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Caffeine content variation in single green Arabica coffee seeds

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Abstract

Coffea arabica L. is highly homozygotic and caffeine content in most cultivars is reported to be similar (approximately 1%). The caffeine content was analysed using individual seeds of cultivars Catuaí Vermelho, Mundo Novo and Bourbon Vermelho, and varieties Mokka and Laurina of *C. arabica*. Seeds were cut transversely in half, a thin slice from each was extracted in a mixture of chloroform and methanol, and caffeine was measured by high performance liquid chromatography. The extraction procedure was validated by comparison with two conventional methods less suited for analysing large numbers of seeds. The results revealed an unexpectedly large variation [Catuaí Vermelho: 3.72–25.9 mg g^{-1} (seeds); Mundo Novo: 5.06–18.59 mg g^{-1}; Bourbon Vermelho: 6.76–16.59 mg g^{-1}] even in types naturally low in caffeine such as Laurina (1.17–9.97 mg g^{-1}) and Mokka (2.01–9.64 mg g^{-1}). The mean values for all cultivars were always in the range of the caffeine content found in the literature. Several studies showed that *C. arabica* has approximately 10% cross-pollination, but the variation observed in caffeine content in individual seeds was too large to be explained by cross-pollination.

Keywords: caffeine, *Coffea arabica*, coffee breeding, genetic variation, seeds

Introduction

The well-known increase in alertness often induced by drinking coffee is attributable to its content of the purine alkaloid caffeine. Other less desirable physiological effects on human health such as sleeplessness and mild addiction (Nawrot *et al.*, 2003; Coffee Board of India, 2004) have led to industrially decaffeinated coffee products using several alternative methods to remove the alkaloid (Katz, 1985; Ramalakshmi and Raghavan, 1999). An alternative approach is to develop coffee plants that produce caffeine-deficient seeds, by either traditional breeding methods (Carvalho, 1988; Mazzafera and Carvalho, 1992; Priolli *et al.*, 2008), genetic modification through plant transformation (Ogita *et al.*, 2003, 2004) or searching gene banks for genetic resources producing naturally low levels of caffeine (Mazzafera *et al.*, 1997; Silvarolla *et al.*, 2000, 2004).

Two coffee species are commercially important: *Coffea arabica,* in which the caffeine content averages 12 mg g^{-1} (seeds); and *Coffea canephora* (syn. *Coffea robusta*), where the caffeine content typically exceeds 20 mg g^{-1}. Genetic variability is expected to be high in allogamous (cross-pollinated) coffee species, such as *C. canephora*, but low in *C. arabica*, a species with approximately 90% self-pollination (Carvalho *et al.*, 1991). Such a high self-pollination rate may be explained by the anthers being positioned at the same level as the stigma, and pollen grains being released by anthers shortly after anthesis in the early morning of hot, sunny days (Carvalho *et al.*, 1991). On rainy days, flower opening may be arrested. In Arabica coffee this commonly gives rise to cleistogamy, self-pollination in the unopened flower. Several studies carried out with different *C. arabica* cultivars and in different countries showed that the extent of cross-pollination in this species was around 10–11% (Carvalho, 1988; Carvalho *et al.*, 1991). This high self-pollination rate promotes genetic homozygosity and has led coffee breeders to seek genetic variability for agronomical traits, including low caffeine content (Mazzafera and Carvalho, 1992; Mazzafera *et al.*, 1992; Barre *et al.*, 1998; Priolli *et al.*, 2008). The usual approach has been to perform interspecific crosses between *C. arabica* and other coffee species (Carvalho, 1988).

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Despite the genetic homozygosity of *C. arabica*, the search for low caffeine-content coffee plants has revealed some variability in the alkaloid content (Mazzafera and Carvalho, 1992; Mazzafera *et al.*, 1992, 1997; Silvarolla *et al.*, 2000, 2004). These reports analysed a mixture of several seeds from single plants. To better understand this variation, we studied the caffeine content in single seeds of single plants of three cultivars and two varieties of *C. arabica*. All seeds were derived from fruits at the berry stage and from one single plant for each cultivar or variety. Surprisingly, a large variation in caffeine content was observed among individual seeds. As *C. arabica* has a high self-pollination rate, the caffeine variation observed between individual seeds of distinct coffee genotypes may not be explained simply by cross-pollination. These points are discussed in this paper.

