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P. Barre · S. Akaffou · J. Louarn · A. Charrier
S. Hamon · M. Noirot

Inheritance of caffeine and heteroside contents in an interspecific cross between a cultivated coffee species *Coffea liberica* var *dewevrei* and a wild species caffeine-free *C. pseudozanguebariae*

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Abstract Coffee species originating from Africa, in particular the two major cultivated species *C. arabica* and *C. canephora*, usually contain caffeine in their beans, whereas almost all Malagasy coffee species are caffeine-free. However, one wild coffee species *C. pseudozanguebariae*, collected near the coast in south Kenya, is also caffeine-free. Beans of this species contain a specific heteroside diterpene (hereinafter referred to simply as heteroside) and give a bitter coffee beverage. We have investigated the inheritance of the caffeine and heteroside contents in the first and second generations of an interspecific cross between *C. pseudozanguebariae* and *C. liberica* var. *dewevrei*, for which the caffeine content is about 1% dmb (dry matter basis). The caffeine content of F_1 hybrids (0.2% dmb) was lower than the parental average (0.47% dmb). Caffeine and heteroside contents appeared to be under polygenic control with a strong genetic effect. Nevertheless, one major gene with two alleles seemed to be involved in the control of both compounds. Absence of caffeine was apparently controlled by one recessive gene. Heteroside content seemed to be controlled by one co-dominant gene, heterozygotes being intermediate between the two different groups of homozygotes.

Key words *Coffea* · Caffeine · Heterosides · Interspecific cross · Polygenic control · Decaffeinated coffee

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P. Barre (✉) · S. Hamon · M. Noirot
Laboratoire de Ressources Génétiques et d'Amélioration des Plantes Tropicales, Centre ORSTOM de Montpellier, B.P. 5045, 34032 Montpellier Cedex 1, France
E-mail: pbarre@mpl.orstom.fr

S. Akaffou · J. Louarn
Station caféière ORSTOM, Man, BP 434, Côte-d'Ivoire

A. Charrier
ENSA.M, UFR Génétique et Amélioration des Plantes, Place Viala, 34060 Montpellier Cedex 1, France

Introduction

The influence of caffeine on the central nervous system is well known (Nehlig et al. 1992), especially in respect of insomnia, and has led coffee companies to provide an artificially de-caffeinated coffee. An alternative could be the breeding of naturally caffeine-free coffee (Charrier 1978). This would be important for *C. canephora*, for which the caffeine content on a dry matter basis (dmb) varies from 1.2% to 3.5% (Charrier and Berthaud 1975). Although genetic improvement using intraspecific diversity is an obvious approach, this approach seems inadequate to obtain caffeine-free coffee.

Interspecific hybridisation has been broadly developed in the genus *Coffea* (Carvalho and Monaco 1967; Charrier 1978; Louarn 1992) and could lead to caffeine-free coffee. Caffeine is lacking in most Malagasy species (Charrier and Berthaud 1975; Clifford et al. 1989) and in the East-African species *C. pseudozanguebariae* (PSE) (Hamon et al. 1984; Rakotomalala et al. 1993). Unfortunately, caffeine-free species are low yielding and give a beverage of poor tasting quality. *C. pseudozanguebariae* yields a highly bitter coffee beverage, which could be due to a specific heteroside, diterpene, found in large amounts in PSE beans (Rakotomalala 1993). Diterpene will hereinafter be simply called heteroside.

Interspecific hybrids were obtained between PSE and two diploid coffee species from tropical lowlands whose beans contain caffeine: the widely cultivated species *C. canephora* (Robusta) and a rarely cultivated species *C. liberica* var. *dewevrei* (DEW). The aim was to obtain a hybrid lacking both caffeine and heteroside. Because we presently lack adult hybrids of the second generation of the cross PSE × *C. canephora*, we describe the inheritance of caffeine content in the first and second generations of an interspecific cross between PSE and DEW. The caffeine content of DEW is about 1%

dmb (Anthony et al. 1993). The genetic inheritance of heteroside was also investigated, as well as its relation to caffeine. The preliminary results presented here constitute a model for the genetic improvement of *C. canephora* using interspecific crosses with PSE.

Materials and methods

Plant material

Plant material was obtained from, and maintained by, J. Louarn at the Coffee Breeding Station ORSTOM-IDEFOR (Man, Côte-d'Ivoire).

