein cholesterol and triglycerides[9].

Variation in diterpenes content in coffee beans depends on the geographical origin and distribution of the *Coffea* species[10]. Determination of diterpenes content in commercial coffee species has proven to be useful in the grouping of *Coffea*. The total diterpene content ranges from 1.3% to 1.9% (w/w) in the beans of *C. arabica* and from 0.2% to 1.5% in *C. canephora* respectively, and the free forms of both cafestol and kahweol are reported to be in the range 0.1–0.4% (ref. 11). It is known that cafestol and kahweol are present in arabica coffee beans, whereas robusta coffee beans contain additionally 16-*O*-methyl cafestol. In India, both arabica and robusta coffee are widely cultivated and consumed. Determination of cafestol and kahweol levels in these species and others will be useful in grading them on the basis of the diterpene levels. Plant tissue culture-mediated selection of low diterpene-yielding plants is a possibility. In view of this, the objective of the present study was to determine the profiles of the major diterpenes cafestol and kahweol in different plant parts of field-grown species, *C. arabica* and *C. canephora* and *in vitro* cultures derived from them. Moreover, profiles of these diterpenes in a range of coffee samples of various *Coffea* species were studied.

Seeds of various *Coffea* species (*C. arabica*, *C. bengalensis*, *C. canephora*, *C. dewevrei*, *C. kapakata* and *C. salvatrix*) used in this study were obtained from the plantations in Central Coffee Research Station, Balehonnur, Karnataka, India. To raise *in vitro* cultures, seedlings were established under aseptic conditions. For this purpose, the collected seeds of *C. canephora* and *C. arabica* were washed in running tap water for 1 h and soaked overnight in water prior to the removal of seed coat and silver skin. The harvested, ripened fruits were treated with 0.1% bavistin (w/v) for 1 h followed by sterile-water wash twice. The seeds were separated from the fruits by removing the pulp and further treated with 1% sodium hypochlorite (v/v) for 20 min followed by copious washing with sterile water. Subsequently, the seeds were soaked in 0.1% (w/v) mercuric chloride for 5 min followed by sterile-water wash five times. The surface-sterilized seeds were aseptically inoculated on quarter-strength Murashige and Skoog (MS) medium[12] supplemented with inositol (100 mg l^{-1}), sucrose (3%) and activated charcoal (8 g ml^{-1}) and kept in dark for one month at 25 ± 2°C. Fully expanded *in vitro* cotyledonary leaves obtained from the seedlings were used as explants for induction of callus and somatic embryos on callus induction medium[13]. Somatic embryos of *C. canephora* and *C. arabica* were incubated for two months in the secondary embryogenesis medium[14] comprising half-strength MS salts with 100 mg l^{-1} inositol, 2% sucrose, 8 mg l^{-1} thiamine HCl, 3.2 mg l^{-1} pyridoxine HCl along with 0.25 mg l^{-1} indole-

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3-acetic acid (IAA) and 0.5 mg l^{-1} N^6-benzyladenine (BA). The pH of the medium was set to 5.6. The regenerated shoots from the somatic embryos were maintained on half-strength MS basal medium.

Analyses of free forms of cafestol and kahweol was done[15] in various tissues, viz. leaves, shoots, roots from *in vitro* grown plantlets, non-embryogenic callus, embryogenic callus and somatic embryos developed from leaf explants of *C. canephora* and *C. arabica*, leaves (young green and old); shoots, roots, seeds, endosperm and zygotic embryos of *in vivo* plants of *C. canephora* and *C. arabica*, and also seeds of wild varieties of coffee, viz. *C. kapakata*, *C. salvatrix* and *C. bengalensis*. Various tissues of *Coffea* weighing 300–500 mg were grounded, transferred into a thimble and extracted with tertiary butyl methyl ether for 4 h. The solvent was evaporated and the residue dried until constant weight. The residue was saponified and evaporated using rotavapor (Laborota-4000, Heidolph, Germany) and then re-dissolved in 1 ml methanol (v/v) prior to the estimation of metabolites.