Materials and methods

Plant materials

Seeds from open-pollinated *C. arabica* plants were used for caffeine content analyses. They were collected from cultivars Catuaí Vermelho, Mundo Novo and Bourbon Vermelho, and varieties Mokka and Laurina grown in experimental plots predominantly occupied by *C. arabica* individuals. They were collected from single trees of each cultivar or variety and the numbers of seeds analysed were: 298 for Mundo Novo, 300 for Bourbon Vermelho, 300 for Laurina and 116 for Mokka. For Catuaí Vermelho, two batches of seeds were analysed. These comprised 453 seeds from a single tree harvested in 2007 and 300 seeds mixed from several trees harvested in 2008. Only fruits at the berry stage were collected from the plants and processed by a dry method (Vincent, 1985). Seeds were kept at approximately 10% relative humidity. The parchment (endocarp) was removed manually before caffeine extraction from seeds.

Caffeine analyses

Seeds were cut transversely in half (one half contained the embryo) by hand using a sharp razor blade. From each half, a thin slice (~0.5 mm thickness) was removed (slice method). These slices were individually weighed and transferred to high performance liquid chromatography (HPLC) vials containing 1.5 ml of a mixture of chloroform and methanol (1:9 in volume). To avoid evaporation, Teflon discs were placed inside the vial caps, which were tightly closed and stored at 8°C for 3 weeks with occasional agitation. Caffeine content was then measured by HPLC (Shimadzu, Kyoto, Japan) without removing the slices from the vials. Caffeine was separated in a C18 reversed-phase column (4 mm × 250 mm, 5 μm; Supelco, Sigma-Aldrich, St. Louis, Missouri, USA), with isocratic elution with 50% (v/v) methanol and 0.5% (v/v) acetic acid in water, at a flow rate of 0.8 ml min^{-1}. Caffeine was detected at 280 nm with a diode array detector. Each vial was analysed twice and the caffeine content was estimated after calibration using pure caffeine standards over an appropriate range of concentrations. Mean values of four replicate analyses of each seed were obtained. To validate the method of extraction, two slices were obtained from 50 seeds of cultivar Mundo Novo and extracted as described above. From the two remaining parts of each seed, one was finely diced with a razor blade, weighed and extracted with 3 ml of 80% (v/v) methanol at 60°C for 1 h [methanol (MtOH) method]. The remaining part was also diced and then extracted with 3 ml of 80% (v/v) methanol in a Polytron grinder (Bioblock Scientific, Geneva, Switzerland) operating at speed 5 for 30 s (Politron + MtOH method). The supernatant of each extraction method was recovered and analysed by HPLC as above. In these ways, the caffeine content of each group of 50 seeds was compared on a dry weight basis using the slice, MtOH and Politron + MtOH methods.

Results

Validation of the slice method

To analyse caffeine content in individual seeds the slice method was used. To validate the method, 50 seeds were individually extracted for caffeine using the slice method and the remnant seed material was extracted by the MtOH method or the Politron + MtOH method. Analyses of individual seeds from different coffee varieties showed variation no greater than 5% between the two slices from the same seed despite inevitable variations in slice thickness arising for cutting these by hand. Comparisons of the three extraction methods revealed that the Politron + MtOH method extracted caffeine with the highest efficiency, probably because of the thorough tissue disruption involved (Fig. 1). The MtOH method differed from the slice method by extracting slightly more caffeine from seeds containing especially high amounts. However, differences were not large. It is clear from Fig. 1 that the results obtained by each method were very similar. A correlation analysis showed R^2 values of 0.975 and 0.988 for slice versus MtOH and slice versus Politron + MtOH methods, respectively. Thus, although the slice method may not be the most appropriate for estimating the absolute caffeine content with the greatest accuracy, it is an acceptable method to evaluate variation in caffeine content among individual seeds.

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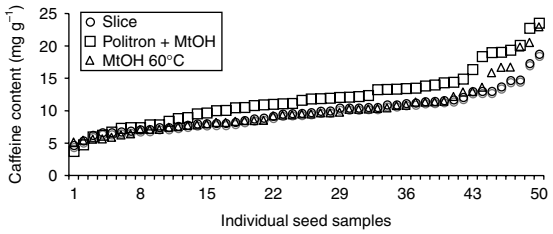

Figure 1. Comparison of three extraction methods of caffeine from 50 individual seeds. Each seed was analysed by HPLC after solvent extraction using the three methods. In the slice method, two transverse slices were taken from the central part of each seed and caffeine was extracted with a mixture of methanol and chloroform. The remaining two parts of the seed were extracted either by the MtOH method, where methanol was heated to 60°C or by the Politron + MtOH method, where methanol was used after tissue maceration with a Polytron homogenizer.