The parental species originated from Central Africa, near Siolo-Nvuna (Berthaud and Guillaumet 1978), and from south Kenya, near Diani (Berthaud et al. 1980), for DEW and PSE, respectively. In 1993, DEW beans were separately harvested from six trees, whereas PSE beans were harvested in bulk.

Four interspecific crosses (8044×5851; 8013×5586; 8053×5586; 8056×5497) were made using PSE as the female parent and DEW as the pollinator. The F_1 generation was studied from five hybrids per cross.

Thirteen F_1 hybrids from the cross 8044×5851 gave by open-pollination the second generation of hybrids. On the basis of molecular markers and phenotypic traits, we identified 82 hybrids as 65 backcrosses on DEW, $F_1 \times$ DEW named BCDEW, and 17 backcrosses on PSE, $F_1 \times$ PSE named BCPSE. Twentynine hybrids of the second generation were harvested in 1993 only, 13 in 1994 only, and 40 in both 1993 and 1994.

Sample preparation

All fruits were harvested at complete maturity and de-pulped using the wet processing method. Caffeine content was evaluated from at least 40 green coffee beans per tree. Each batch of beans was crushed in a ball mill (Dangoumeau) for 8 min. The powder was divided into nine samples, three to estimate water content and six to extract the caffeine and the heteroside.

Caffeine and heteroside extraction

Caffeine and heteroside were extracted using a slightly modified version of the method of Vithmutz et al. (1974). About 0.25 g of powder was mixed with 1 g of magnesium oxide (MgO) in about 25 ml of distilled water in a 50-ml tube with a cap. Alkaline hydrolysis was carried out by autoclaving at 110°C for 30 min. The final volume was adjusted to 250 ml with water. Samples were filtered through a 0.45-μm filter and stored at −20°C before analysis. No loss of accuracy was recorded due to the simplification of Vithmutz's method.

High-performance liquid chromatography (HPLC) measurements

The HPLC system comprised two Waters Associates Model 510 pumping units, an autosampler (Waters 717 plus autosampler), a variable-wavelength UV detector (Waters 996 Photodiode Array Detector), a C_{18} pre-column and a 250 mm × 4 mm Merck Superspher 100 RP 18 column, with a 5 μm particle size. The elution program used two solvents. Solvent A was 2 mM phosphoric acid, pH 2.7, containing 5% methanol. Solvent B was methanol containing 5% 2 mM phosphoric acid, pH 3.9. These two mobile phases were filtered (0.2 μm) and de-gassed by sonication. Analysis was done at room temperature using the following elution program: A-B mixture (65/35) for 15 min, 5 min to reach 100% of solvent B by linear gradient, 5 min with 100% of solvent B and 5 min to return to initial conditions (65/35). The flow rate was 1 ml/min. UV detection at wavelengths from 200 to 300 nm was used to check the purity of the caffeine and heteroside peaks. The spectrum of the heteroside is shown Fig. 1. The 273-nm wavelength was used to estimate caffeine and heteroside contents. The sample processing order was fully randomised for HPLC analysis.

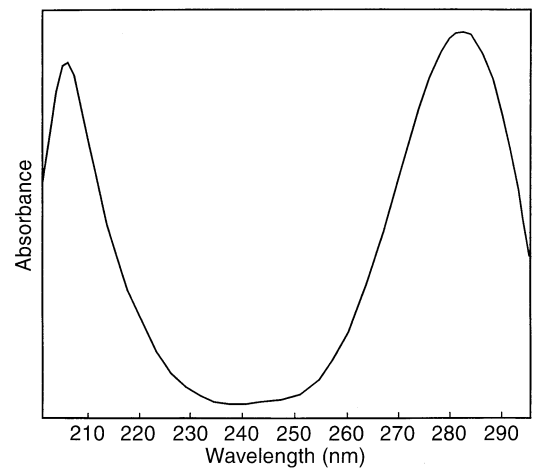

Fig. 1 UV spectrum of the heteroside diterpene specific to *C. pseudozanguebariae*

Caffeine was quantified using a calibration curve, and its content was expressed as a percent of the dry matter basis (dmb). Following every ten samples, a caffeine standard (10 mg/l) was analysed to check possible variations. Because of the absence of purified heteroside, the relative content of heteroside was calculated with regard to the content in PSE. Error variances were similar (0.004) for caffeine and heteroside.