High performance liquid chromatography (HPLC) analysis was performed on a NUCLEOSIL® 100-5, C_{18} column of size 4.6 μm × 250 mm (HPLC Trennsaule, Macherey-Nagel Gmbh & Co, Germany). Parameters were controlled by a Shimadzu LC 10-A liquid chromatograph equipped with a dual pump and a UV spectrophotometer detector (model SPD-10A) and the recorder, Shimadzu C-R7A Chromatopac was set at a chart speed of 2.5 cm/min. An aliquot of 10 μl was injected with Rheodyne 7125 injector. The UV detector was set at 220 nm for cafestol and 280 nm for kahweol. Peaks were identified by comparing with the retention time of reference standards and by spiking. Analyses were carried out in ten samples, and values expressed as average. The eluent solvent system used was acetonitrile : water : glacial acetic acid (70 : 29 : 1). The mean ± SE values are given in Tables 1 and 2.

Diterpene levels in the seed germplasm of various *Coffea* species were compared (Table 1). In nature, the two diterpenes cafestol and kahweol are esterified to various fatty acids. In order to analyse the total amount of individual diterpenes, the coffee oil was saponified and the diterpenes levels determined in the unsaponifiable matter using HPLC[15] with some modifications in the solvent system (mobile phase ratio). It is evident that cafestol levels ranged from 0.95 to 1.68 mg g^{-1} and kahweol from 0.02 to 0.21 mg g^{-1} respectively, in six tested species. The levels of cafestol and kahweol were the highest in seeds of *C. arabica* (Table 1). Though *C. canephora* is known for high caffeine and chlorogenic acids, its diterpenes levels were significantly less than those in *C. arabica*. The variation in free diterpene profiles in *Coffea* species in the present study could have a bearing on the eco-agronomic conditions. Moreover, the extraction methods also influence the diterpenes profile. Methyl tert-butyl ether used in our study for the extraction of diterpenes as an alternative to hexane, was advantageous for significant yield of diterpenes[15].

Shoot and leaves of both *C. arabica* and *C. canephora* showed considerable amount of cafestol and kahweol. Endosperm of *C. arabica* alone contained 60% of total seed diterpenes, i.e. 1.02 mg g^{-1} of cafestol and 0.14 mg g^{-1} of kahweol respectively. Young green leaf contained higher amounts of cafestol and kahweol than old leaf (Table 2) and roots were devoid of them.

Both cafestol and kahweol were found in somatic embryos raised on half-strength MS medium containing 0.25 mg l^{-1} BA and 0.5 mg l^{-1} IAA (Table 2). In somatic embryos of *C. arabica*, 0.72 mg g^{-1} cafestol and 0.063 mg g^{-1} kahweol were present compared to 0.57 and 0.064 mg g^{-1} respectively, in somatic embryos of *C. canephora*. But in both non-embryogenic and embryogenic calluses the levels of cafestol were less than in the somatic embryos, whereas kahweol content did not vary significantly. Similar trend was also noticed in *C. arabica* (Table 2). It is interesting to note that the cafestol content increased during differentiation of non-embryogenic callus to embryogenic callus and then to somatic embryos. The significance of enhancement of cafestol during somatic embryogenesis is worth investigating.

Zygotic embryos of *C. canephora* had lower levels of cafestol and kahweol compared to somatic embryos. The levels of cafestol and kahweol in endosperm increased during maturity in both *C. arabica* and *C. canephora*. Similar increase in diterpenes levels was observed in the ontogeny of zygotic embryo (Table 2). The role of diterpenes in differentiation of *Physcomitrella patens* protonema has been reported recently[16]. Similarly, cafestol

Table 1. Diterpenes in different species of *Coffea*

Seed sample	Cafestol (mg g^{-1} dry wt)	Kahweol (mg g^{-1} dry wt)
Coffea arabica	1.68 ± 0.14	0.21 ± 0.007
Coffea bengalensis	0.95 ± 0.02	0.02 ± 0.004
Coffea canephora	1.1 ± 0.012	0.034 ± 0.002
Coffea dewevrei	0.58 ± 0.01	0.080 ± 0.015
Coffea kapakata	1.06 ± 0.04	0.023 ± 0.001
Coffea salvatrix	1.26 ± 0.15	0.089 ± 0.001

Number of samples analysed = 10 (± SE).