Comparison of cultivars and varieties

The frequency distribution of the caffeine content showed that wide variations exist (Fig. 2) both between varieties and cultivars and between individual seeds within any one variety or cultivar. For example, Bourbon Vermelho and Catuaí Vermelho contained the most caffeine (~11.6 mg g^{-1}) and Mokka the least (5.61 mg g^{-1}), a difference of over 50%. Analyses of the frequency (percentage of seeds) in each class differing by 1 mg g^{-1} revealed several important features. The numbers of seeds falling into each class were normally distributed for each of the six types. The class with mean caffeine content was the most populous class in each case, but caffeine content within any one type varied considerably (Fig. 2). The ranges for seeds taken from one individual plant were 5.06–18.59 mg g^{-1} for Mundo Novo; 2.01–9.64 mg g^{-1} for Mokka; 6.76–16.59 mg g^{-1} for Bourbon Vermelho; 1.17–1.97 mg g^{-1} for Laurina and 3–25.9 mg g^{-1} for Catuaí Vermelho. When mixed seeds from several plants of Catuaí Vermelho harvested in 2008 were compared (Fig. 2F), the distribution of caffeine content among individual seeds was smaller (6.91–17.19 mg g^{-1}) than that found among seeds that were harvested from one plant in the previous year (Fig. 2E).

Discussion

In the present paper we reveal a wide variation in caffeine concentration between individual seeds of *C. arabica*. Most reports in the literature indicate that caffeine in commercial Arabica coffee varies between 10 and 12 mg g^{-1}. These data, however, come from determinations of caffeine content in several seeds from the same plant. The only types with less caffeine (~5–6 mg g^{-1}, Fig. 2) are those such as Mokka and Laurina that carry the *laurina* genetic factor (Clifford, 1985). The slice method of extraction that we used was slightly less efficient than two more conventional but

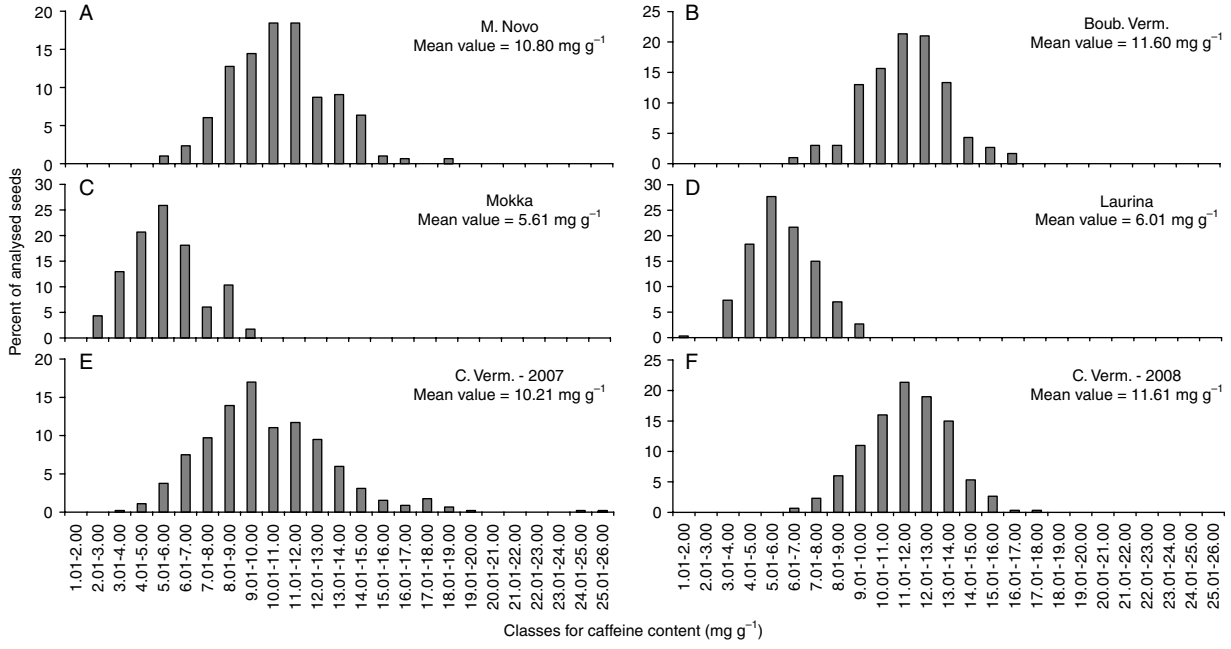

Figure 2. Variations in caffeine content between single seeds of cultivars 'Mundo Novo' (A), 'Bourbon Vermelho' (B) and 'Catuaí Vermelho' harvested in 2007 (E) and 2008 (F), and varieties Mokka (C) and Laurina (D) of *C. arabica*. Individual seeds were extracted with the slice method and analysed for caffeine content by HPLC. In the graphs, seeds were grouped into classes each differing in caffeine content by 1 mg g^{-1}.