Statistical analysis

All data were analysed using Statistica software.

A nested model of ANOVA with random effects was used to test between-cross and between-hybrid (within-cross) variations in caffeine and heteroside contents in F_1 hybrids. Significant variations were subsequently quantified using the mathematical expectation of the respective mean square.

A partly nested model of ANOVA was used to test differences between years (fixed effect), between female parents (random effect) and between hybrids (random effect) nested in the female parent. Analysis involved 2 harvest years (1993, 1994), six progenies and four hybrids per progeny.

Results

Parental species

Caffeine content (CAF) in *C. liberica* var. *dewevrei* (DEW) was 0.9% dmb on average. Values ranged from 0.7 to 1.1% dmb ($F_{5,30} = 6.2$; $P = 0.0004$). Heteroside was not detected in DEW, and caffeine was not detected in *C. pseudozanguebariae* (PSE). As mentioned

Table 1 Results of the partly nested model of ANOVA used to test for differences between 2 harvest years (fixed effect), between six progenies (each progeny is derived from a different F_1 female parent) (random effect), and between four hybrids per progeny (random effect nested in progeny) for caffeine content (CAF) and relative heteroside content (RHET)

Effects	*df*	CAF		RHET	
		F	*P*	*F*	*P*
Harvest year	1	4	0.102	5.4	0.070
Progeny (female parent)	5	2	0.133	0.9	0.490
G_2 hybrid	**18**	**201**	**0.000**	**202**	**0.000**
Harvest year × progeny	5	0.5	0.760	1.4	0.290
Harvest year × G_2 hybrid	**19**	**7.3**	**0.000**	**9.4**	**0.000**
Error	240				

earlier, the relative heteroside content (RHET) in PSE was standardised to 1.

F_1 hybrids

CAF differed between crosses ($F_{3,16} = 3.54$; $P = 0.04$) and between hybrids within crosses ($F_{16,100} = 8.14$; $P < 0.001$). Cross means varied from 0.16% dmb to 0.23% dmb. Variation between crosses and within crosses accounted for 36% and 64%, respectively, of the total variation between genotypes.

The CAF of F_1 hybrids (0.17% dmb) was 60% lower than the parental average (0.47% dmb) implying that CAF could not be an additive trait. Nevertheless, additivity was respected if we consider the square root of CAF (0.41 and 0.48 for F_1 hybrids and parental average, respectively).

By contrast with CAF, crosses did not differ in RHET ($F_{3,16} = 1.09$; $P = 0.38$), while the between-hybrid variation was still very highly significant ($F_{16,99} = 8.56$; $P < 0.001$) and ranged from 0.38 to 0.80. In addition, the RHET of F_1 hybrids (0.55) was close to the parental average (0.5).

Second-generation hybrids

Heteroside content inheritance

RHET was estimated using 2 harvest years for 24 hybrids of the second generation (four hybrids per progeny). RHET differed between hybrids derived from the same female parent (Table 1). These differences accounted for 92% of variance. "Harvest year × hybrid" interactions accounted for 8% of variance, whereas no difference was noted between harvest years. The absence of year-effect and the weakness of the interaction allowed the pooling of the 1993 and 1994 results.

The distribution of RHET in the backcross on PSE (BCPSE) shows two groups (Fig. 2 a) suggesting the control of this trait by one major gene with two codominant alleles (h1 and h2). The group with an average (1.25) close to those of PSE (1) would include homozygotes with two alleles from PSE (h1h1), whereas the group with an average (0.53) close to those of F_1 hybrids (0.55) would include heterozygotes (h1h2). The h1h1:h1h2 distribution was 8 : 9 and did not differ significantly ($\chi^2 = 0.06$; $P < 0.81$) from the expected 1:1.