Table 2. Cafestol and kahweol in different parts of *Coffea arabica* and *Coffea canephora*

	Coffea arabica				*Coffea canephora*			
	In vivo		*In vitro*		*In vivo*		*In vitro*	
Sample	Cafestol	Kahweol	Cafestol	Kahweol	Cafestol	Kahweol	Cafestol	Kahweol
Plant parts								
Leaves (young, green)	1.25 ± 0.05	0.175 ± 0.024	1.532 ± 0.018	0.194 ± 0.015	0.242 ± 0.009	0.108 ± 0.005	0.335 ± 0.012	0.039 ± 0.006
Leaves (old, yellow)	–	–	0.515 ± 0.012	0.022 ± 0.003	–	–	–	–
Flower	–	–	0.031 ± 0.005	0.011 ± 0.001	–	–	0.026 ± 0.002	0.009 ± 0.001
Endosperm (immature fruit)	–	–	0.958 ± 0.009	0.42 ± 0.008	–	–	0.640 ± 0.018	0.020 ± 0.003
Zygotic embryos (immature seed)	–	–	0.358 ± 0.014	0.022 ± 0.008	–	–	0.216 ± 0.007	0.01 ± 0.002
Endosperm (mature fruit)	–	–	1.022 ± 0.004	0.142 ± 0.007	–	–	0.875 ± 0.018	0.012 ± 0.002
Zygotic embryos (mature fruit)	–	–	0.732 ± 0.004	0.055 ± 0.002	–	–	0.196 ± 0.004	0.025 ± 0.001
Seedling								
Cotyledonary leaf	0.425 ± 0.012	0.008 ± 0.001	–	–	0.145 ± 0.014	0.003 ± 0.0001	–	–
Hypocotyl	–	–	0.861 ± 0.018	0.0058 ± 0.0004	–	–	0.524 ± 0.0021	0.026 ± 0.003
Roots	ND	ND	ND	ND	ND	ND	ND	ND
In vitro cultures								
Non-embryogenic callus	0.469 ± 0.012	0.0035 ± 0.0002	–	–	0.242 ± 0.004	0.032 ± 0.002	–	–
Embryogenic callus	0.567 ± 0.014	0.035 ± 0.005	–	–	0.352 ± 0.004	0.036 ± 0.004	–	–
Somatic embryos	0.723 ± 0.007	0.063 ± 0.014	–	–	0.570 ± 0.009	0.064 ± 0.006	–	–
Regenerated plant stem	0.856 ± 0.018	0.059 ± 0.006	–	–	0.382 ± 0.016	0.012 ± 0.006	–	–
Green beans	–	–	1.689 ± 0.035	0.216 ± 0.012	–	–	1.10 ± 0.012	0.034 ± 0.006
Roasted beans	–	–	–	–	–	–	1.04 ± 0.005	0.020 ± 0.002

Values (mg g^{-1} dry wt) are an average of 10 samples (± SE); ND, Not detected.