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less convenient methods. However, our correlation analyses (Fig. 1) convince us that these comparisons are reliable, in spite of slight underestimation of the highest caffeine content values by this method.

Catuaí Vermelho harvested in 2007 had a slightly higher caffeine content than that of Catuaí Vermelho harvested in 2008 (Fig. 2E and F). This may be not only because of the difference in harvest methods (i.e. seeds from several plants in 2008 versus seeds from a single plant in 2007), but also because of caffeine content variation from year to year, as shown in a previous study (Mazzafera *et al.*, 1992). Two seeds of Catuaí Vermelho from 2007 showed caffeine content higher than 24 mg g^{-1}, a value usually found in *C. canephora*.

The biosynthesis (Ashihara and Suzuki, 2004) and biodegradation (Mazzafera, 2004) of caffeine in coffee involve several steps. The final concentration of caffeine is inevitably a result of the balance between the two pathways. The balance between synthesis and degradation is affected by the availability of the substrates for the several enzymes involved and the expression of the related genes, which are likely to be modulated by biotic and abiotic factors. Little research has been carried out on the effects of these possible factors on coffee fruits.

As caffeine seems to be uniformly distributed in the coffee seed tissues (Baumann *et al.*, 1998), a stochastic expression of each allele of genes associated with caffeine content in each cell may determine the final caffeine content in tissues and a whole seed. The seed-to-seed variation observed here seems too large to be explained by the 10% cross-pollination reported for coffee, or based on the knowledge available on the genetic control of caffeine biosynthesis (Ashihara *et al.*, 2008). The data for Mokka and Laurina support this conclusion, as these plants were surrounded in the field by other *C. arabica* varieties with double caffeine content. Then, a cross-pollination of these varieties with Mundo Novo, for example, would yield seeds containing at least one *Lr* (high caffeine content allele) and therefore with caffeine content similar to Mundo Novo seeds. However, the variation was between much smaller values and values close to 10 mg g^{-1} found in Mundo Novo.

The results obtained for Mokka and Laurina varieties are of particular interest, as a single dominant allele in the endosperm (*Lrlrlr* and *LrLrlr* genotypes) increases the caffeine content to values around 12 mg g^{-1} caffeine (Monaco *et al.*, 1975). However, this information comes from a study where several seeds were mixed and used; it would be interesting to investigate the contribution of the *lr* genetic factor to the caffeine content in the endosperm of individual seeds of the three possible genotypes (*LrLrlr*, *Lrlrlr* and *lrlrlr*).

Baumann *et al.* (1998) analysed individual seeds of somaclonal and non-somaclonal plants of Laurina and observed that seeds from the former clearly had less seed-to-seed variation and a lower caffeine content. Such differences may be due to somaclonal genetic variation caused by the propagation procedure (tissue culture regeneration from leaf segments), although this possibility was not discussed by the authors. Unfortunately, only a small number of seeds (7–11) were analysed, which might have been unrepresentative of the larger population, as we have shown in the present study. A significant implication of our findings is that future studies must take account of the large seed-to-seed variation in caffeine concentration. This would involve sampling from populations of seeds that are sufficiently large for caffeine content measurement to reflect the true mean value of any given genotype reliably.

An important question raised by Baumann *et al.* (1998), which is also valid here, is the stability of the low caffeine content found in some seeds. Does a coffee plant originating from a seed with low caffeine content keep this characteristic in both leaves and seeds? The coffee seed is predominantly formed by the triploid endosperm (2*n* from the female parent and 1*n* from the male parent), the reserves of which are used by the tetraploid embryo during germination. Thus, the caffeine content in the endosperm may not predict the caffeine content of the plant of the next generation, originating from the embryo. This certainly could be answered by monitoring the caffeine content in plants obtained after germination. This would open a new approach for breeding coffee for low caffeine content.

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