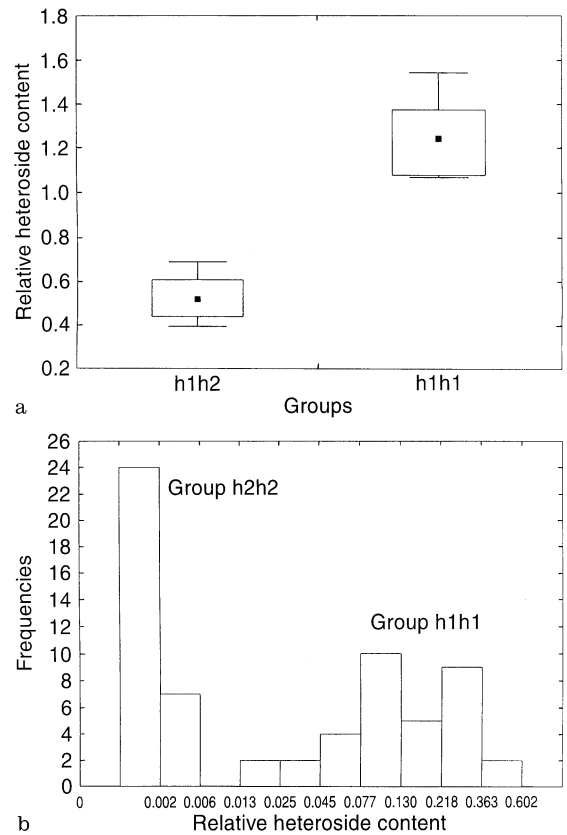

Fig. 2 a Distribution of relative heteroside content in the backcross on *C. pseudozanguebariae* (BCPSE). Two types of hybrids: h1h2 (nine genotypes) close to the F_1 average and h1h1 (eight genotypes) close to the PSE average. **b** Distribution of relative heteroside content (RHET) in the backcross to *C. liberica* var. *dewevrei* (BCDEW)

The products of the backcross to DEW (BCDEW) (Fig. 2 b) were also distributed in two groups. One group included 31 hybrids, with or without traces of heteroside (RHET ≤ 0.006; mean = 0.0011), which should be homozygotes with two alleles from DEW (h2h2). The other group comprised 34 hybrids, for

which the heteroside content ranged between 0.014 and 0.45 (mean = 0.170). These hybrids were assumed to be heterozygotes (h1h2). The h2h2 : h1h2 distribution was 31 : 34 and did not differ significantly ($\chi^2 = 0.24$; $P < 0.71$) from the expected 1 : 1 distribution. Note, nevertheless, that the RHET of heterozygote hybrids (h1h2) in the backcross to DEW (BCDEW) (0.17) was clearly lower than those of heterozygotes in the backcross to PSE (BCPSE) (0.53) and those of F_1 heterozygotes (0.55).

Caffeine content inheritance

CAF variation was mainly due (94.6 %) to differences between hybrids (Table 1). Interaction between the harvest year and hybrids represented only 5.9 % of the variation, while no difference was apparent between harvest years. As for RHET, the absence of a year effect and the weakness of the interaction allowed the pooling of the 1993 and 1994 results.

The 17 backcrossed hybrids to PSE (BCPSE) were distributed in two groups (Fig. 3 a). One group included eight hybrids without caffeine and two hybrids with a CAF less than 0.004% dmb. This group of ten hybrids with a mean CAF of 0.00055% dmb was considered caffeine-free. The other group comprised seven hybrids with a CAF ranging between 0.006% dmb and 0.132% dmb (mean = 0.053% dmb). The 65 backcrossed hybrids to DEW (BCDEW) contained caffeine (0.16% dmb–1.22% dmb) and the distribution was unimodal with an average of 0.55 (Fig. 3 b).

If we consider the trait presence vs absence of caffeine, all F_1 and BCDEW hybrids contained caffeine and about half of the BCPSE were caffeine-free. These results suggested that absence of caffeine is controlled by one recessive gene. In this case, BCPSE hybrids without caffeine should be recessive homozygotes (cc), the F_1 and BCPSE hybrids with caffeine should be heterozygotes (Cc), and the BCDEW hybrids should be either heterozygotes (Cc) or dominant homozygotes (CC). Note that the cc : Cc distribution in BCPSE (10 : 7) did not differ significantly ($\chi^2 = 0.53$; $P < 0.47$) from the expected 1 : 1 distribution.

The variation in caffeine content in hybrids which contain caffeine suggested that this trait was under polygenic control. As in F_1 hybrids, the trait square-root of caffeine was additive in the backcross to PSE (BCPSE) with an average of 0.21 compared to 0.24, as well as in the backcross to DEW (BCDEW) with an average of 0.73 compared to 0.72.

Relationship between caffeine and heteroside contents

In second-generation hybrids with caffeine higher than 0.004 and with RHET higher than 0.006, a hyperbolic relationship (Y = 0.03/X + 0.08) between RHET (Y) and CAF (X) was apparent (Fig. 4). This relationship could explain the lower RHET (than expected assuming additivity) in Hh hybrids with caffeine higher than 0.004.