and kahweol could be of relevance in the differentiation in coffee tissues. *Coffea* species found in West and Central African forests had a low concentration of cafestol and kahweol, whereas species originating from East Africa were reported to have higher levels of these compounds. Variation in total diterpenes levels has been reported in arabica and robusta coffee from different geographical regions[10,11,17,18]. In general, the secondary metabolite profile varies with the genotypes and cultivars in other plant species too, viz. soybean for isoflavones[19] and capsaicin in *Capsicum* sp.[20]. Similarly, in *Coffea*, variation in diterpenes could be possibly attributed to genetic peculiarities of the bean, geographic and cultivation conditions. Closely related *Coffea* species exhibit variation in caffeine levels under the same eco-geographical conditions[21]. The diterpenes profile too could be of significance. Cafestol and kahweol profiles in green beans and roasted beans of *C. arabica* and *C. canephora* did not vary significantly. Biochemical diversity in the genus *Coffea* has been well documented, particularly with reference to chlorogenic acids and caffeine[22]. The biochemical profile variation in beans is the main factor that contributes to coffee drink quality. This study demonstrated variations in free diterpenes in Indian coffee beans. Further, the diversity for

diterpene content both at the interspecific[11,23] and intraspecific levels[24] in *Coffea* sp. suggests the existence of genetic polymorphism of the enzymes controlling the cafestol/kahweol biosynthesis pathways. Flowers of both *Coffea* species exhibited diterpenes levels lower than those in seeds and leaves. Probably their levels start increasing with the onset of fruiting leading to high accumulation in beans of ripened fruits. Significance of diterpenes in flowers of coffee is yet to be studied. Earlier studies reported the presence of caffeine and theobromine in different floral parts of tea plant[25]. In general, caffeine biosynthesis is active in the leaves followed by its transport to the fruits[26]. The key enzymes of the biosynthetic pathway of cafestol and kahweol from ent-kaurene are not yet established[26]. Comprehensive biosynthetic and physiological studies on diterpenes would unravel their role in coffee-bean development.

The present study has shown the presence of diterpenes cafestol and kahweol in commercial coffee germplasm grown in India. Their profiles in *in vitro* cultures have implications in selection of somaclonal variants for *Coffea* improvement with respect to antinutritional diterpenes. Moreover, implications of diterpenes production in ontogeny of fruit and seed maturation will be of physiological and ecological significance.