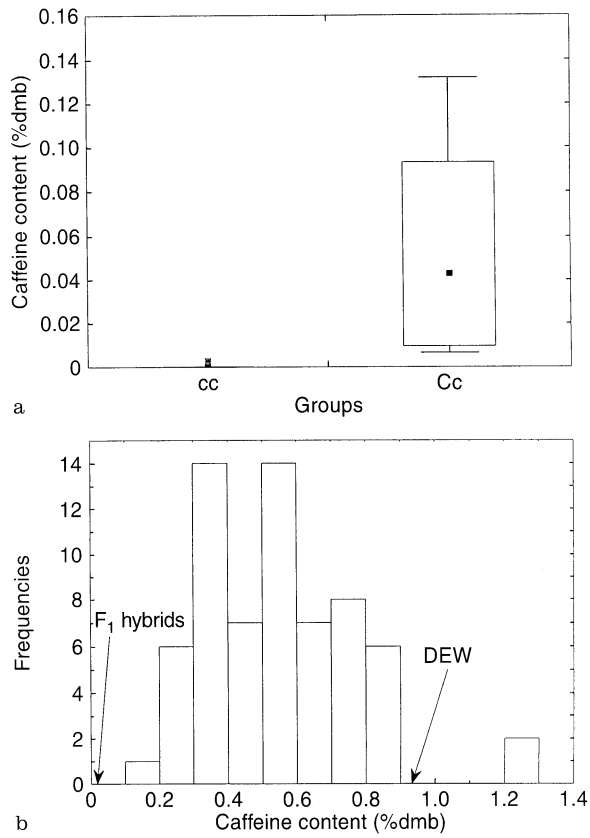

Fig. 3 a Distribution of caffeine in the backcross to *C. pseudozanguebariae* (BCPSE). Two types of hybrids: ten genotypes close to zero (cc) and seven genotypes with a higher amount of caffeine (Cc). **b** Distribution of caffeine content in the backcross to DEW (BCDEW)

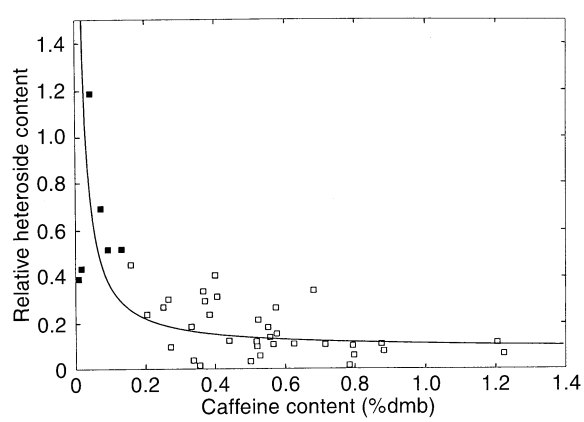

Fig. 4 Relationship between caffeine (CAF) and relative heteroside (RHET) contents in second-generation hybrids with caffeine higher than 0.004 and with RHET higher than 0.006. A hyperbolic function y = 0.03/x + 0.08 was fitted to the curve. *Filled squares* (■) indicate BCPSE hybrids and *empty squares* (□) indicate BCDEW hybrids

In the backcross to PSE, three hybrids had a RHET close to the F_1 average and were caffeine-free (h1h2/cc), seven had a RHET close to PSE and were caffeine-free (h1h1/cc), six hybrids had a RHET close to the F_1 average and contained caffeine (h1h2/Cc) and one hybrid had a RHET close to PSE and contained caffeine (h1h1/Cc). The distribution of presence vs absence of caffeine was not independent of the distribution of RHET ($\chi^2 = 5.13$, $P = 0.024$), with absence of caffeine being linked to higher RHET values.

Discussion

Genetic control of caffeine content

Studies of intraspecific variation in caffeine content in many species from Africa and Madagascar indicate that despite the large range of caffeine content observed in some coffee species, such as *C. canephora*, a caffeine-free coffee tree in a species usually with caffeine or a caffeine-containing coffee tree in a species usually caffeine-free has yet to be found (Charrier and Berthaud 1975; Clifford et al. 1989; Anthony et al. 1993; Rakotomalala et al. 1993).

The present study deals with a cross between a coffee species with caffeine – *C. liberica* var. *dewevrei* (DEW) – and one without – *C. pseudozanguebariae* (PSE). The major result is that the presence vs absence of caffeine seems to be determined by one major gene. The absence of caffeine appeared to be recessive so that hybrids without caffeine should be recessive homozygotes.

In species with caffeine, quantitative studies have mainly concerned cultivars of *C. canephora* or *C. arabica*, crosses within *C. canephora*, and F_1 interspecific hybrids (Charrier and Berthaud 1975; Louarn 1976; Le Pierres 1987; Mazzafera and Carvalho 1992). These quantitative studies led to two main results, confirmed here in second-generation hybrids with caffeine: 1) caffeine content is a quantitative trait with a polygenic inheritance, and (2) genetic effects are preponderant.

Caffeine content is often recorded as additive in intra- and inter-specific hybrids involving coffee species with caffeine, whatever the ploidy level (Capot 1972; Charrier and Berthaud 1975; Louarn 1976; Berthaud 1977; Le Pierres 1987). Nevertheless, a study involving a large number of coffee species showed that caffeine content in F_1 hybrids could, or could not, be additive depending on the cross (Mazzafera and Carvalho 1992). In our interspecific cross between species with and without caffeine, additivity was clearly rejected in F_1 hybrids, which all contain caffeine. However, the square root of caffeine content was additive in F_1 hybrids and in caffeine-containing hybrids of backcrosses to both PSE and DEW. We deduce that caffeine could depend linearly on either a squared variable X^2 or a product of variables XY, X and Y being additive. Absence of additivity would only be revealed when parental differences for X, Y, or both, are substantial. Additional information on the relationships between caffeine and other traits (leaf size, ripening time, and bean size) in species with caffeine are necessary to interpret our results.

Genetic control of heteroside content

Data presented here are the first on the inheritance of the specific heteroside content from PSE. This trait seems to be determined by one major gene with two co-dominant alleles named h1 for the allele from PSE and h2 for the allele from DEW. Parental species should be homozygotes: h1h1 for PSE and h2h2 for DEW. The F_1 hybrids consisted of heterozygotes (h1h2) and had a mean value intermediate between the parental species. As expected, in both backcrosses to PSE and DEW we found the same number of homozygotes and heterozygotes.

In addition to this major gene, the variation in heteroside content in each genotype may involve other genes. The low heteroside content observed in heterozygotes (h1h2) of the backcross to DEW (obs. 0.17 vs exp. 0.5) could be partly explained by the relationship between caffeine and heteroside content.

In the backcross to PSE, the RHET of the homozygotes h1h1 (1.25) was higher than the PSE value estimated from a batch of beans harvested from several PSE trees. Similarly, the F_1 average was slightly higher than expected (0.55 vs 0.50). This suggests that there are genetic variations in RHET in PSE, and that the RHET of the batch of beans was lower than that of the PSE parents of the F_1 hybrids as well as that of the PSE parents of the backcross to PSE.

Relationship between caffeine and heteroside contents

A hyperbolic relationship between CAF and RHET was noted in the second-generation hybrids with caffeine and heteroside. The hyperbolic shape could represent a trade-off. The higher the CAF, the lower the RHET, and vice versa. Interpretation of the relationship between caffeine and heteroside contents is not simple. This relationship may derive from genetic linkages and/or interaction during the synthesis of the two compounds. Further studies on the biosynthetic pathways appear necessary to understand this relationship.

In the backcross to PSE, the major gene controlling the absence of caffeine seemed to be linked to the major gene controlling heteroside content. More coffee trees are needed to estimate the degree of this linkage.

Consequences for breeding

One aim of this study was to transfer the absence of caffeine from *C. pseudozanguebariae* into coffee species with caffeine, such as *C. canephora*, without transferring other undesirable traits, such as the presence of heteroside which could be involved in the bitterness of the coffee beverage. This transfer should be greatly facilitated if the simple genetic determinism of absence of caffeine is confirmed.

Recessiveness of the absence of caffeine requires the help of linked molecular markers to follow the allele through generations of successive backcrosses. Indeed, this should allow the distinction in successive backcrosses to DEW or *C. canephora* between the dominant homozygotes and the heterozygotes. The selection of heterozygotes could be done at the seedling stage.

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