1. Sridevi, V. and Giridhar, P., Recent trends in coffee biotechnology towards quality improvement. *Indian J. Bot. Res.*, 2008, **4**(1), 5–12.
2. Garattini, S., Coffee and health. In *Monographs of the Mario Negri Institute for Pharmaceutical Research* (ed. Garattini, S.), Raven Press, New York, 1993, p. 432.
3. Urgert, R. and Katan, M. B., The cholesterol raising factor from coffee beans. *Annu. Rev. Nutr.*, 1997, **17**, 305–324.
4. Grollier, Jean, F. (Paris, F. R.), Plessis, Sophie (Paris, F.R.), Use of coffee bean as a sun filter. U.S. Patent No. 4793990, US Patent and Trade Mark Office, 1988.
5. Bertholet, R., Preparation of cafestol. US Patent No. 4692534, US Patent and Trade Mark Office, 1988.
6. Schilter, B., Perrin, I., Cavin, C. and Huggett, C. A., Placental glutathione *S*-transferase (GST-P) induction as a potential mechanism for the anti-carcinogenic effect of the coffee-specific components cafestol and kahweol. *Carcinogenesis*, 1996, **17**, 2377–2384.
7. Pelle, E., Topical composition and method for enhancing lipid barrier synthesis. US Patent No. 5855897, US Patent and Trademark Office, 1999.
8. Urgert, R., Schulz, A. G. M. and Katan, M. B., Effects of cafestol and kahweol from coffee grounds on serum lipids and serum liver enzymes in humans. *Am. J. Clin. Nutr.*, 1995, **61**, 149–154.
9. Hekers, H., Gobel, U. and Kleppel, U., End of the coffee mystery: diterpene alcohols raise serum low density lipoprotein cholesterol and triglyceride levels. *J. Int. Med.*, 1994, **235**, 192–193.
10. Lercker, G., Frega, N., Bocci, F. and Rodriguez-Estrada, M. T., High resolution gas chromatographic determination of diterpene alcohols and sterols in coffee lipids. *Chromatographia*, 1995, **41**, 29–33.
11. de Roos, B., vander Weg, G., Urgert, R., van de Bovenkamp, P., Charrier, A. and Katan, M. B., Levels of cafestol, kahweol and related diterpenoids in wild species of the coffee plant *Coffea*. *J. Agric. Food Chem.*, 1997, **45**, 3065–3069.
12. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 1962, **15**, 473–497.
13. Yasuda, T., Fujii, Y. and Yamaguchi, T., Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. *Plant Cell Physiol.*, 1985, **26**, 595–597.
14. Giridhar, P., Indu, E. P., Vijayaramu, D. and Ravishankar, G. A., Effect of $AgNO_3$ on *in vitro* shoot growth of coffee. *Trop. Sci.*, 2003, **43**, 144–146.
15. Kolling-Speer, I., Strohscneider, S. and Speer, K., Determination of free diterpenes in green and roasted coffees. *J. High Resol. Chromatogr.*, 1999, **22**, 43–46.
16. Hayashi, K. *et al.*, Endogenous diterpenes derived from *ent*-kaurene, a common gibberellin precursor, regulate protonema differentiation of the moss *Physcomitrella patens*. *Plant Physiol.*, 2010, **153**, 1085–1097.
17. Viani, R., Physiologically active substance in coffee. In *Coffee* (eds Clarke, R. J. and Macrae, R.), Elsevier, New York, 1988, vol. 3, pp. 1–31.
18. Ratnayake, W. M. N., Hollywood, R., O'Grady, E. and Stavrie, B., Lipid content and composition of coffee brew prepared by different methods. *Food Chem. Toxicol.*, 1993, **31**, 263–269.
19. Sakthivelu, G. *et al.*, Isoflavone composition, phenol content, and antioxidant activity of soybean seeds from India and Bulgaria. *J. Agric. Food Chem.*, 2008, **56**, 2090–2095.
20. Prasad, B. C. N., Gururaj, H. B., Vinod Kumar, Giridhar, P. and Ravishankar, G. A., Valine pathway is more crucial than phenyl propanoid pathway in regulating capsaicin biosynthesis in *Capsicum frutescens* Mill. *J. Agric. Food Chem.*, 2006, **54**, 6660–6666.
21. Hamon, S., Anthony, F. and Le Pierrès, D., La variabilité génétique des caféine's spontanés de la section *Mozambicoffea* 1/Précisions sur deux espéces affines *Coffea pseudozanguebariae* et C. spj A. Adansonia (Genetic variability of wild Coffee plants of the section Mazambicoffea 1/Details on two related species: *Coffea pseudozanquebariae* and C sp. A. Bridson). *Bull. Mus. Natn. Hist. Nat., Paris*, 4e serie (6) section B. *Adansonia*, 1984, **2**, 207–223.
22. Mazzafera, P., Soave, D., Zullo, M. A. T. and Guerreiro, F. O., Oil content of green beans from some coffee species. *Bragantia*, 1998, **57**, 45–48.
23. Anthony, F., Clifford, M. N. and Noirot, M., Biochemical diversity in the genus *Coffea* L.: chlorogenic acids, caffeine and mozambioside contents. *Gen. Res. Crop Evol.*, 1993, **40**, 61–70.
24. Speer, K. and Kölling-Speer, I., Lipids. In *Coffee: Recent Developments* (eds Clarke, R. J. and Vitzthum, O. G.), Blackwell Science, Oxford, 2001, pp. 33–49.
25. Fujimori, N. and Ashihara, H., Biosynthesis of caffeine in flower buds of *Camellia sinensis*. *Ann. Bot.*, 1993, **71**, 279–284.
26. De Castro, R. D. and Marraccini, P., Cytology, biochemistry and molecular changes during coffee fruit development. *Braz. J. Plant Physiol.*, 2006, **18**, 175–199.

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